MECANISMES TROMBÒTICS A LA SÍNDROME ANTIFOSFOLIPÍDICA I A LES VASCULITIS

TESI DOCTORAL

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TAULES i FIGURES

Pàgines

1. ABREVIATURES

β_2GPI: β_2-glucoproteïna I

F1+2: fragment 1+2 de la protrombina

EFG: factor de creixement epidèrmic (*epidermal growth factor*).

kD: kilodaltons.

MTHFR: metilen-tetra-hidrofolat reductasa.

PAI: inhibidor de l'activador del plasminogen (*plasminogen activator inhibitor*).

PAI-1: inhibidor tipus 1 de l'activador del plasminogen.

PAI-2: inhibidor tipus 2 de l'activador del plasminogen.

PAI-3: inhibidor tipus 3 de l'activador del plasminogen.

TAFI: inhibidor de la fibrinolisi activable per la trombina (*thrombin activatable fibrinolysis inhibitor*).

TFPI: inhibidor de la via del factor tissular (*tissue factor pathway inhibitor*).

t-PA: activador tissular del plasminogen (*tissue-type plasminogen activator*).

u-PAR: receptor de l'activador del plasminogen tipus uroquinasa (*urokinase-type plasminogen activator receptor*).

2. AGRAÏMENTS

Els treballs que formen part d'aquesta memòria no es podrien haver portat a terme sense la col·laboració de moltes persones que de manera personal i professional han participat en la seva realització. Entre elles vull mostrar el meu agraïment especial:

Al Prof. Miguel Ingelmo, co-director d'aquesta tesi pel seu constant estímul en la investigació en el camp de les malalties autoimmunes, especialment de les vasculitis.

A la Dra. Dolors Tàssies, co-directora d'aquesta tesi, pel seu esperit crític, rigor científic, recolzament constant i permanent disposició.

Al Dr. Josep Font, Cap de Servei de Malalties Autoimmunes Sistèmiques per la confiança dipositada en mi per realitzar la present tesi.

Al Dr. Joan Carles Reverter, per la seva manera de fer les coses, la seva gran disponibilitat tant a nivell personal com professional, la seva paciència i per la seva amistat incondicional durant aquests anys.

Al Dr. Joan Monteagudo, per les seves suggerències i col·laboració en el desenvolupament d'aquesta tesi.

Al Dr. Ricard Cervera, pels comentaris en l'anàlisi dels treballs publicats.

Al personal del Servei d'Hemoteràpia i Hemostàsia, en especial a Carolina Freire, Josefina Hernando, Josefina Pijoan, Montse Corbella, Antonia Guerrero, Teresa Martínez i Marc Pino per la paciència mostrada per les nombroses extraccions i citacions realitzades durant els mesos de treball d'aquesta tesi. A més, a la primera d'elles li he d'agrair especialment la seva inestimable ajuda en la realització de les tècniques de laboratori.

Al Dr. Antonio Vidaller del Servei de Medicina Interna de l'Hospital Prínceps d'Espanya de Barcelona per la seva permanent col·laboració en el camp de la malaltia de Behçet.

Al Dr. Alfons López Soto del Servei de Medicina Interna de l'Hospital Clínic de Barcelona per la tasca investigadora iniciada ja fa uns quants anys en la malaltia de Behçet.

Al doctor Munther A. Khamashta i al Dr. Graham R.V. Hughes de la Lupus Unit del St Thomas' Hospital de Londres per la seva col·laboració en els treballs en el camp de la síndrome antifosfolipídica.

A la Dra. Teresa Gallart del Servei d'Immunologia de l'Hospital Clínic de Barcelona per la seva desinteressada col·laboració en la investigació amb anticossos anticardiolipina monoclonals.

Al Dr. Ramon Deulofeu del Servei de Bioquímica de l'Hospital Clínic de Barcelona per la seva col·laboració en les determinacions genètiques dels pacients amb malaltia de Behçet.

Als Dr. Santi Maragall, Dr. Ginés Escolar i Dr. Antoni Ordinas del Servei d'Hemoteràpia i Hemostàsia per la seva col·laboració en els treballs d'aquesta tesi.

Als companys del Servei de Malalties Autoimmunes Sistèmiques, Medicina Interna i Urgències, en especial, a Albert Bové, Ramon Bataller, Paco Muñoz, Ernest Bragulat, Miquel Sánchez, Josep Hernández i Josep Mª Nicolás pel seu desinteressat recolzament i amistat incondicional durant tots aquests anys.

Al Departament d'Universitats i Recerca de la Generalitat de Catalunya i a l'Hospital Clínic de Barcelona per haver-me concedit les ajudes que han permès portar a terme tots aquests treballs.

A tots els malalts que constitueixen el material humà d'aquesta tesi per la seva col·laboració desinteressada.

L'estat d'hipercoagulabilitat o trombofília es defineix com la tendència a desenvolupar coàguls a les venes o artèries (1). Actualment, es reconeix la trombofília com una entitat multifactorial i multigènica, de manera que existeixen una sèrie d'anormalitats genètiques i adquirides que provoquen i augmenten el risc de trombosi i la presència de més d'una d'aquestes anormalitats incrementa aquest risc (2,3).

La síndrome antifosfolipídica, la malaltia de Behçet i l'arteritis de cèl·lules gegants són tres entitats englobades en el grup de malalties autoimmunes sistèmiques. Malgrat que es caracteritzen per la possible aparició de fenòmens trombòtics el mecanisme patogènic en cadascuna d'elles és diferent.

L'objectiu d'aquesta tesi és l'estudi dels mecanismes patogènics que originen les manifestacions trombòtiques dels pacients amb síndrome antifosfolipídica, malaltia de Behçet i arteritis de cèl·lules gegants. Concretament, s'analitza el paper que els factors de risc trombòtic, en especial els que afecten la fibrinolisi plasmàtica, juguen en el desenvolupament d'aquests fenòmens trombòtics. Per l'estudi s'han escollit precisament aquestes tres entitats ja que, com hem esmentat abans, en cadascuna d'elles predomina un fenomen patogènic diferent. Així, a la síndrome antifosfolipídica predomina la trombosi i l'oclusió vascular, mentre que el substrat patogènic de les altres dues entitats és una vasculitis. Aquest terme defineix un procés clínic-patològic caracteritzat per inflamació, necrosi i secundàriament trombosi de la paret vascular que provoca oclusió i isquèmia dels teixits irrigats pels vasos afectats (4). Quant a les síndromes vasculítiques, es poden diferenciar diverses entitats segons predomini el fenomen inflamatori o el trombòtic. A l'arteritis de Horton o arteritis de la temporal, la inflamació de la paret vascular és la dada anatomo-patològica més rellevant i la isquèmia es presenta de forma secundària degut a la hiperplàsia de l'íntima, mentre que la malaltia de Behçet és la vasculitis en la qual, a més dels fenòmens inflamatoris, les

trombosis són més freqüents. En aquesta entitat, a més de la infiltració dels *vasa vasorum* per neutròfils, s'ha detectat la presència de trombes.

Malgrat que existeix una clara associació epidemiològica entre els anticossos antifosfolipídics i les trombosis, el mecanisme pel qual es relacionen no es coneix amb exactitud (5). En aquest sentit, se n'han proposat diversos entre els quals destaquen l'acció sobre components cel·lulars relacionats amb l'hemostàsia, bàsicament sobre la producció plaquetària de tromboxà (6) i prostaciclina (7) o activant directament les plaquetes incrementant la seva capacitat d'agregació (8). Els anticossos antifosfolipídics també poden actuar sobre els monòcits i les cèl·lules endotelials augmentant l'expressió de factor tisular (9) o de les molècules d'adhesió (10). S'han descrit, per altra banda, alteracions en la via de la proteïna C (11) i de la proteïna S (12), així com inhibició de l'antitrombina (13) o de l'acció anticoagulant de la β_2-glucoproteïna I (β_2GPI) (14). Finalment també s'han relacionat amb una inhibició de la fibrinolisi ja que augmenten els nivells d'inhibidor tipus-1 de l'activador tisular del plasminogen (PAI-1) (15). Pel què fa a la síndrome antifosfolipídica, les nostres investigacions s'han centrat en el paper que alguns dels factors genètics de risc trombòtic, en especial relacionats amb les alteracions de la fibrinolisi, poden jugar com coadjuvants en el desenvolupament de les trombosis. Concretament, ens hem interessat en el polimorfisme 4G/5G del gen del PAI-1 i la seva relació amb les trombosis arterials i venoses. Per altra part, en el camp de la síndrome antifosfolipídica, no només hem analitzat les característiques dels pacients sinó també la dels anticossos antifosfolipídics i la relació amb el seu potencial trombòtic. En aquest sentit, hem avaluat el paper que la β_2GPI juga en la interacció plaquetar amb el subendoteli promoguda pels anticossos anticardiolipina.

Actualment es coneixen alguns dels mecanismes pels que els processos inflamatoris poden estimular la coagulació plasmàtica. Mediadors inflamatoris com l'endotoxina o el factor de necrosi tumoral incrementen *in vitro* l'expressió de factor tisular en monòcits

i, possiblement en les cèl·lules endotelials (16). Al mateix temps, els mediadors inflamatoris poden augmentar els nivells del PAI-1, inhibint la fibrinolisi plasmàtica i afavorint l'aparició de fenòmens trombòtics (17). És a dir, existeixen evidències indirectes dels nexes d'unió entre inflamació (vasculitis) i coagulació i fibrinolisi plasmàtica (fenòmens trombòtics). Tot i així, aquestes troballes realitzades al laboratori no sempre tenen la seva expressió a la pràctica clínica. Això suggereix el fet que, malgrat que el procés inflamatori pot interaccionar amb els sistemes de la coagulació i fibrinolisi, requereix d'altres factors afegits per tal que es pugui desenvolupar la trombosi. Aquests poden ser els factors genètics i adquirits de risc trombòtic, alguns dels quals han estat també objecte del nostre estudi en el camp de les vasculitis.

Com hem comentat anteriorment, la malaltia de Behçet és la vasculitis que s'associa amb major freqüència a l'aparició de fenòmens trombòtics (18). Existeixen nombrosos treballs que han intentat relacionar les trombosis amb alteracions de la coagulació (19,20) i de la fibrinolisi (21,22), per bé que el mecanisme final de producció d'aquestes trombosis és, actualment, desconegut. En aquest cas i a banda d'analitzar el paper que els principals factors genètics i adquirits de risc trombòtic poden tenir en l'aparició de les trombosis d'aquests pacients, també ens hem interessat per l'estat d'activació de la coagulació i la fibrinolisi, a més de valorar el grau de lesió endotelial existent, intentant establir en aquests pacients, un nexe d'unió entre aquests paràmetres i les trombosis.

Finalment, a l'arteritis de Horton, la presentació clínica dels pacients amb predomini de fenòmens isquèmics (afectació ocular i claudicació mandibular) o dels inflamatoris (febre, síndrome tòxica o elevació dels reactants de fase aguda) s'ha relacionat amb un determinat perfil de citocines (23), mentre que el paper dels factors genètics i adquirits de risc trombòtic i la fibrinolisi plasmàtica en el quadre clínic d'aquests pacients, tot just ha estat analitzat.

A la primera part de la tesi es realitza una revisió bibliogràfica en la què es repassen els sistemes de l'hemostàsia primària, de la coagulació i de la fibrinolisi plasmàtica, i els factors de risc trombòtic. A continuació, es revisa l'estat actual del coneixement dels principals factors genètics i adquirits de risc trombòtic i l'estat de la fibrinolisi plasmàtica en cadascuna de les tres entitats estudiades, s'exposen la hipòtesi de treball i els objectius plantejats. Finalment, es presenten els articles publicats amb un breu comentari de cadascun d'ells i s'exposen les conclusions globals que d'ells es desprenen.

4.A. HEMOSTÀSIA PRIMÀRIA

El sistema hemostàtic, constituït per components cel·lulars i proteïnes plasmàtiques solubles, manté la sang en estat fluid (24). En resposta a una lesió endotelial, les plaquetes s'adhereixen al subendoteli exposat, s'activen, agreguen altres plaquetes circulants i proporcionen la superfície fosfolipídica adequada per a la unió de diferents compostos enzimàtics de la cascada de la coagulació que generen gran quantitat de trombina en aquesta zona.

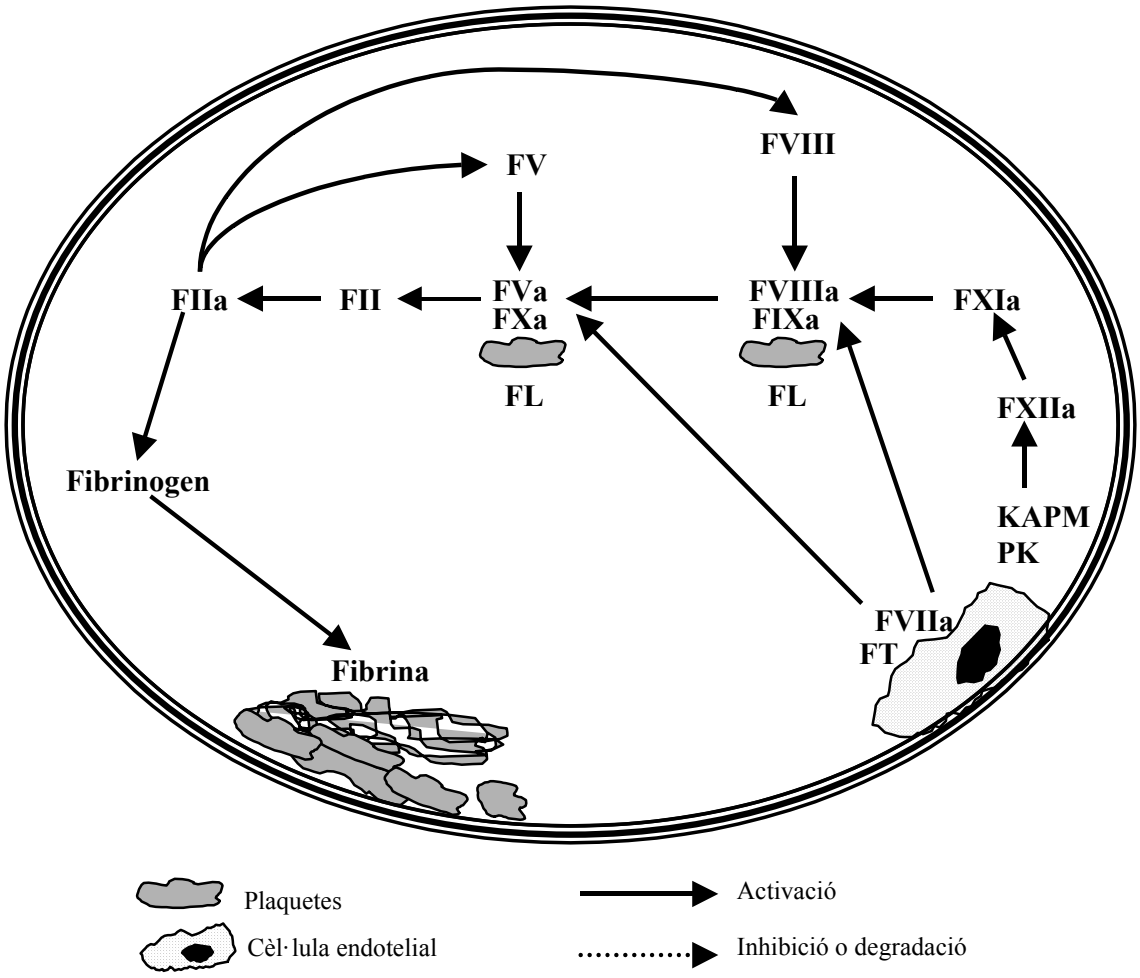
4.B. COAGULACIÓ PLASMÀTICA

La coagulació sanguínia és el conjunt de reaccions que donen lloc en el punt de lesió vascular a la trombina, enzim clau en aquest procés (25) (Figura 1). Aquest sistema s'inicia en la superfície de les cèl·lules endotelials a través de l'exposició del factor tisular al torrent sanguini. Aquest s'uneix al factor VII activat, i el complex enzimàtic resultant activa els factors IX i X (26). Els factors VII, IX i X activats intervenen en un sistema de retroalimentació positiva que activa el factor VII unit al factor tisular. Per altre part, el factor IX activat per la via del factor tisular, activa el factor X, en una reacció que és accelerada per un cofactor, el factor VIII. L'exposició del factor tisular desencadena la coagulació per l'anomenada via extrínseca, i és el mecanisme pel qual s'inicia *in vivo* la coagulació en resposta a la lesió vascular.

Una altre via de la coagulació és la via intrínseca formada pel factor XII, el quininogen d'alt pes molecular, la prekalicreïna i el factor XI. El paper fisio-patològic d'aquesta via no està completament aclarit ja que no intervé en la coagulació a partir de la lesió vascular i, a més, dèficits congènits de les proteïnes d'aquest sistema no provoquen problemes hemorràgics excepte la deficiència del factor XI. El resultat final d'aquestes dues vies és l'activació del factor X. El factor X activat forma amb el seu cofactor, el

factor V, el complex protrombinasa que converteix el factor II en factor II activat (trombina).

Figura 1: Esquema de les vies intrínseca i extrínseca de la coagulació plasmàtica



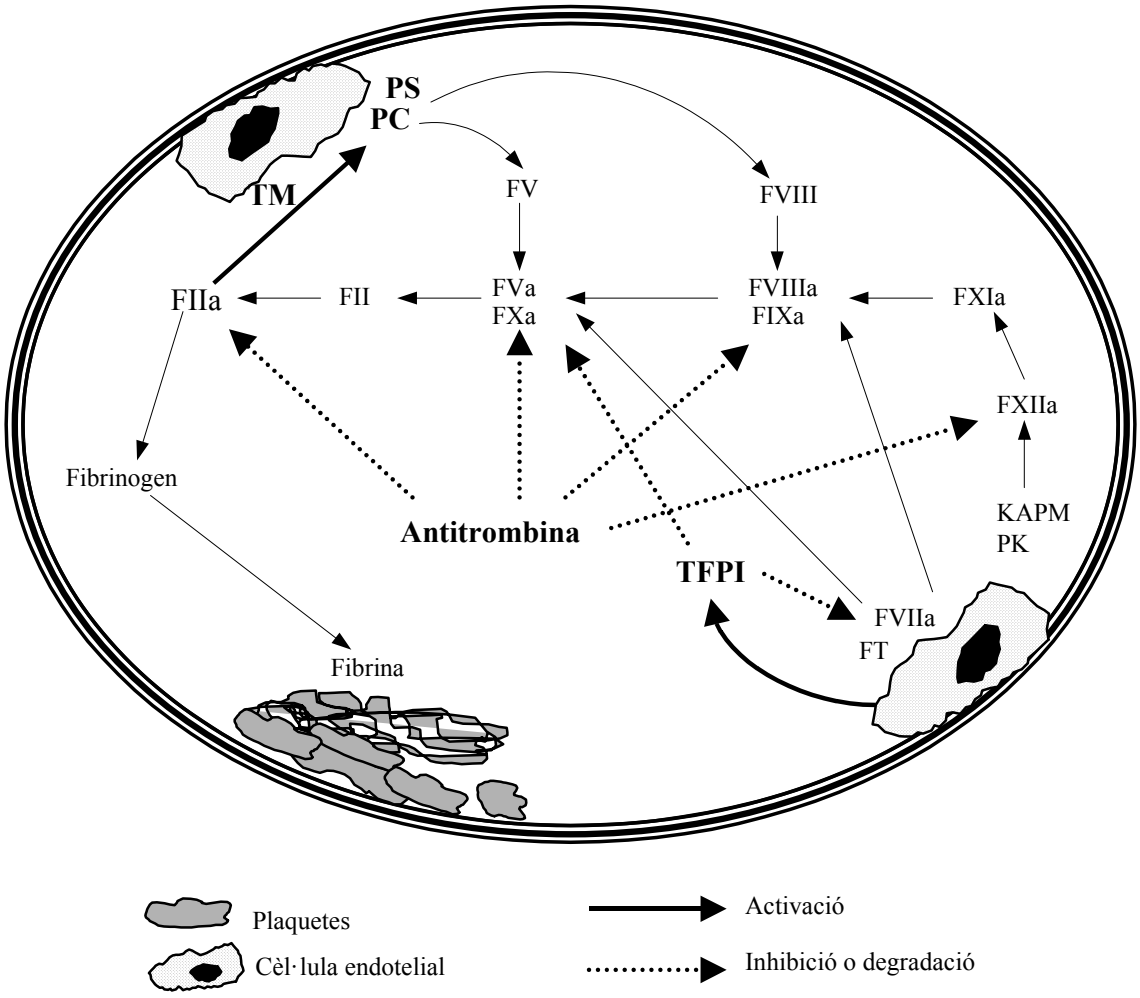
FT: factor tisular; **KAPM:** kininogen d'alt pes molecular; **PK:** prekalicreïna; **FL:** fosfolípids.
Els diferents factors de la coagulació estan representats per una F majúscula i el nombre corresponent. En cas d'estar activats s'hi ha afegit una a minúscula.

L'activació del factor V es produeix pel mateix factor X activat. A l'etapa final de la coagulació, la trombina trenca el fibrinogen per generar monòmers de fibrina que es polimeritzen, unint-se els uns als altres per formar un coàgul químicament estable. La trombina també intervé en un sistema de retrolimentació positiva activant els factors VIII i IX. El factor VIII circula unit al factor von Willebrand (27) i, un cop activat, se separa d'aquest i forma, en la superfície de les plaquetes, amb el factor IX activat un

complex que activa al factor X. L'activació del factor XI per la trombina genera un altre sistema de retroalimentació que dóna lloc a la formació de factor IX activat, que activa al mateix temps al factor X (28).

La cascada de la coagulació sanguínia té la capacitat d'amplificar un petit estímul inicial per poder formar un coàgul de fibrina. En qualsevol cas, la naturalesa explosiva d'aquest sistema no pot existir sense mecanismes de regulació (o anticoagulants naturals) que evitin una coagulació massiva (Figura 2).

Figura 2: Esquema dels sistemes anticoagulants naturals de la coagulació plasmàtica



TM: trombomodulina; **PC:** proteïna C; **PS:** proteïna S; **TFPI:** tissue factor pathway inhibitor; **FT:** factor tisular; **KAPM:** kininogen d'alt pes molecular; **PK:** prekalicreïna; **FL:** fosfolípids.
Els diferents factors de la coagulació estan representats per una F majúscula i el nombre corresponent. En cas d'estar activats s'hi ha afegit una a minúscula.

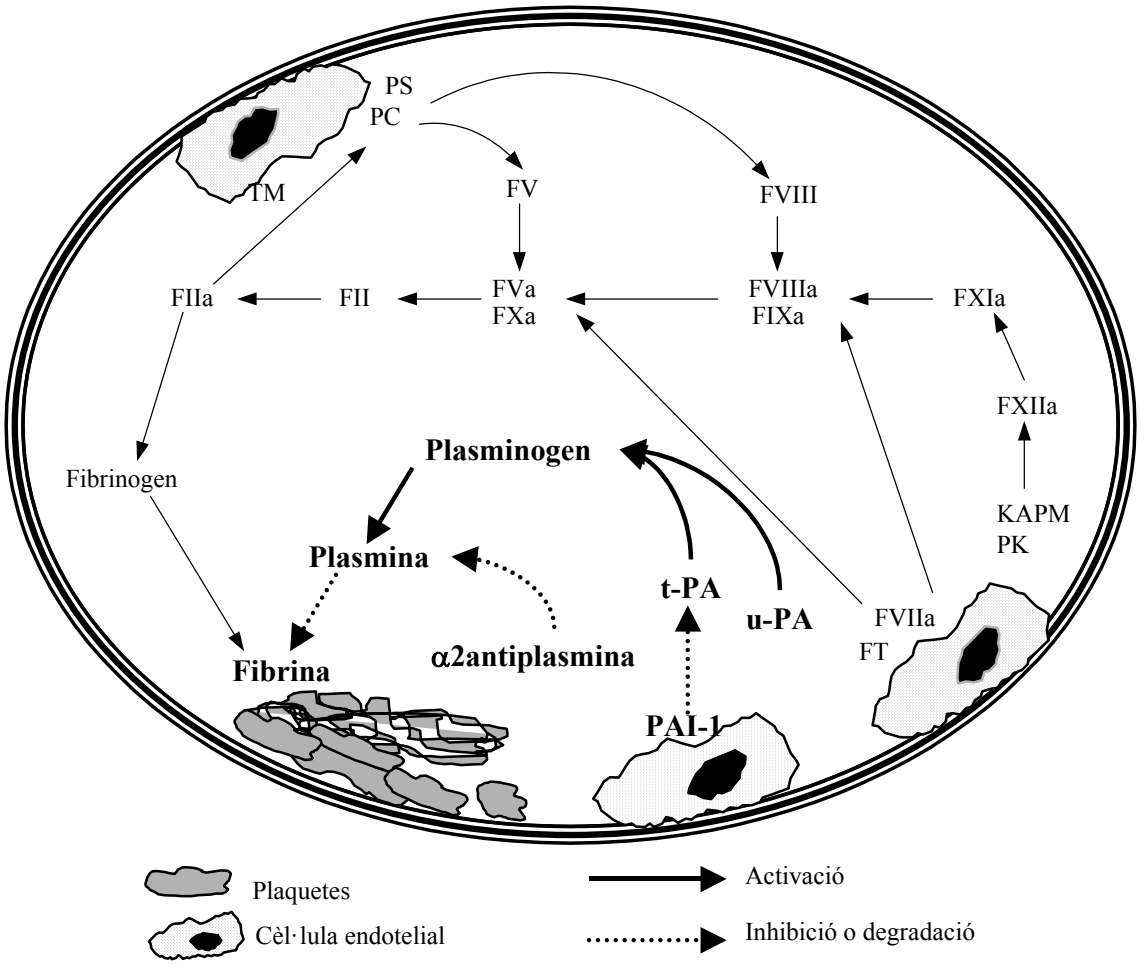
Així, el *tissue factor pathway inhibitor* (TFPI) és una proteïna plasmàtica associada a una lipoproteïna que forma un complex quaternari amb el factor tisular i inhibeix els

factors VII i X, inhibint per tant, la via extrínseca de la coagulació (29). Molts dels enzims generats durant l'activació de la coagulació són inhibits per l'antitrombina. Es tracta d'una proteasa serínica que inhibeix l'activitat dels enzims de la via intrínseca i comú de la coagulació. La trombina, en presència de trombomodulina unida a les cèl·lules endotelials, activa la proteïna C que, a la vegada, inactiva els factors V activat i VIII activat (30). Com altres reaccions de l'hemostàsia, aquesta és accelerada per un cofactor, en aquest cas, la proteïna S. Finalment, diferents reaccions del sistema de la fibrinolisi culminen amb la formació de la plasmina, proteasa serínica que elimina el coàgul de fibrina del vas. Per tant, el correcte funcionament del sistema hemostàtic depèn de l'adequat balanç entre reaccions procoagulants per una banda i anticoagulants i fibrinolítiques per l'altra. Qualsevol anomalia que afecti el sistema pot alterar aquest equilibri i ocasionar estats de risc trombòtic.

4.C. FIBRINOLISI PLASMÀTICA

El sistema de la fibrinolisi és una cascada enzimàtica que consta d'una sèrie d'activadors i inhibidors que regulen la conversió del plasminogen en plasmina. La generació de plasmina lliure a la superfície del trombe condueix a la lisi de la fibrina, donant lloc als productes de degradació de la fibrina (Figura 3). La regulació del sistema de la fibrinolisi està mediada per interaccions moleculars específiques entre els seus principals components, i per la síntesi i posterior alliberació a partir de les cèl·lules endotelials dels activadors del plasminogen i dels seus inhibidors. Per tant, un increment de l'activitat del sistema de la fibrinolisi afavoreix l'aparició de trastorns hemorràgics, mentre que el defecte de l'activitat fibrinolítica pot predisposar a la trombosi (31,32).

Figura 3: Esquema de la fibrinolisi plasmàtica



PAI-1: inhibidor tipus-1 de l'activador tisular del plasminogen; **t-PA:** activador tisular del plasminogen; **u-PA:** activador del plasminogen tipus uroquinasa; **TM:** trombomodulina; **PC:** proteïna C; **PS:** proteïna S; **TFPI:** tissue factor pathway inhibitor; **FT:** factor tisular; **KAPM:** kininogen d'alt pes molecular; **PK:** prekalicreïna; **FL:** fosfolípids.
Els diferents factors de la coagulació estan representats per una F majúscula i el nombre corresponent. En cas d'estar activats s'hi ha afegit una a minúscula.

4.C.1 Components del Sistema de la Fibrinolisi Plasmàtica

Els enzims del sistema de la fibrinolisi són proteases del tipus serina, és a dir, el seu *locus* actiu està format pels aminoàcids serina, àcid aspàrtic i histidina, donant lloc a l'anomenada regió catalítica. Aquest *locus* actiu es localitza a la regió carboxi-terminal de les molècules, mentre que les regions amino-terminal contenen un o més dominis estructurals i funcionals, com els dominis *finger* (per analogia amb els *finger* de la fibronectina), el domini *epidermal growth factor* (EFG) i els dominis *kringle*. Els inhibidors del sistema de la fibrinolisi són membres de la superfamília de les serpines (inhibidors de la serina). En aquest cas, a l'extrem carboxi-terminal posseeixen un

pèptid reactiu específic (Arginina-X o Lisina-X) que està unit al seu enzim. Això dóna lloc a una estructura inactiva amb una funció d'inhibició enzimàtica. Les característiques químiques i genètiques dels principals components del sistema de la fibrinolisi queden resumides a la Taula 1.

Taula 1: Característiques químiques i genètiques dels components de la fibrinolisi plasmàtica.

	PM (kD)	**Nombre d' aminoàcids**	**Regió catalítica o *locus* reactiu**	**Cromosoma**
Plasminogen	92	791	-	6
t-PA	68	530	$His^{322}Asp^{371}Ser^{478}$	8
u-PA	54	411	$His^{204}Asp^{255}Ser^{356}$	10
α_2-antiplasmina	70	464	$Arg^{364}Met^{365}$	18
PAI-1	52	379	$Arg^{364}Met^{347}$	7
PAI-2	47,60	393	$Arg^{358}Thr^{359}$	18
u-PAR	50-60	313	-	19

PM: pes mol·lecular (expresat en kilodaltons); **t-PA:** activador tisular del plasminogen; **u-PA:** activador del plasminogen tipus uroquinasa; **PAI-1:** inhibidor tipus-1 de l'activador del plasminogen; **PAI-2:** inhibidor tipus-2 de l'activador del plasminogen; **u-PAR:** receptor de l'activador del plasminogen tipus uroquinasa.

4.C.1.a. *Plasminogen*

El plasminogen humà és una glicoproteïna de cadena simple de 92 kD formada per 791 aminoàcids i 24 ponts disulfur (33). La molècula s'organitza en set dominis estructurals, de manera que els dominis *kringle* contenen lisina en els punts específics que controlen la unió del plasminogen a la fibrina i la interacció de la plasmina amb l'α_2-antiplasmina (34). El plasminogen es converteix en plasmina al trencar-se la unió Arg^{561}-Val^{562}. El gen del plasminogen està localitzat al braç llarg del cromosoma 6, concretament a les bandes q26 o q27 (35).

4.C.1.b. *Activador tisular del plasminogen*

L'activador tisular del plasminogen (t-PA) és una proteasa serínica de 70 kD, formada per 527 aminoàcids (36). La molècula està organitzada en diversos dominis que controlen diferents funcions de l'enzim. Per altre part, el t-PA és degradat per la plasmina, per hidròlisi del pont Arg^{275}-Ile^{276}, a una estructura inactiva de dues cadenes. El gen del t-PA es localitza en el cromosoma 8 (bandes p12-q11.2) (37).

4.C.1.c. ***Activador del plasminogen tipus uroquinasa***

El scu-PA o prouroquinasa és una glicoproteïna de 54 kD que conté 411 aminoàcids (38). Com en altres serinproteases, la triada catalítica es localitza a l'extrem carboxi-terminal. El domini EFG és el responsable de la unió del scu-PA al seu receptor, present a la superfície de varis tipus cel·lulars. La scu-PA es transforma en tcu-PA pel trencament de la unió Lis^{158}-Ile^{159}. El gen de l'activador del plasminogen tipus uroquinasa (u-PA) es localitza al cromosoma 10 (36).

4.C.1.d. ***Receptor de l'activador del plasminogen tipus uroquinasa***

El receptor del u-PA (u-PAR) és una glucoproteïna de 50-60 kD formada per 313 aminoàcids que s'uneix a la u-PA mitjançant els dominis EFG d'aquesta. Està format per tres dominis estructurals homòlegs, essent el fragment aminoterminal el que s'uneix a la u-PA (39). La unió de scu-PA al u-PAR sembla que és crucial per l'activació de la u-PA. El gen del u-PAR es localitza en el cromosoma 19, bandes q13.1-q13.2 (40).

4.C.1.e. ***α-2-antiplasmina***

L'α_2-antiplasmina és una glicoproteïna de 70 kD formada per 464 aminoàcids (41). Posseeix una característica única entre les serpines ja que el seu extrem carboxi-terminal conté un *locus* secundari d'unió que reacciona amb els llocs d'unió de lisina

dels dominis *kringle* 1-3 del plasminogen i de la plasmina (42). El gen de l'α_2-antiplasmina està localitzat al cromosoma 18, bandes p11.1-q11.2 (43).

4.C.1.f. *Inhibidor de l'activador del plasminogen*

Els dos principals inhibidors de l'activador del plasminogen (PAI) són el PAI-1 i el PAI-2. El PAI-1 és una glicoproteïna de cadena única de 52 kD formada per 379 aminoàcids (44). La molècula del PAI-1 s'estabilitza mitjançant la seva unió amb la proteïna S o amb la vitronectina. El gen del PAI-1 es localitza al cromosoma 7, bandes q21.3-q22 (45). El PAI-2 és una serpina de 393 aminoàcids de la què existeixen dues formes diferents amb propietats cinètiques similars; una forma intracel·lular no glicosilada de 47 kD i una forma glicosilada de 60 kD (46). La funció del PAI-2 intracel·lular és desconeguda ja que el seu enzim d'unió, la u-PA, és extracel·lular. Es creu que es podria comportar com un *pool* del qual PAI-2 es podria secretar en cas de lesió cel·lular. El gen de PAI-2 es localitza en el cromosoma 18, bandes q21-q23 (47).

4.C.1.g. *Inhibidor de la fibrinolisi activable per la trombina*

Recentment s'ha caracteritzat el *thrombin activatable fibrinolysis inhibitor* (TAFI). Es tracta d'una proteïna plasmàtica de cadena única de 60 kD sintetitzada a nivell hepàtic en forma de procarboxipeptidasa U. Estudis recents han demostrat que el complex thrombina-trombomodulina, per proteòlisi a la posició Arg^{92}, dóna lloc a la seva forma activa o procarboxipeptidasa B (48). Aquest complex és, probablement, l'activador fisiològic del TAFI (49). Una vegada activat, el TAFI inhibeix la fibrinolisi ja que actua sobre l'activació del plasminogen mediada per la fibrina (50). El gen del TAFI es localitza al cromosoma 13, bandes q14.11.

4.C.2. Activació del Plasminogen a Plasmina

Tots els activadors del plasminogen el converteixen a plasmina mitjançant el trencament de la unió Arg^{561}-Val^{562}. La molècula de plasmina que en resulta és de doble cadena i està constituïda per una cadena pesada que conté els cinc dominis *kringle* (extrem amino-terminal del plasminogen) i una cadena lleugera (extrem carboxi-terminal) que conté la regió catalítica, formada per His^{603}, Asp^{646} y Ser^{741} (33).

4.C.3. Inhibició de la Plasmina per l'α_2-Antiplasmina

L'α_2-antiplasmina forma juntament amb la plasmina un complex 1:1 inactiu. Aquesta inhibició es porta a terme mitjançant dues reaccions consecutives: la primera, ràpida, dóna lloc a un complex inactiu reversible i és seguida per una segona reacció, més lenta, de la qual en resulta un complex inactiu irreversible.

4.C.4. Mecanisme d'Acció de l'Activador tisular del Plasminogen

4.C.4.a. *En presència de fibrina*

El t-PA és un enzim amb poca activitat en absència de fibrina. En canvi, en presència d'aquesta augmenta de manera important el grau d'activació sobre el plasminogen. La formació dels monòmers de fibrina mediada a partir de la trombina i la seva posterior polimerització són essencials per a l'estimulació del plasminogen a partir del t-PA (51). Existeixen dades que suggereixen que la fibrina actua com la superfície en la què el t-PA i el plasminogen formarien un complex ternari. La formació d'aquest complex dóna lloc a un augment de l'afinitat del t-PA pel plasminogen (52).

4.C.4.b. *A la superfície cel·lular*

Algunes cèl·lules poden unir els activadors del plasminogen i el mateix plasminogen a la seva superfície, donant lloc a un augment de l'activació d'aquest (53) i, a la vegada, protegeix a la plasmina unida de la seva inhibició per l'α_2-antiplasmina (54). Alguns

estudis han demostrat que l'endoteli regula la fibrinolisi pericel·lular al modular l'expressió dels receptors del plasminogen (55). Una proteïna de membrana de 40 kD s'ha proposat com el receptor funcional del t-PA (56), de manera que el t-PA unit a la superfície cel·lular manté la seva activitat enzimàtica i a més queda protegit de la inhibició del PAI-1. Per tant, la unió del plasminogen i dels seus activadors a l'endoteli afavoreix la generació de plasmina i pot jugar un paper important en el manteniment del flux sanguini. Aquests receptors cel·lulars també poden intervenir en l'eliminació ràpida del t-PA del torrent circulatori. Les cèl·lules endotelials hepàtiques posseeixen uns receptors que reconeixen els domini *kringle* i les cèl·lules del parènquima hepàtic contenen un receptor calci-dependent capaç d'interaccionar amb els dominis *finger* i/o EFG del t-PA (57). Els hepatòcits contenen a més receptors d'alta afinitat per la unió i degradació dels complexos t-PA/PAI essent també capaços d'unir-se encara que amb menor afinitat al t-PA circulant (58).

4.C.5. Mecanisme d'Acció de l'Activador del Plasminogen Tipus Uroquinasa

4.C.5.a. *En presència de fibrina*

Al plasma i en absència de fibrina, scu-PA és estable i no activa el plasminogen. En presència del coàgul de fibrina i a diferència de tcu-PA, scu-PA indueix la lisi específica d'aquest coàgul (59). A més, l'α_2-antiplasmina impedeix la conversió de scu-PA a tcu-PA fora del coàgul i manté la seva especificitat per la fibrina. El fragment E-2 de la fibrina estimula específicament l'activació del plasminogen per la scu-PA, essent, per tant, un activador deficient del plasminogen quan aquest es presenta unit als residus de lisina de la fibrina intacta, però presenta una alta activitat pel plasminogen unit a residus de lisina de l'extrem carboxi-terminal formats a partir de la fibrina parcialment degradada (60).

4.C.5.b. *A la superfície cel·lular*

La unió de scu-PA a u-PAR juga un paper crucial en la seva activació en condicions fisiològiques. Com s'ha mencionat anteriorment, aquesta unió provoca un augment en la generació de plasmina degut per una part a l'activació del plasminogen (61) i per altra part, a l'activació per un mecanisme de retroalimentació de scu-PA a tcu-PA per la plasmina generada (62). La plasmina unida a la cèl·lula queda protegida de la seva degradació per la α_2-antiplasmina i a més, afavoreix l'activació de la scu-PA unida al seu receptor (61). Aquest sistema es pot inhibir eficaçment pel PAI-1 i PAI-2. Malgrat que s'ha suggerit que l'activació del plasminogen es produiria a partir de la formació d'un complex que dependria de u-PAR, els mecanismes de funcionament d'aquest receptor són, ara per ara, desconeguts.

4.C.6. Inhibició dels Activadors del Plasminogen

En la inhibició del t-PA humà s'han involucrat nombrosos mecanismes. PAI-1 és un inhibidor ràpid del t-PA que en condicions normals es troba al plasma a baixes concentracions. A més, el t-PA s'inhibeix de forma més lenta per la α_2-antiplasmina, α_1-antitripsina i el C1-inhibidor (63). Amb tot, la principal via d'eliminació del t-PA del torrent circulatori és l'hepàtica.

Al plasma humà, tcu-PA és inhibit lentament per diverses proteases com α_2-macroglobulina, α_1-antitripsina, antitrombina, α_2-antiplasmina i el PAI-3, que correspon a l'inhibidor de la proteïna C activada, encara que PAI-1 i PAI-2 són responsables de la seva inhibició de forma més ràpida i específica. A diferència de tcu-PA, scu-PA no s'inhibeix per proteases plasmàtiques si no que el principal mecanisme d'eliminació és la via hepàtica (64). El PAI-1 reacciona amb el t-PA de cadena simple i de doble cadena i amb tcu-PA però no amb scu-PA (65). Les regions de la molècula de t-PA i de u-PA amb càrrega positiva són les responsables d'aquesta ràpida reacció. El PAI-1 s'elimina del torrent circulatori mitjançant la via hepàtica (66). Per la seva part,

el PAI-2 inhibeix més lentament el tcu-PA que el PAI-1, actua de forma eficaç sobre el t-PA de doble cadena, de forma menys eficaç sobre el t-PA de cadena simple i no inhibeix el scu-PA (65).

4.C.7. Inhibició de l'Activació del Plasminogen Mediada per la Fibrina

La inhibició de l'activació del plasminogen mediada per la fibrina es produeix per l'acció del TAFI. El TAFI activat actua com un potent inhibidor de la fibrinolisi ja que elimina els residus lisina carboxi-terminals de la fibrina parcialment degradada per la plasmina, eliminant els llocs d'unió del plasminogen i inhibint, per tant, l'activació del plasminogen per part del t-PA (67).

4.D. FACTORS CAUSANTS DE TROMBOFÍLIA

Els factors que predisposen a la trombosi venosa són diferents dels de la trombosi arterial (factors aterogènics com el tabac, la hipertensió arterial, o la dislipèmia que no incrementen el risc de trombosi venosa). Clàssicament, Virchow postulà tres causes principals de trombosi: l'estasi sanguínia, els canvis a la paret vascular i els canvis en la composició de la sang (68). Els factors de risc coneguts per la trombosi venosa pertanyen al primer grup (estasi venosa) i al tercer (canvis en la composició).

Actualment i degut a l'ampli desenvolupament de les tècniques de biologia molecular els factors causants de trombofília es poden dividir en genètics i adquirits (69). Els factors causants de trombofília de caire adquirit inclouen l'edat, la immobilització, la cirurgia, el traumatisme, l'embaràs i el puerperi, les neoplàsies, els anticonceptius orals i la teràpia hormonal substitutiva i els anticossos antifosfolipídics (70-72). Per altra part, existeixen unes anomalies protrombòtiques ben establertes, la majoria de les quals són congènites i hereditàries, que conformen els factors causants de trombofília de caire genètic. La prevalença estimada d'aquestes alteracions genètiques a la població general

varia del 0,02 al 5%, mentre que una o més d'aquestes anomalies trombofíliques pot trobar-se en un 40-60% dels pacients amb un primer episodi de trombosi venosa (73)(Taula 2). En canvi, la prevalença d'aquests factors en pacients amb trombosi és molt variable i va des del 20% del factor V Leiden fins el 1% del dèficit d'antitrombina (73).

Taula 2: Prevalença estimada de factors genètics causants de trombofília en pacients amb trombosi venosa profunda i en la població general:

	Pacients amb trombosi venosa (%)	**Població general (%)**
Dèficit d'antitrombina	1	0,02
Dèficit de proteïna C	3	0,2-0,4
Dèficit de proteïna S	1-2	Desconegut
Factor V Leiden	20	5
Protrombina G20210A	6	2

Adaptat de: Rosendaal FR. Venous thrombosis: a multicausal disease. Lancet 1999;353:1167--1173.

Actualment s'estan avaluant nous polimorfismes dels gens dels factors de la coagulació (II, VII, VIII, XII, XIII), de les proteïnes C i S i del t-PA i de la u-PA (74). Entre ells, un dels que sembla més prometedor és el que fa referència al polimorfisme 4G/5G del gen del PAI-1.

4.D.1. Dèficit d'Antitrombina

Es transmet de forma autosòmica dominant i la seva prevalença en donants de sang s'ha estimat en un 0,02% (75) mentre que en pacients amb trombosi no seleccionats varia del 0,5% al 1% (76,77). Característicament, els pacients afectats desenvolupen trombosis abans dels 25 anys (78,79). Menys de l'1% de tots els fenòmens trombòtics són deguts a aquest dèficit d'antitrombina (3).

4.D.2. Dèficit de Proteïna C

La deficiència heterozigota de proteïna C s'ha associat a un augment del risc trombòtic (80,81). Es transmet de forma autosòmica dominant i la forma homozigota és incompatible amb la vida. La prevalença de trombosi en aquests pacients és del 3% (76,77,82) i la prevalença del dèficit de proteïna C a la població general és del 0.2% (83). Tot això fa que el risc relatiu de trombosi associat al dèficit de proteïna C sigui de 15 (3). Entre l'1%-2% de tots els fenòmens trombòtics es deuen a aquest dèficit de proteïna C (3).

4.D.3. Dèficit de Proteïna S

Des de 1984 s'han descrit famílies amb aquest dèficit (84). La seva herència és autosòmica dominant i la seva freqüència és similar al dèficit de proteïna C. El mateix defecte genotípic pot donar lloc a variables fenotípiques com el tipus I (proteïna S total i proteïna S lliure baixes) o el tipus III (proteïna S lliure baixa amb proteïna S total normal) (85,86). Si a això hi unim l'efecte de les variables adquirides (sobretot l'edat), l'espectre clínic de trombosi en aquest dèficit és molt ample (86). Només l'1% de tots els fenòmens trombòtics són deguts a aquest dèficit (3).

4.D.4. Resistència a la Proteïna C Activada i Factor V Leiden

El 1993, Dahlbäck i cols (87) van descriure tres pacients amb trombosi que eren portadors d'un nou defecte a la via anticoagulant de la proteïna C en forma d'una disminució de l'activitat anticoagulant del plasma enfront de la proteïna C activada o resistència a la proteïna C activada. Un any més tard, Bertina y cols (88) van descobrir que el 80% de tots els individus amb resistència a la proteïna C activada eren portadors d'una mutació a l'exó 10 del gen del factor V, constituint l'anomenat factor V Leiden.

La mutació consisteix en un canvi d'una guanina per una adenina a la posició 1691 del gen que codifica el factor V (88), de forma que a la població caucàsica constitueix la mutació genètica que dóna lloc a trombosis amb més freqüència (89-91). La resistència a la proteïna C activada es presenta en el 20% dels pacients amb trombosi (92) i és, per tant, l'anormalitat genètica causant de trombofília més freqüent. La seva prevalença a la població sana d'origen caucàsic varia entre el 2% i el 15% (3). La presència de la mutació està associada a un augment del risc de trombosi de 3 a 8 vegades a la forma heterozigota i d'almenys 80 vegades a la forma homozigota (90).

L'estat de resistència a la proteïna C activada també pot aparèixer en absència del factor V Leiden. En aquest cas pot ser secundària a causes d'origen genètic o adquirides. Entre les primeres destaca la recent descripció d'una altra mutació en el factor V (factor V Cambridge) (93), mentre que l'embaràs (94), els anticonceptius orals (95) o l'existència d'un anticoagulant lúpic (96) són causes adquirides de resistència a la proteïna C activada.

4.D.5. Mutació G20210A del Gen de la Protrombina

Descrita el 1996, aquesta mutació constitueix la segona causa genètica de risc trombòtic més freqüent i està present en el 6% dels pacients amb trombosis (97). La mutació no afecta a la funció de la protrombina si no que s'associa a un augment de la concentració plasmàtica d'aquesta. La seva prevalença a la població de raça blanca és del 2% (98) i s'ha descrit un augment del risc trombòtic de 2 a 5 vegades en relació amb la seva presència (99).

4.D.6. Hiperhomocisteïnèmia

Els nivells plasmàtics elevats d'homocisteïna s'associen amb un augment del risc de trombosis venoses (100,101). La hiperhomocisteïnèmia pot ser conseqüència de factors

adquirits o genètics (102), de manera que la majoria dels individus amb un augment de la mateixa no són portadors de cap variació genètica, si no que presenten un deteriorament del metabolisme de la metionina, en el què la hiperhomocisteïnèmia està produïda per un consum alimentari insuficient d'àcid fòlic i vitamines B_6 o B_{12} (103-105). Entre les variacions genètiques destaquen les mutacions de la cistationina β-sintetasa (106) o de la metilen-tetra-hidrofolat reductasa (MTHFR) (107). Un polimorfisme freqüent d'aquesta última, el C677T, s'associa amb la presència d'un enzim termolàbil i nivells elevats d'homocisteïna (107-109), encara que el seu paper com factor de risc trombòtic és controvertit (107-109).

4.D.7. Síndrome Antifosfolipídica

Constitueix un factor causant de trombofília adquirit en el què la presència d'anticossos antifosfolipídics (anticoagulant lúpic i anticossos anticardiolipina) al plasma es relaciona amb trombosis arterials, venoses i pèrdues fetals recurrents (110). Actualment se sap que en realitat aquests anticossos estan dirigits contra un complex de fosfolípid i proteïna (o cofactor), de les que s'han reconegut la β2-glucoproteïna I (111) i la protrombina (112). L'anticoagulant lúpic estaria present entre el 2% i el 14% del total de les trombosis (3).

4.D.8. Anomalies Congènites del Fibrinogen

Aproximadament un 12% de les anomenades disfibrinogenèmies presenten episodis trombòtics, de manera que s'han descrit quadres de trombosis greus, venoses o arterials, a 34 famílies mentre que unes altres 6 variants presentaven simultàniament hemorràgies i trombosis (113). Els mecanismes descrits en aquestes anomalies consisteixen en un augment de la resistència de la fibrina a la lisi per la plasmina o bé en un defecte de l'activació del plasminogen pel t-PA en presència de fibrina.

4.D.9. Anomalies Congènites del Plasminogen

El 1978 es va detectar l'existència d'una displasminogenèmia en una família japonesa amb tendència trombòtica greu (114). Des d'aleshores s'han descrit diverses anomalies moleculars del plasminogen, amb alteració a nivell del centre actiu i activació defectuosa a plasmina, associades a trombosis venoses recurrents (115). En la majoria dels casos en els què es coneix el defecte molecular, aquest consisteix en una substitució a nivell del lloc actiu de l'aminoàcid lisina en posició 600 per treonina. En altres casos es tracta de molècules de plasminogen amb menor afinitat pels seus activadors.

Com hem mencionat anteriorment, actualment s'està avaluant el possible paper protrombòtic de nous polimorfismes que afecten alguns dels factors de la coagulació (factors II, VII, VIII, XII, XIII), a alguns dels anticoagulants naturals (proteïnes C i S) i a components de la fibrinolisi (t-PA i u-PA) (74). De totes maneres, de tots aquests nous factors, farem referència només al polimorfisme 4G/5G del gen del PAI-1, un dels objectius de l'estudi de la present tesi.

4.D.10. Polimorfisme 4G/5G del Gen del PAI-1

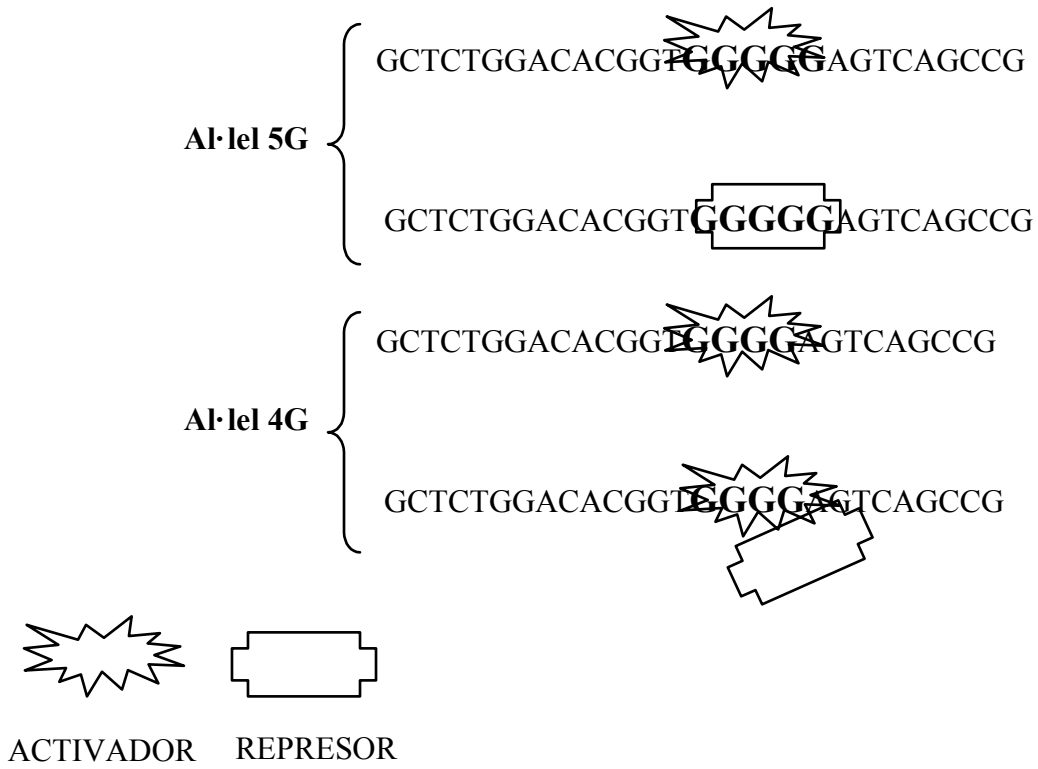
L'element més estudiat de la ruta fibrinolítica ha estat el PAI-1 degut al constatat risc coronari que s'associa a l'elevació dels seus nivells (116,117). Els darrers anys, s'han descrit variacions polimòrfiques en el gen del PAI-1 relacionades amb els seus nivells circulants.

L'expressió de PAI-1 està regulada per factors com les citocines, els glucocorticoids o la trombina (118). Aquesta regulació es produeix en la transcripció del gen, el promotor del qual respon als diferents reguladors. Inicialment es van descriure dos polimorfismes que podrien estar relacionats amb els nivells de PAI-1: una repetició dinucleòtida (CA)n en el quart intró del gen (119) i un polimorfisme en la longitud dels fragments de

restricció per l'enzim Hind III a l'extrem 3' (120), però no s'ha trobat relació d'aquests dos polimorfismes amb manifestacions clíniques vasculars o trombòtiques (120).

En canvi, recentment els nivells de PAI-1 s'han relacionat amb un nou polimorfisme de deleció/inserció (4G/5G) d'una base situada a la regió promotora del gen, -675bp abans del lloc on s'inicia la transcripció (121). La freqüència d'al·lels a la població general és de 0,47/0,53 i els homozigots 4G/4G presenten majors nivells de PAI-1 que els homozigots 5G/5G i que els heterozigots (122-124). La localització d'aquest polimorfisme a la regió promotora del gen el dota d'un especial interès ja que estudis *in vitro* han identificat precisament en aquesta regió, el punt d'unió de proteïnes reguladores de la transcripció (123,124). Així, la transcripció estaria regulada per una nucleoproteïna activadora i una nucleoproteïna repressora, que competirien pel lloc d'unió en la variant al·lèlica amb cinc guanines (5G). La deleció d'una guanina (4G) en el lloc d'unió, fa que aquest no sigui reconegut com a tal per la proteïna repressora (123) (Figura 4). En absència d'unió de la proteïna repressora (4G) la transcripció de PAI-1 augmenta. Aquest polimorfisme 4G/5G no només s'ha relacionat amb els nivells de PAI-1 circulant (122-124) sinó que també s'ha demostrat la relació entre el genotip 4G/4G i la malaltia vascular coronària en pacients joves i en diabètics tipus II (119,125-127).

Figura 4: Regulació de la transcripció del PAI-1



A l'al·lel 5G es poden unir una proteïna activadora i una repressora. En canvi, en el cas de l'al·lel 4G la proteïna repressora no reconeix el lloc d'unió i, per tant, només s'hi uneix la proteïna activadora. En absència d'aquesta proteïna repressora la transcripció del PAI-1 augmenta.

4.E. ELS FACTORS CAUSANTS DE TROMBOFÍLIA I L'ESTAT DE LA FIBRINOLISI A LA SÍNDROME ANTIFOSFOLIPÍDICA, A LA MALALTIA DE BEHÇET I A L'ARTERITIS DE HORTON

En aquest apartat, revisarem breument les dades publicades en referència als factors de risc trombòtic i a l'estat de la fibrinolisi en les tres entitats objecte d'estudi. A més a l'apartat de la síndrome antifosfolipídica revisem breument les dades existents respecte al paper patogènic de la β_2-glicoproteïna I.

4.E.1. Síndrome Antifosfolipídica

A la síndrome antifosfolipídica, l'estudi dels factors de risc trombòtic s'han avaluat en un intent d'explicar l'heterogeneïtat clínica d'aquests pacients. Deixarem de banda els articles en què es descriuen casos individuals de pacients amb fenòmens trombòtics en els que s'objectiva la coexistència dels anticossos antifosfolipídics i un altre factor protrombòtic i, en canvi, ens centrarem en les sèries publicades fins el moment. La majoria dels estudis mostren que la presència de les mutacions del factor V Leiden, G20210A del gen de la protrombina, C677T de la MTHFR i del polimorfisme 4G/5G del gen del PAI-1 no es correspon amb un risc augmentat de trombosi en aquests pacients. Els resultats més importants queden reflectits a la Taula 3.

La mutació més ben estudiada ha estat el factor V Leiden, de manera que la majoria de les sèries demostren una prevalença similar en pacients amb síndrome antifosfolipídica i controls sans. Només alguns estudis han relacionat la presència d'aquesta mutació amb un risc augmentat de trombosi. Concretament, Simantov i cols (128) l'han relacionat amb les trombosis arterials i venoses, i Montaruli i cols (129) i Ames i cols (130) amb un risc augmentat de fenòmens trombòtics. Un altre grup d'investigadors ha proposat que en pacients amb anticoagulant lúpic, el factor V Leiden podria definir un subgrup que presentaria un risc augmentat de trombosi venosa (131). En un altre estudi s'ha

objectivat que en pacients amb síndrome antifosfolipídica, les combinacions d'algunes d'aquestes mutacions trombofíliques són més freqüents (132). A l'article més recent, si bé la prevalença del factor V Leiden era més elevada en pacients amb síndrome antifosfolipídica i trombosi, no arribava a presentar una significació estadística (133).

Taula 3. Resum dels principals estudis de mutacions genètiques protrombòtiques en pacients amb anticossos antifosfolipídics.

Autor (ref)	Casos	Factor V (G1691A)	Protrombina (G20210A)	MTHFR (C677T) T/T	PAI-1 (4G/5G) 4G/4G
Davies (134)	43 SAF	8%	NR	NR	NR
Bokarewa (135)	45 SAFP	40%	NR	NR	NR
	39 T sense aaf	36%			
Dizon-Townson (136)	30 SAFP	0	NR	NR	NR
Biousse (137)	15 SAFP	7%	NR	NR	NR
Fijnheer (138)	25 SAFS	8%	NR	NR	NR
Simantov (128)	53 SAFP	8%	NR	NR	NR
Montaruli (129)	24 SAF + TV	21%	NR	NR	NR
	13 SAF + TA	0			
Bentolilla (139)	45 SAFP	NR	2%	NR	NR
	134 controls		4%		
Bertolaccini (140)	74 SAF	NR	1%	NR	NR
Ames (130)	28 SAF	14%		25%	
	70 TV sense aaf	20%	NR	18%	NR
	193 controls	4%		17%	
Ruiz-Argüelles (141)	14 SAFP	NR	0	NR	NR
Reshetniak (142)	20 SAF	10%	0	NR	NR
Galli (131) #	152 AL+	5%	11%	18%	NR
Forastiero (132)	69 SAF	3%	9%	22%	28%
	200 controls	3%	2%	13%	24%
Torresan (143)	40 SAF	0%	5%	2,5%	NR
	298 controls	0,7%	0,7%	5,4%	
Chopra (133)	69 SAF	13%	0%	NR	NR
	88 aaf sense T	5%	6%		

MTHFR: metilen-tetra-hidrofolat reductasa; **PAI-1:** Inhibidor tpius 1 de l'activador tisular del plasminogen; **SAF:** síndrome antifosfolipídica; **SAFP:** SAF primària; **SAFS:** SAF secundària; **aaf:** anticossos antifosfolipídics; **T:** trombosi; **TV:** trombosi venosa; **TA:** trombosi arterial; **AL+:** presència d'anticoagulant lúpic; **NR:** no reportat.
En aquest estudi el nombre de pacients estudiats va ser de 152, però en el cas de la mutació G20210A del gen de la protrombina va ser de 145 i en el de la C677T de la MTFHR de 83.

Pel que fa a la prevalença de la mutació G20210A del gen de la protrombina en pacients amb síndrome antifosfolipídica, alguns estudis l'han trobat similar a la de la població general (131,139,140), mentre que en altres s'ha trobat una prevalença major d'aquesta

mutació en el grup de pacients (132,143).

Per últim farem referència al polimorfisme 4G/5G del gen del PAI-1. En dos estudis molt recents s'ha avaluat la presència d'aquest polimorfisme en una població de pacients amb síndrome antifosfolipídica. En el primer d'ells (132), la freqüència del genotip homozigot per l'al·lel 4G fou similar entre els diferents grups de pacients estudiats. Per altra part, si bé els autors no donen una informació més concreta, si que comenten la tendència d'una major freqüència del genotip 4G/4G en els pacients amb síndrome antifosfolipídica amb trombosi arterial envers els que tenen trombosi venosa. El segon estudi no va trobar diferències significatives en les freqüències al·lèliques entre els pacients i el grup control (144). A més, els autors no pogueren establir cap relació entre aquest polimorfisme i les manifestacions trombòtiques (arterials i venoses) dels pacients amb síndrome antifosfolipídica.

Respecte a la fibrinolisi, la primera descripció de la seva disminució associada a trombosi i als anticossos antifosfolipídics al lupus eritematós sistèmic data de fa 20 anys (145). Des d'aleshores, nombrosos articles han confirmat aquestes troballes (146-148). En línies generals, aquesta disminució de la fibrinolisi es deu a un excés del seu principal inhibidor, el PAI-1 (15,146,149-151), si bé en alguns treballs també s'ha descrit un descens del t-PA o defectes de la seva alliberació després de l'estimulació mitjançant oclusió venosa (145,152). Per altra part, el paper etiològic dels anticossos antifosfolipídics en aquest defecte de la fibrinolisi no s'ha pogut demostrar (147,149-151,153) i a més, aquestes alteracions de la fibrinolisi també poden aparèixer en pacients amb conectivopaties sense història de trombosi (151,154). En contraposició a aquestes dades, en altres articles, la fibrinolisi en pacients amb anticossos antifosfolipídics s'ha trobat normal o fins i tot augmentada (155-158).

Com hem comentat a la introducció, els nostres estudis a la síndrome antifosfolipídica s'han centrat, per una part, en les característiques dels pacients pel què fa als factors de risc trombòtic amb especial èmfasi en el polimorfisme 4G/5G del gen del PAI-1 i, per una altra part, en l'avaluació de les característiques dels anticossos antifosfolipídics per tal d'establir el seu potencial patogènic. Això ho hem portat a terme analitzant la contribució de la β_2GPI en el desenvolupament dels fenòmens trombòtics a la síndrome antifosfolipídica.

L'any 1990 es descobrí que els anticossos antifosfolipídics no van dirigits directament contra els fosfolípids de membrana, sinó contra un complex de fosfolípid-proteïna (també anomenada cofactor)(111). D'aquests cofactors, el més ben identificat i estudiat és la β_2GPI. Avui se sap que els anticossos anticardiolipina "patogènics" de la síndrome antifosfolipídica s'uneixen a aquest complex format per la β_2GPI i els fosfolípids aniònics de membrana, mentre que els anticossos anticardiolipina "no patogènics" que apareixen en altres entitats (infeccions o neoplàsies) reconeixen la cardiolipina independentment de la β_2GPI. Per tant, es considera que la principal diana antigènica dels anticossos anticardiolipina "patogènics" seria la mateixa β_2GPI. A més s'ha correlacionat la presència dels anticossos anti-β_2GPI (tant d'isotip IgG, IgM com inclús IgA) amb l'aparició de trombosis en els pacients amb síndrome antifosfolipídica (159,160).

La β_2GPI s'ha involucrat en els diferents mecanismes patogènics que s'han plantejat en relació als anticossos antifosfolipídics. Així, ha estat descrit com el cofactor que s'uneix als fosfolípids aniònics de la membrana plaquetar. Els anticossos antifosfolipídics reconeixerien aquest complex i interaccionarien a través de la seva porció Fc amb els receptors FcγRII de la superfície plaquetar (161). Mitjançant aquesta interacció, les plaquetes podrien activar-se iniciant el fenomen trombòtic (162). Un procés similar s'ha

descrit amb els fosfolípids de membrana de les cèl·lules endotelials (163). En aquest sentit, les dades existents a la bibliografia demostren que la unió de la β_2GPI a les cèl·lules endotelials es produeix tant a la macro com a la microcirculació i que la unió dels anticossos antifosfolipídics al cofactor és capaç d'activar les cèl·lules endotelials (164). La β_2GPI també s'ha involucrat en la via de la proteïna C. S'ha objectivat que la β_2GPI inhibeix la unió de la proteïna C als fosfolípids molt millor que la unió de la protrombina, el que provoca un efecte procoagulant (165). Els anticossos antifosfolipídics reconeixen la proteïna C només en presència de β_2GPI. Aquests resultats suggereixen que la disfunció de la proteïna C induïda pels anticossos antifosfolipídics està mediada per la β_2GPI. El nostre grup va demostrar que anticossos anticardiolipina monoclonals amb activitat anti-β_2GPI (obtinguts de dos pacients amb síndrome antifosfolipídica i antecedents de trombosis) eren capaços d'incrementar l'expressió de factor tissular per part dels monòcits (166). Finalment, també s'ha demostrat que els anticossos anticardiolipina, inhibint l'efecte de la β_2GPI, inhibirien l'activitat fibrinolítica del plasma a través d'incrementar l'activitat del PAI-1 (167).

4.E.2. Malaltia de Behçet

Existeixen nombrosos treballs en pacients amb malaltia de Behçet en els quals s'han investigat les alteracions de la coagulació en general i de la fibrinolisi en particular i la seva relació amb els fenòmens trombòtics. Si bé s'han descrit casos aïllats de pacients amb malaltia de Behçet, trombosi i dèficit de proteïna C, proteïna S i antitrombina (168,169), en les sèries de pacients analitzades no s'ha pogut trobar una relació entre aquests dèficits i les manifestacions trombòtiques (19,170-172). En referència a les mutacions genètiques del factor V Leiden, mutació G20210A del gen de la protrombina i la mutació C677T de la MTHFR, els resultats queden plasmats a la Taula 4. En aquest

cas, mentre alguns autors han demostrat una relació entre la presència del factor V Leiden i de la mutació G20210A i les trombosis venoses a la malaltia de Behçet (173,174), en altres estudis no s'han confirmat aquestes troballes (175,176). La prevalença de la mutació C677T de la MTHFR a la malaltia de Behçet ha estat avaluada per un sol estudi aparegut amb posterioritat a l'inici de la present tesi (176). En aquest s'ha objectivat que la prevalença de la mutació és similar en els pacients amb malaltia de Behçet amb i sense trombosi. El polimorfisme 4G/5G del gen del PAI-1 no ha estat avaluat fins ara en aquesta entitat.

Refrent als anticossos antifosfolipídics, la seva prevalença a la malaltia de Behçet varia entre el 0% i el 47% (probablement degut a la diferent metodologia dels ELISA emprats en la seva detecció) (19,179-189). A la majoria de les sèries es conclou que els anticossos antifosfolipídics no tenen un efecte causal en la clínica trombòtica d'aquests pacients (19, 179-181,183-189).

Taula 4: Resum dels principals estudis de mutacions genètiques protrombòtiques en pacients amb malaltia de Behçet.

Autor (ref)	**Casos***	**Factor V Leiden**		**G20210A**		**MTHFR C677T**	
		T+	**T-**	**T+**	**T-**	**T+**	**T-**
Lesprit (175)	15 (15)	0%		NR		NR	
Mammo (173)	23 (8)	38%	0%	NR		NR	
Oner (177)	44 (5)	30%	6%	NR		NR	
Gurgey (178) #	34 (NR)	24%		NR		NR	
Mader (19) #	25 (8)	8%		NR		NR	
Gül (174)	64 (32)	38%	9%	31%	3%	NR	
Toydemir (176)	60 (30)	33%	20%	2%	0%	22%	25%

*Nombre de casos: entre parèntesi els pacients amb trombosi.
MTHFR: metilen-tetra-hidrofolat reductasa; **T+:** pacients amb trombosi; **T-:** pacients sense trombosi;
NR: no reportat
El percentatge del factor V Leiden correspon a la sèrie en general.

Pocs estudis han analizat la fibrinolisi i, en aquest cas, els resultats són també no concloents com ho demostra el fet que s'han trobat valors de t-PA antigen normals (21,190-191) o disminuïts (192), i d'activitat de t-PA normals (190) o elevats (191). Pel

què fa al PAI-1 antigen s'han descrit valors elevats en pràcticament tots els estudis (21,190,191), mentre que l'activitat de PAI-1 s'ha trobat dins dels valors normals (192) o elevada (190,191). Com en els casos anteriors, la diferent metodologia emprada en els estudis però també la diferent fase d'activitat en què van ser recollides les mostres dels pacients, poden explicar aquesta discordància en els resultats. De totes maneres, aquests estudis no han demostrat relació entre aquests resultats i els fenòmens trombòtics en els pacients amb malaltia de Behçet (21,190-192).

4.E.3. Arteritis de Horton

Els elements de l'hemostàsia pràcticament no han estat avaluats en aquesta entitat. Les investigacions s'han centrat sobretot en el paper de les citocines i les molècules d'adhesió com resposta a l'alteració de l'endoteli per la vasculitis existent (23).

Ara bé, a la bibliografia s'ha referenciat la prevalença dels anticossos antifosfolipídics i la seva relació amb els fenòmens isquèmics de l'arteritis de cèl·lules gegants, és a dir, l'afectació visual, la claudicació mandibular i els accidents vasculars cerebrals. La freqüència dels anticossos anticardiolipina a l'arteritis de cèl·lules gegants varia entre el 7,5% i el 54% (193-204)(Taula 5). Si bé poden haver diferències metodològiques que expliquin aquestes discrepàncies, el que es repeteix en pràcticament tots els articles és que els anticossos anticardiolipina estan augmentats en relació a la població general sana.

Taula 5: Prevalença dels anticossos antifosfolipídics en pacients amb arteritis de cèl·lules gegants.

Autor (ref)	Casos	Anticoagulant lúpic	Anticossos anticardiolipina	Anticossos anti-β2GPI

Aguilar (193)	50	NR	54%	NR
Font (194)	24	NR	8%	NR
Cid (195)	40	NR	7.5%	NR
McHugh (196)	22	NR	50%	NR
Espinoza (197)	50	NR	48%	NR
Kerleau (198)	20	NR	50%	NR
Liozon (199)	86	NR	50%	NR
Chakvarty (200)	98	NR	20%	NR
Liozon (201)	41	NR	51%	0%
Meyer (202)	22	NR	18%	11%
Manna (203)	40	NR	30%	NR
Duhaut (204)	266	NR	20%	NR

NR: no reportat.
Els percentatges corresponen a les sèries en total

Pel què fa al seu origen i al seu paper en les manifestacions isquèmiques, els darrers treballs que inclouen un nombre elevat de pacients estan d'acord en el fet que probablement es comporten només com marcadors de la lesió endotelial existent en aquesta vasculitis (204). Fins ara, només dos estudis han analitzat la prevalença dels anticossos anti-β_2GPI a l'arteritis de cèl·lules gegants. En un d'ells, cap dels 45 pacients analitzats va presentar positivitat per aquests anticossos (201). En el segon, només 2 dels 19 pacients tenien anticossos anti-β_2GPI (202).

Fins el moment actual, no existeix cap treball que hagi analitzat el paper de la fibrinolisi a les manifestacions isquèmiques de l'arteritis de Horton.

Els mecanismes patogènics dels fenòmens trombòtics apareguts a la síndrome antifosfolipídica, la malaltia de Behçet i l'arteritis de Horton no són completament coneguts. En aquestes entitats, la clínica trombòtica té un grau important d'heterogeneïtat en la seva presentació. A la síndrome antifosfolipídica existeixen pacients que només trombosen al territori venós, d'altres ho fan de forma exclusiva a nivell arterial i un altre grup pateixen trombosis a ambdós territoris. Igualment, hi ha pacients que només presenten clínica obstètrica i d'altres recidiven malgrat un tractament anticoagulant correcte. Per altra part, s'ha descrit en determinades patologies infeccioses (lues o síndrome d'immunodeficiència adquirida) sense trombosi i en individus sans que no desenvolupen fenòmens trombòtics (els anomenats portadors assimptomàtics), l'existència d'anticossos antifosfolipídics "no patogènics". En relació a la malaltia de Behçet, les trombosis només apareixen en una quarta part dels pacients i són més freqüents al territori venós. Finalment, a l'arteritis de Horton, les manifestacions isquèmiques es presenten en aproximadament el 20% dels pacients amb un clar predomini de l'arbre vascular arterial.

La possible implicació de la fibrinolisi plasmàtica en aquesta heterogeneïtat clínica, resulta molt atractiva per la important relació existent entre les seves vies d'activació i inhibició i els fenòmens inflamatoris que tenen lloc en les malalties autoimmunes. En pacients amb malalties sistèmiques, la fibrinolisi pot estar disminuïda degut a un augment dels nivells plasmàtics del PAI-1, el seu principal inhibidor fisiològic. Com hem esmentat a la revisió bibliogràfica, els nivells del PAI-1 poden estar, en part, determinats genèticament per un polimorfisme de deleció/inserció d'una base (4G/5G) a la regió promotora del seu gen.

Aquests antecedents ens han portat a plantejar la possible relació entre el polimorfisme 4G/5G del gen del PAI-1 i les manifestacions trombòtiques a les tres entitats analitzades. Aquesta relació seria secundària a la major resposta d'alliberació del PAI-1

que es produiria en els pacients portadors de l'al·lel 4G, quan la cèl·lula endotelial s'estimula durant els fenòmens autoimmunes o el procés inflamatori. Aquest increment del PAI-1 en els individus amb l'al·lel 4G podria inhibir la fibrinolisi d'una manera excessiva i exercir un efecte protrombòtic additiu al propi de la malaltia de base augmentant el risc de trombosi. Per tant, la variabilitat de l'expressió clínica trombòtica en aquestes entitats es deuria a la seva associació amb un substrat genèticament predeterminat.

D'altra banda, avui sabem que els anticossos antifosfolipídics van dirigits realment contra un complex de fosfolípid i proteïna (o cofactor). D'aquestes, la més ben estudiada és la β_2GPI, de manera que s'ha involucrat en els diferents mecanismes patogènics en relació als anticossos antifosfolipídics. Així, ha estat descrit com el cofactor que s'uneix als fosfolípids aniònics de la membrana plaquetar. Els anticossos antifosfolipídics podrien reconèixer aquest complex, induint l'activació plaquetar i iniciant el fenomen trombòtic. Aquestes evidències indirectes ens han portat a plantejar la hipòtesi que els anticossos anticardiolipina requeririen tenir activitat anti-β_2GPI per tal de promoure la interacció plaquetar amb el subendoteli. És a dir, els anticossos antifosfolipídics amb activitat anti-β_2GPI serien capaços de generar el procés trombòtic, mentre que aquells que no reconeguessin la β_2GPI constituirien el grup dels "no patogènics". Aquestes diferències en les característiques dels anticossos antifosfolipídics podrien explicar part de l'heterogeneïtat clínica que presenten els pacients amb aquests anticossos.

6.A. OBJECTIUS GENERALS:

1. Determinar en els pacients amb síndrome antifosfolipídica, malaltia de Behçet i arteritis de Horton la prevalença dels principals factors genètics i adquirits causants de trombofília i correlacionar la presència d'aquests amb les manifestacions trombòtiques.

2. Determinar en els mateixos grups de pacients l'estat d'activació de la fibrinolisi plasmàtica, els marcadors d'activació de la coagulació i de generació *in vivo* de trombina i correlacionar-los amb les manifestacions trombòtiques.

6.B. OBJECTIUS PARTICULARS:

6.B.1. Primer Estudi:

The 4G/5G polymorphism of the type 1 plasminogen activator inhibitor gene and thrombosis in patients with antiphospholipid syndrome. Arthritis Rheum 2000;43:2349-58.

1. Correlacionar el polimorfisme 4G/5G del gen del PAI-1 amb les manifestacions trombòtiques arterials i venoses dels pacients amb síndrome antifosfolipídica.

2. Correlacionar la resta dels principals factors genètics causants de trombofília amb les manifestacions trombòtiques arterials i venoses dels pacients amb síndrome antifosfolipídica.

3. Determinar els marcadors d'activació de la fibrinolisi plasmàtica i els marcadors de generació *in vivo* de trombina en els pacients amb síndrome antifosfolipídica.

6.B.2. Segon Estudi:

Effects of β_2-glycoprotein I and monoclonal anticardiolipin antibodies in platelet interaction with subendothelium under flow conditions. Arthrtitis Rheum 2002;46:3283-9.

1. Determinar la importància de l'activitat anti-β_2GPI dels anticossos anticardiolipina per incrementar la interacció plaquetar amb el subendoteli en condicions de flux.

2. Estudiar els efectes de la β_2GPI sobre l'acció dels anticossos anticardiolipina en la interacció plaquetar amb el subendoteli en condicions de flux.

6.B.3. Tercer Estudi:

Vascular involvement in Behçet's disease: relation with thrombophilic factors, coagulation activation, and thrombomodulin. Am J Med 2002;112:37-43.

1. Determinar la prevalença dels principals factors genètics i adquirits causants de trombofília en pacients amb malaltia de Behçet i correlacionar-los amb les seves manifestacions trombòtiques.

2. Determinar en els mateixos pacients els marcadors de generació *in vivo* de trombina, de la fibrinolisi plasmàtica i de lesió endotelial, correlacionant aquests paràmetres amb les manifestacions trombòtiques de la malaltia.

3. Determinar els marcadors d'activació de les vies intrínseca i extrínseca de la coagulació en els pacients amb malaltia de Behçet.

6.B.4. Quart Estudi:

Antiphospholipid antibodies and thrombophilic factors in giant cell arteritis. Semin Arthritis Rheum 2001;31:12-20.

1. Determinar la prevalença dels anticossos antifosfolipídics en pacients amb arteritis de cèl·lules gegants.

2. Correlacionar en els mateixos pacients aquests anticossos antifosfolipídics amb les manifestacions isquèmiques i trombòtiques.

3. Determinar en els mateixos pacients la prevalença dels principals factors genètics causants de trombofília i els paràmetres d'activació de la fibrinolisi plasmàtica i correlacionar-los amb les manifestacions isquèmiques i trombòtiques.

ARTHRITIS & RHEUMATISM
Vol. 43, No. 10, October 2000, pp 2349–2358

THE 4G/5G POLYMORPHISM OF THE TYPE 1 PLASMINOGEN ACTIVATOR INHIBITOR GENE AND THROMBOSIS IN PATIENTS WITH ANTIPHOSPHOLIPID SYNDROME

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Objective. **To investigate the relationship between the 4G/5G polymorphism of the type 1 plasminogen activator inhibitor (*PAI-1*) gene and thrombotic manifestations in patients with antiphospholipid syndrome (APS).**

Methods. **We studied a total of 247 patients included in the following 4 groups: 70 patients with primary APS, 104 patients with systemic lupus erythematosus (40 with antiphospholipid antibodies [aPL] and clinical [secondary] APS, 13 with aPL but without clinical APS, and 51 with neither detectable aPL nor a history of thrombosis), 14 asymptomatic individuals with aPL, and 59 patients with thrombosis but without known thrombosis risk factors. A control group of 100 healthy individuals was also analyzed. *PAI-1* 4G/5G polymorphism was determined by polymerase chain reaction and endonuclease digestion.**

Results. **The allele frequency of 4G/5G in controls was 0.47/0.53. There were no differences in allele distribution among patient groups or between patients and controls. However, a higher frequency of the 4G allele was observed in APS patients with versus those without thrombosis (0.57 versus 0.39; $P < 0.05$) (odds ratio [OR] 2.83, 95% confidence interval [95% CI] 1.18–6.76). This higher frequency of the 4G allele was attributable to the higher frequency in patients with versus those without arterial thrombosis (0.64 versus 0.43; $P < 0.01$) (OR 5.96, 95% CI 1.67–21.32), while patients with venous thrombosis had an allele distribution similar to that of those without venous thrombosis (0.49 versus 0.50; *P* not significant). There was a trend toward higher PAI-1 antigen and activity levels in APS patients and controls with the 4G/4G genotype, but this did not reach statistical significance.**

Conclusion. **The presence of the 4G allele of the 4G/5G polymorphism of the *PAI-1* gene may be an additional risk factor for the development of arterial thrombosis in APS.**

Patients with antiphospholipid syndrome (APS) have an increased risk of venous and arterial thrombosis, recurrent pregnancy loss, and/or thrombocytopenia. These clinical manifestations are associated with the presence of antiphospholipid antibodies (aPL), including anticardiolipin antibodies (aCL) and lupus anticoagulant (LAC), in patients with systemic lupus erythematosus (SLE), secondary APS, or primary APS (1). Despite the strong association between aPL and thrombosis, the pathogenic role of aPL in the development of thrombosis has not been fully elucidated. Several mechanisms have been proposed, including inhibition of the release of prostacyclin by the endothelium (2), alterations in the protein C–protein S pathway (3), a direct procoagulant effect on platelets (4), and impairment of fibrinolysis (5–7).

The possible contribution of fibrinolysis to the development of thrombosis in APS is very intriguing due

Supported by Spanish grants Fis 98/0171, Fis 00/1048, Fis 99/0148, Fis 97/0525, and Fis 96/0580. Dr. Espinosa's work was supported by a grant from the Hospital Clínic of Barcelona (1998) and grant 1999FI 00684 CIRIT (Generalitat de Catalunya) (1999/2000).

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Submitted for publication January 28, 2000; accepted in revised form June 9, 2000.

to the interrelation between the fibrinolysis activation pathways and the inflammatory events accompanying autoimmune diseases (8). In fact, it is known that the expression of the major regulator of fibrinolysis, type 1 plasminogen activator inhibitor (PAI-1), is modulated by agents such as cytokines (9). Several studies have suggested that in patients with systemic diseases, including APS, fibrinolysis may be decreased, due mainly to an excess of PAI-1 (5,6,10,11). Nevertheless, some investigators have found discordant results, with normal or increased fibrinolysis in these diseases (12–15). Although these discrepancies can be partially explained by methodology, they may also be related to a predetermined genetic substrate for the fibrinolytic response.

Genetic factors involving PAI-1 synthesis have recently been investigated. In particular, plasma PAI-1 levels have been found to be associated with a single-basepair guanosine deletion/insertion polymorphism (4G/5G) located in the promoter region, 675-bp upstream from the start of transcription of the *PAI-1* gene (16). Furthermore, it has been demonstrated that the 4G allele is associated with enhanced PAI-1 expression due to differential binding to the polymorphic site of nuclear proteins involved in the activation and inhibition of gene transcription (17,18). In addition, the *PAI-1* 4G/5G polymorphism has been found to be associated with some thrombotic manifestations (19), suggesting that this polymorphism may be an additional thrombotic genetic factor. Because of the variability of the clinical expression of APS, potential predictive factors such as the 4G/5G polymorphism should be investigated to help identify patients with a high risk of thrombosis. The aim of the present study was to investigate the relationship between the 4G/5G polymorphism of the *PAI-1* gene and thrombotic manifestations in patients with APS.

PATIENTS AND METHODS

Patients. We studied a total of 247 patients, 174 of whom were included in the following 2 groups: 70 patients diagnosed with primary APS on the basis of clinical and laboratory data (1) (59 women and 11 men, mean ± SD age 36.8 ± 11.9 years) and 104 patients who fulfilled the revised criteria of the American College of Rheumatology for the diagnosis of SLE (20). Patients in the second group were further classified into 3 groups, consisting of 40 who had aPL and clinical (secondary) APS (38 women and 2 men, mean ± SD age 39.9 ± 13.6 years), 13 who had aPL but not clinical APS (12 women and 1 man, mean ± SD age 36.4 ± 9.0 years), and 51 who had neither detectable aPL nor a history of thrombosis (48 women and 3 men, mean ± SD age 41.3 ± 15.6 years).

The remaining 73 patients were in the following 2 groups: 14 asymptomatic individuals (11 women and 3 men, mean ± SD age 34.2 ± 11.8 years) with aPL detected in laboratory tests in at least 2 determinations performed 3 months apart, and 59 patients (32 women and 27 men, mean ± SD age 35.9 ± 17.5 years) with thrombosis (33 with venous thrombosis and 26 with arterial thrombosis) and without known thrombophilia as defined by the absence of protein C, protein S, antithrombin or plasminogen deficiencies, and the absence of aPL, protein C resistance, factor V Leiden mutation, hyperhomocysteinemia, or prothrombin gene *G20210A* mutation.

We also analyzed a control group of 100 healthy individuals (74 women and 26 men, mean ± SD age 41.3 ± 18.0 years) without autoimmune disease, bleeding disorders, thrombosis, or a history of pregnancy loss.

All the thrombotic events were assessed clinically and confirmed by objective methods. Diagnosis of deep venous thrombosis was confirmed by Doppler ultrasonographic scans or by venography. Pulmonary embolism was diagnosed by ventilation/perfusion scanning or by pulmonary angiography. Cerebrovascular ischemic episodes were confirmed by computed tomography scanning or by magnetic resonance imaging techniques. Myocardial infarctions were confirmed by electrocardiographic studies and by elevated levels of cardiac enzymes. The time between thrombosis and blood sampling was at least 6 months (range 6–41 months). The followup of patients ranged from 2 years to 12 years (median 5.9 years). Body mass index (BMI) was calculated as weight (kg)/(height [m])^2, and history of smoking was recorded.

The study was approved by the Human Experimental Committee of the Hospital Clínic and was performed according to the principles of the Declaration of Helsinki. Informed consent was obtained from all participants.

Sampling. Venous blood samples were drawn in the morning from the sitting and resting patient with a clean antecubital venipuncture without venocclusion. Samples for coagulation and fibrinolysis studies were obtained in tubes containing 3.8% trisodium citrate (1/9 volume/volume; Becton Dickinson, Rutherford, NJ), and platelet-free plasma was immediately obtained by double centrifugation, first at 2,000*g* for 10 minutes at 22°C, and then at 5,000*g* for 10 minutes at 4°C. Plasma was aliquoted, snap-frozen in a mixture of dry ice/ethanol (1/2 v/v), and stored. For tissue-type plasminogen activator (tPA) activity, samples were obtained in strong acidic citrate tubes (Stabilyte; Biopool, Umeå, Sweden). For genotype studies, samples were drawn in trisodium EDTA tubes (Becton Dickinson), and 100 μl of whole blood was immediately transferred into tubes containing lysis buffer (5*M* guanidine thiocyanate, 1.3% [weight/volume] Triton X-100, and 50 m*M* Tris HCl, pH 6.4) and frozen at −70°C. Sera for biochemistry and for antibody studies were drawn in tubes containing no anticoagulants (Becton Dickinson).

Hemostasis studies. *General.* Prothrombin and activated partial thromboplastin times were determined in an automated coagulometer CA-6000 (Dade Behring, Marburg, Germany) using standard reagents (Thromboplastin IS and Actin FSL; Dade Behring) and were expressed as ratios (patient time:control time). Fibrinogen level was measured by the Clauss technique.

Risk factors for thrombosis. Protein C activity was quantified by a colorimetric assay (Chromogenix, Möndal, Sweden). Free and total protein S were quantified by enzyme-linked immunosorbent assay (ELISA) using specific monoclonal antibodies (Stago, Asnières, France). Antithrombin activity was measured using a chromogenic assay (Dade Behring). Homocysteine levels were measured by high-performance liquid chromatography. Activated protein C resistance was determined in coagulation assays, including the direct test and after 1:5 dilution in factor V–immunodepleted plasma (Dade Behring) (21). Individuals with phenotypic activated protein C resistance or with LAC, which may interfere with activated protein C resistance measurements, were tested for the presence of factor V Leiden mutation using a nucleic acid sequence–based amplification assay (Organon Teknika, Boxtel, The Netherlands) (22). Briefly, the region of the factor V RNA carrying the Leiden mutation was isothermally amplified at 41°C for 90 minutes using specific primers and the action of 3 enzymes (AMV retrotranscriptase, RNase H, and T7 RNA polymerase), and detection was performed by acrylamide gel electrophoresis after hybridization with specific probes for both the wild-type and the mutant factor V RNA.

For the prothrombin gene *G20210A* mutation, DNA samples were analyzed by polymerase chain reaction (PCR), as reported by Poort et al (23). Briefly, a 345-bp fragment from the 3′-untranslated region of the prothrombin gene was amplified using a mutagenic oligonucleotide that introduces a novel *Hind* III restriction site into the amplified fragments from the less-frequent 20210A allele. After digestion with *Hind* III, the DNA fragments were detected by agarose gel electrophoresis. The 20210G allele lacks the restriction site and generates only a 345-bp fragment, while the 20210A allele generates a 322-bp fragment and a 23-bp fragment.

Serum levels of cholesterol and triglycerides as well as lipoprotein fractions were determined by enzymatic and colorimetric methods as previously reported (24). Serum glucose was evaluated by standard methods in an autoanalyzer (Bayer, Tarrytown, NY), and glycosylated hemoglobin (HbA_{1c}) was assessed by spectrophotometric chromatography in an ionic exchange microcolumn (Biosystem, Barcelona, Spain).

Fibrinolysis parameters. Plasminogen was evaluated using a chromogenic assay (Chromogenix). Antigen related to tPA was measured by means of ELISA (Stago), and tPA activity was measured by a chromogenic assay (Chromogenix). PAI-1 activity was measured by the method described by Chmielewska et al (Chromogenix) (25), and plasma antigen related to PAI-1 was measured by ELISA (Biopool), based on a double antibody principle (26). Inactive tPA–PAI-1 complexes were measured by ELISA using a monoclonal antibody that binds tPA–PAI-1 complexes and a conjugated monoclonal antibody against a different epitope of the complex (Technoclone, Vienna, Austria).

Thrombin and plasmin generation markers. The prothrombin fragment 1+2 (F_{1+2}), as a thrombin generation marker, was assessed by ELISA (Enzygnost-F_{1+2}; Dade Behring). Plasma levels of plasmin–α_2-antiplasmin complexes were quantified as a plasmin generation marker by ELISA (Dade Behring).

Antiphospholipid antibodies. LAC was detected using activated partial thromboplastin time, dilute Russell's viper venom time, and the tissue thromboplastin inhibition test. Tests were also performed in mixtures with control plasmas or phospholipids following the guidelines of the Subcommittee for the Standardization of Lupus Anticoagulants of the International Society of Thrombosis and Hemostasis (27). Anticardiolipin antibodies were measured using a standardized ELISA (28). Results were expressed in IgG and IgM phospholipid units and reported as negative ($\leq$15 units), low positive (16–25 units [+]), moderate positive (26–40 units [++]), and high positive (>40 units [+++]). Detection of anti–β_2-glycoprotein I (anti-β_2GPI) was done by a previously described ELISA (29) using microtiter plates coated with human β_2GPI. In each assay, 2 IgG and 2 IgM anti-β_2GPI sera and 10 negative sera were used as controls. Values of optical density at 492 nm (OD_{492}) that were $\geq$5 SD above the mean OD_{492} values of negative control sera were considered positive. OD_{492} values of 5–7 SD, 7–9 SD, and >9 SD above the mean OD_{492} values of negative control sera were considered low positive (+), moderate (++), and high (+++), respectively.

PAI-1 4G/5G polymorphism genotyping. Genomic DNA was extracted from 100 μl of whole blood by a silica-based method (30). For detection of the *PAI-1* 4G/5G polymorphism, a recently described protocol based on a PCR technique and endonuclease digestion was used with minor modifications (31). For DNA amplification, we used a mutated oligonucleotide that inserts a site for the *Bsi* YI enzyme in the product of amplification. This restriction site enables the identification of the extra G base. The following primers were used: 5′-CAC-AGA-GAG-AGT-CTG-GC*CACG-T-3′, forward primer, position −697/−676, with a C→A substitution (*) in position −681; and 5′-CCA-ACA-GAG-GAC-TCT-TGG-TCT-3′, reverse primer, position −598/−619. PCR was performed on 50 μl–volume samples in a Techne Progene thermal cycler (Techne, Cambridge, UK) with 30 cycles at 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, and a final extension cycle at 72°C for 7 minutes. The expected size of the amplified products was 99 bp for the 5G allele and 98 bp for the 4G allele.

After amplification, 20 μl of the PCR product was digested for 150 minutes at 55°C with 5 units of the *Bsi* YI restriction enzyme (Roche Diagnostics, Basel, Switzerland). The digested product was analyzed by 4% agarose gel electrophoresis (Metaphor; FMC Bioproducts, Rockland, ME) and visualized under ultraviolet light after staining with ethidium bromide. The digested fragments were expected to consist of a single 98-bp band for the 4G allele and 2 bands (77 bp and 22 bp) for the 5G allele.

Cytokine levels. Plasma levels of tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), and IL-6, the main cytokines related to PAI-1 secretion (32), were determined. Samples were analyzed by ELISA according to the manufacturer's recommendations (Quantikine; R&D Systems, Minneapolis, MN).

Statistical analysis. Results are shown as the mean ± SD. Comparisons were performed by chi-square test or analysis of variance. The parameter values of each group were compared with those of the control group by Dunnett's multiple comparison test (33). The Hardy-Weinberg equation was used to calculate the expected genotype distribution under equilibrium assumptions. The observed numbers of each genotype

Table 1. Clinical and immunologic characteristics of patients with antiphospholipid syndrome (APS)*

Characteristic	Primary APS (n = 70)	Secondary APS (n = 40)
Clinical		
Thrombosis	43	23
Arterial	24	12
Venous	21	13
Thrombocytopenia ($<100 \times 10^9$ platelets/liter)	22	18
Fetal miscarriages, no./no. women	34/59	22/38
Immunologic		
Lupus anticoagulant	39	24
aCL†	49	26
IgG, +/++/+++	5/9/30	3/6/14
IgM, +/++/+++	5/8/11	6/4/6
Anti-β_2GPI‡	32	18
IgG, +/++/+++	5/8/13	2/2/12
IgM, +/++/+++	4/8/11	1/3/10

* Values are the number of patients with the clinical or immunologic manifestation. aCL = anticardiolipin antibodies; anti-β_2GPI = anti–β_2-glycoprotein I.
† + = low positive; ++ = moderate positive; +++ = high positive (see Patients and Methods for details).
‡ + = low positive; ++ = moderate; +++ = high (see Patients and Methods for details).

were compared with those expected for a population in Hardy-Weinberg equilibrium by chi-square test (34). Odds ratios (OR) were determined by contingency tables, and 95% confidence intervals (95% CI) were calculated (35).

RESULTS

General findings. The immunologic profiles and clinical characteristics of the APS patients are shown in Table 1. Sixty-one percent of patients with primary APS and 58% of patients with secondary APS had thrombotic events. Fifty-six percent of the patients with thrombosis in primary APS and 52% of those with thrombosis in secondary APS had arterial thrombosis (Table 1). The most frequent thromboses in the arterial territory were ischemic stroke, myocardial infarction, and peripheral thrombosis (Table 2). Twenty-four patients had a single arterial thrombotic event, and 12 patients had ≥2 arterial thromboses. Deep venous thrombosis in the legs and pulmonary thromboembolism were the most frequent thromboses in the venous territory. No significant differences were observed in clinical or immunologic profiles between primary and secondary APS patients.

Thrombosis risk factors. BMI was <30 kg/m^2 in all patients. There were no significant differences in BMI among patient groups. Serum levels of cholesterol, triglycerides, and lipoproteins were within the normal ranges and did not show differences among the groups. Patients and controls had normal values of serum glucose and HbA_{1c}. A total of 10.6% of the patients with primary or secondary APS, 9.8% of SLE patients without APS, 22.0% of patients with unexplained thrombosis, and 18.0% of controls were smokers.

Fibrinogen was within the normal range in both patients and controls. Low values of protein C (<60%) were found in 3 patients (1 with primary APS and previous venous thrombosis, 1 with secondary APS and 2 previous venous thromboses, and 1 with SLE and aPL without thrombosis). All patients had normal values of protein C antigen. Total protein S was within the normal range in patients and in controls. Low values of free protein S (<60%) were found in 10 patients (3 with primary APS [2 with venous thrombosis and 1 with stroke plus deep venous thrombosis, pulmonary embolism, and fetal miscarriages], 5 with secondary APS [3 with venous thrombosis and 2 with fetal miscarriages], 1 with SLE and aPL but without APS, and 1 with SLE but without aPL or thrombosis). Antithrombin values were within the normal range in all patients and controls. Mild hyperhomocysteinemia was identified in 2.7% of APS patients and in 3% of controls, with no relationship with thrombosis.

The heterozygous factor V Leiden mutation was detected in 1 primary APS patient with stroke, 2 secondary APS patients (1 with pulmonary embolism and stroke and 1 with recurrent fetal miscarriages), 1 SLE patient without aPL or thrombosis, and 2 controls. The heterozygous prothrombin gene *G20210A* mutation was found in 1 primary APS patient with venous thrombosis, 1 secondary APS patient with recurrent fetal miscar-

Table 2. Characteristics of thrombosis in patients with antiphospholipid syndrome (APS)*

	Primary APS (n = 70)	Secondary APS (n = 40)
Arterial thrombosis		
Ischemic stroke	15	7
Myocardial infarction	6	3
Peripheral thrombosis	6	3
Spleen infarction	3	1
Thrombotic renal microangiography	3	2
Venous thrombosis		
Deep venous thrombosis	17	11
Pulmonary thromboembolism	5	3
Retinal thrombosis	2	2
Portal thrombosis	1	0

* Values are the number of patients with the clinical manifestation.

Table 3. Fibrinolysis laboratory results and type I plasminogen activator inhibitor (*PAI-1*) genotype distribution in patient and control groups*

	Primary APS (n = 70)	Secondary APS (n = 40)	SLE without APS		Asymptomatic aPL+ (n = 14)	Unexplained thrombosis (n = 59)	Controls (n = 100)
			aPL+ (n = 13)	aPL− (n = 51)			
Plasminogen, %	106.4 ± 15.0	102.6 ± 14.9	105.1 ± 17.0	98.8 ± 13.9	98.4 ± 14.3	102.1 ± 13.6	104.5 ± 14.7
tPA Ag, ng/ml	5.8 ± 1.7†	6.2 ± 2.0†	5.6 ± 1.9	5.9 ± 1.4†	5.0 ± 1.6	4.8 ± 1.7	4.8 ± 2.1
tPA activity, IU/ml	2.1 ± 0.9	2.0 ± 0.9	2.2 ± 1.0	2.3 ± 1.0	2.7 ± 0.9	2.6 ± 1.2	2.4 ± 1.1
PAI-1 Ag, ng/ml	21.6 ± 12.4	22.7 ± 12.2	20.9 ± 11.3	22.5 ± 13.5	18.3 ± 16.7	19.3 ± 10.2	20.1 ± 11.5
PAI-1 activity, units/ml	15.4 ± 9.4	15.7 ± 8.4	16.7 ± 7.8	15.3 ± 9.7	13.0 ± 7.2	12.9 ± 9.6	12.0 ± 8.2
tPA–PAI-1, ng/ml	3.9 ± 3.0	3.6 ± 2.7	3.4 ± 3.1	3.5 ± 3.1	2.6 ± 1.7	2.8 ± 1.8	2.8 ± 2.1
F_{1+2}, nmoles/liter	1.3 ± 0.7†	1.2 ± 0.6†	1.0 ± 0.5	0.9 ± 0.5	0.7 ± 0.4	1.1 ± 0.5†	0.8 ± 0.4
Plasmin–α_2-antiplasmin complexes, μg/liter	196.3 ± 89.4	204.3 ± 87.3	210.1 ± 76.0	208.4 ± 95.1	218.6 ± 81.4	210.6 ± 77.4	214.1 ± 92.2
No. with genotype, 4G/4G, 4G/5G, 5G/5G	18, 33, 19	10, 20, 10	3, 6, 4	13, 25, 13	3, 7, 4	13, 29, 17	21, 52, 27
Allele frequency, 4G/5G	0.49/0.51	0.50/0.50	0.46/0.54	0.50/0.50	0.46/0.54	0.47/0.53	0.47/0.53

* Except where otherwise indicated, values are the mean ± SD. APS = antiphospholipid syndrome; SLE = systemic lupus erythematosus; aPL = antiphospholipid antibody; tPA = tissue-type plasminogen activator; Ag = antigen; tPA–PAI-1 = tPA–PAI-1 complexes; F_{1+2} = prothrombin fragment 1+2.
† $P < 0.05$ versus controls.

riages, 1 SLE patient without aPL or thrombosis, and 1 control.

Fibrinolysis parameters and *PAI-1* polymorphism. The fibrinolysis parameters evaluated are shown in Table 3. Plasminogen was within the normal range in all patients and controls, and no differences were seen among the groups of patients. Levels of the tPA antigen were significantly elevated in both primary and secondary APS patients, as well as in SLE patients without aPL or thrombosis, compared with controls ($P < 0.05$). Similarly, higher levels of tPA antigen were observed in SLE patients with aPL without thrombosis, but the difference from controls was not significant, probably due to the low number of patients in this group. In asymptomatic patients with aPL and in patients with unexplained thrombosis, tPA antigen levels were similar to those observed in the control group. Activity of tPA was slightly lower in patients with APS and in SLE patients, but these differences did not reach statistical significance compared with controls. PAI-1 antigen levels and PAI-1 activity did not differ significantly among patient groups or controls.

Levels of tPA–PAI-1 complexes did not differ significantly among groups, although these were slightly higher in patients with autoimmune diseases (APS or SLE). When considered together, patients with APS (primary plus secondary) had higher PAI-1 activity than controls (mean ± SD 15.5 ± 9.0 units/ml versus 12.0 ± 8.2 units/ml; $P < 0.01$).

Plasma levels of plasmin–α_2-antiplasmin complexes did not differ among the groups, although levels of the thrombin generation marker F_{1+2} were significantly elevated in both primary and secondary APS patients and in patients with unexplained thrombosis (but not in SLE patients without aPL or thrombosis) (Table 3).

Distribution of the 4G/5G alleles of the *PAI-1* gene in the different groups is shown in Table 3. The allele frequency of 4G/5G was 0.47/0.53 in controls, not significantly different from its similar distribution in the patient groups. No relationship was found between the levels or the isotype of aCL, anti-β_2GPI, or the presence or absence of LAC and the *PAI-1* polymorphism. There was a trend for PAI-1 antigen and activity levels to be higher in APS patients and controls with the 4G/4G genotype and lower in those with the 5G/5G genotype (Figure 1), although these differences were not statistically significant. No differences were seen between primary and secondary APS patients in either 4G/5G genotype distribution or fibrinolysis parameters.

The distribution of 4G/5G genotypes in APS patients according to their clinical manifestations is shown in Table 4. No differences were seen in 4G/5G genotype distribution when we considered patients with and without fetal miscarriages or thrombocytopenia. However, APS patients with thrombosis had a higher frequency of the 4G allele compared with APS patients without thrombosis (0.57 versus 0.39; $P < 0.05$). The OR for thrombosis in APS patients with at least 1 4G allele was 2.83 (95% CI 1.18–6.76). This excess of the 4G allele in thrombotic patients was due to the higher frequency of the 4G allele observed in patients with versus those

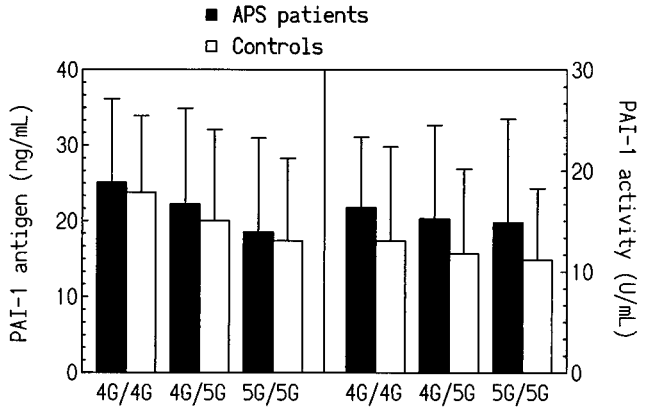

Figure 1. Type 1 plasminogen activator inhibitor (PAI-1) antigen and activity levels in controls and in patients with antiphospholipid syndrome (APS). PAI-1 antigen and activity levels tended to be higher in patients and controls with the 4G/4G genotype and lower in those with the 5G/5G genotype, although the differences were not statistically significant.

without arterial thrombosis (0.64 versus 0.43; $P < 0.01$) (the OR for arterial thrombosis in APS patients with at least 1 4G allele was 5.96 [95% CI 1.67–21.32]). However, the frequency of the 4G allele was not higher in APS patients with versus those without venous thrombosis (0.49 versus 0.50; *P* not significant [NS]) (OR 0.99, 95% CI 0.39–2.48).

Table 5 shows the risk of arterial thrombosis in APS patients according to 4G/5G genotype. Both 4G/4G and 4G/5G patients had a similar higher risk of thrombosis than did 5G/5G patients (OR 7.51 and 5.25 in 4G/4G and 4G/5G patients, respectively). Levels of PAI-1 antigen, PAI-1 activity, and tPA–PAI-1 complexes were 23.4 ± 13.1 ng/ml, 16.1 ± 8.1 units/ml, and 4.0 ± 2.9 ng/ml, respectively, in APS patients with arterial thrombosis, and 21.3 ± 11.9 ng/ml, 15.2 ± 9.4 units/ml, and 3.7 ± 2.9 ng/ml, respectively, in APS patients without arterial thrombosis (*P* NS). No significant differences were seen in the remaining fibrinolysis parameters in APS patients when we considered thrombotic history. Levels of PAI-1 antigen and PAI-1 activity according to 4G/5G genotype in patients with and those without arterial thrombosis are shown in Figure 2. In both groups, PAI-1 antigen and activity levels tended to be higher in patients with the 4G/4G genotype and lower in those with the 5G/5G genotype, showing a pattern similar to that of the controls in Figure 1. No differences were found in 4G/5G polymorphism or in fibrinolysis parameters among the patient groups when we consid-

Table 4. 4G/5G *PAI-1* genotype and clinical manifestations in APS patients*

	Genotype, 4G/4G, 4G/5G, 5G/5G, no. of patients	Allele frequency 4G/5G
Thrombosis		
Yes (n = 66)	21, 33, 12†	0.57/0.43†
No (n = 44)	7, 20, 17	0.39/0.61
Arterial thrombosis		
Yes (n = 36)	13, 20, 3‡	0.64/0.36‡
No (n = 74)	15, 33, 26	0.43/0.57
Venous thrombosis		
Yes (n = 34)	8, 17, 9	0.49/0.51
No (n = 76)	20, 36, 20	0.50/0.50
Thrombocytopenia		
Yes (n = 40)	12, 18, 10	0.53/0.47
No (n = 70)	16, 35, 19	0.48/0.52
Fetal miscarriages		
Yes (n = 56)	15, 27, 14	0.51/0.49
No (n = 41)	10, 20, 11	0.49/0.51

* See Table 3 for definitions.
† $P < 0.05$ versus patients without thrombosis.
‡ $P < 0.01$ versus patients without arterial thrombosis.

Table 5. Arterial thrombosis and 4G/5G genotype in patients with antiphospholipid syndrome

Genotype	Arterial thrombosis, no. (%)		OR (95% CI) for arterial thrombosis*
	Yes	No	
4G/4G (n = 28)	13 (46)	15 (54)	7.51 (1.84–30.57)†
4G/5G (n = 53)	20 (38)	33 (62)	5.25 (1.41–19.69)†
5G/5G (n = 29)	3 (10)	26 (90)	Referent
Total (n = 110)	36 (33)	74 (67)	

* OR = odds ratio; 95% CI = 95% confidence interval.
† $P < 0.01$ versus referent.

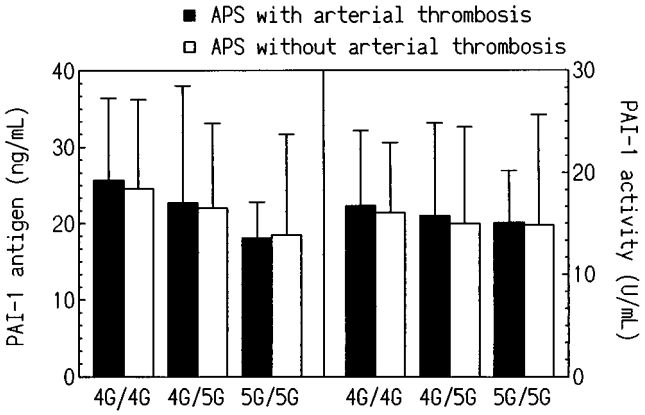

Figure 2. PAI-1 antigen and activity levels in APS patients with or without arterial thrombosis. PAI-1 antigen and activity levels tended to be higher in patients with the 4G/4G genotype and lower in those with the 5G/5G genotype, although the differences were not statistically significant. See Figure 1 for definitions.

ered we considered the site of arterial thrombosis (stroke, myocardial infarction, or other arterial thrombosis).

Cytokines. In all patients and controls, plasma levels of TNFα and IL-1β were within the normal range. Only 4 patients had elevated IL-6 levels (2 with primary APS [1 with both arterial and venous thromboses, fetal miscarriages, and thrombocytopenia and 1 with venous thrombosis and thrombocytopenia] and 2 with secondary APS [1 with venous thrombosis and fetal miscarriages, and 1 with only venous thrombosis]).

DISCUSSION

APS is an autoimmune disorder in which the presence of aPL is associated with an increased risk of arterial or venous thrombosis, recurrent pregnancy loss, and/or thrombocytopenia. Although there is a strong association between aPL and thrombosis, the heterogeneity of thrombotic manifestations in APS suggests that other additional factors may contribute to a "thrombophilic profile" in these patients. Impairment of fibrinolysis has been reported as a pathogenic mechanism for thrombosis in APS (5–7). In fact, fibrinolysis activation pathways are interrelated with inflammatory phenomena associated with autoimmune diseases, via cytokines (8). Inflammatory responses are mediated mainly by TNFα, IL-1β, and IL-6; on the other hand, these cytokines are important modulators of PAI-1 synthesis by vascular endothelium (32).

Circulating PAI-1 levels have recently been found to be associated with polymorphic variation in the human *PAI-1* gene (16). In particular, a 4G/5G deletion/insertion polymorphism in the promoter region has been shown to influence the expression of PAI-1 in patients with cardiovascular disease (17,18), metabolic diseases (19), and deep vein thrombosis (36). Experimental studies have demonstrated that both the 4G allele and the 5G allele bind an activator of gene transcription. The 5G allele also binds a repressor protein to an overlapping binding site in competition with the activator protein, while the 4G allele lacks the binding site for the repressor (18). Thus, in patients with the 4G allele, *PAI-1* gene transcription may be increased. This increased fibrinolysis inhibition response in the 4G genotype has been demonstrated in vitro under cytokine stimulation, but has not been observed under basal conditions (17). In the present study, we found an association between the 4G allele of the 4G/5G polymorphism of the *PAI-1* gene and arterial thrombosis in patients with APS. This association may be at least partially due to the inhibitory effect on fibrinolysis derived from the presence of the 4G allele. We did not find statistically significant differences in levels of either PAI-1 antigen or activity among the different genotype groups, although we did find a trend toward higher levels of PAI-1 antigen and activity in 4G homozygous patients and controls.

This lack of correlation between the *PAI-1* 4G/5G genotype and plasma PAI-1 levels in our series may be attributable to two facts. First, PAI-1 measurements were made in samples obtained under basal conditions. Therefore, these results may not reflect PAI-1 levels at the time of thrombus development. In this sense, experimental studies have demonstrated that the presence of the 4G allele enhances PAI-1 transcription only under conditions of stimulation, but not under basal conditions, suggesting that individuals with the 4G genotype may have an altered PAI-1 response only during the acute phase (17). For these reasons, PAI-1 levels in our patients may have been lower than those found in the acute phase and may not have reflected the effect of *PAI-1* 4G/5G polymorphism.

Second, plasma PAI-1 levels may not reflect key changes in local vascular concentrations. In fact, it has been demonstrated that PAI-1 messenger RNA may be locally expressed in atherosclerotic lesions (37). Cells within atheroma express and secrete PAI-1 into the surrounding matrix. A number of atheroma-associated factors increase PAI-1 expression by vascular cells in vitro, including cytokines and growth factors (9). Therefore, the presence of the 4G allele, under endothelial cell stimulation by cytokines, may determine a local increase in PAI-1 transcription mainly at the site of vessel injury (as in cases of atheromatosis or plaque rupture) and may be locally important in thrombus formation, while not necessarily being reflected in plasma PAI-1 levels.

Similarly, the results obtained in the plasma levels of cytokines, with normal values in nearly all cases, may be attributed to the fact that samples were drawn under basal conditions and not during thrombotic episodes; in addition, the samples may not reflect locally important variations. Further, although tPA antigen levels were slightly elevated in APS patients, tPA activity was not increased. The increase of tPA antigen levels may be explained, at least in part, by the increase of PAI-1–complexed tPA.

There is evidence that thrombosis is a multifactorial disease in which genetic and environmental factors are interrelated. In APS, aPL are an important risk factor for thrombosis, but the heterogeneity of throm-

botic manifestations in this syndrome suggests that aPL are probably not the only factor implicated in the pathogenesis of thrombosis.

In recent years, the association of thrombosis affecting the venous territory with several genetic factors has been demonstrated (38). In contrast, genetic risk factors implicated in the development of arterial thrombosis are less well characterized (38,39). In APS, arterial territory is frequently affected (>50% of thromboses in our series), and thromboses affecting arteries (as in myocardial infarction or ischemic stroke) are sources of great morbidity and mortality in this syndrome. In our series, we found several alterations in the protein C–protein S system together with thrombosis, mainly venous, in APS patients. Interestingly, however, we did not find any additional risk factor for arterial thrombosis, with the exception of the presence of the 4G allele of *PAI-1*.

The significance of the 4G/5G polymorphism of *PAI-1* as a thrombophilic factor is not yet fully elucidated. Some investigators have found the presence of the 4G allele to be associated with myocardial infarction in young patients (18), or with the development of coronary artery disease in patients with non–insulin-dependent diabetes mellitus (19). Others have found an association of the 4G allele with a family history of coronary artery disease (40) or with a rapid progression to acute coronary syndromes in subjects with coronary atherosclerosis (41), or have found the allele in patients with coronary atheroma and previous myocardial infarction (42). However, the association of the 4G allele with myocardial infarction has not been found in large multicenter studies (43–45). In patients with venous thrombosis, the 4G allele has been related to impaired fibrinolytic capacity (36), although the relationship of the 4G allele to venous thrombosis has not been confirmed (46). On the whole, the data available in the literature suggest that, although the 4G/5G *PAI-1* polymorphism by itself does not seem to be a major risk factor for thrombosis, the presence of the 4G allele may enhance the phenotypic expression of thrombophilia when it is associated with other prothrombotic factors of genetic, environmental, or metabolic origin (47–49).

APS patients have a tendency to thrombosis, and in the present study we have confirmed our previous observation (50) that these patients have increased thrombin generation even under basal conditions. This may be due to increased tissue factor expression (50) or to the inhibition of coagulation regulatory factors (3). In these patients with activated coagulation, the added effect of an impairment of the fibrinolytic system, genetically determined in the case of the 4G allele, may locally strengthen thrombus extension. This may help to explain the localization of thrombi in the arterial region, where fibrinolytic response and platelet function seem to play the major roles, similar to the protein C–protein S pathway in the venous region (38,39). Moreover, as deduced from our study, in APS patients only 1 4G allele seems sufficient to increase the risk of arterial thrombosis. This contrasts with findings of studies of other groups of patients with venous thrombosis and protein S deficiency, in which the presence of 2 4G alleles is necessary (47). These observations may reflect the different importance of emerging genetic factors in the development of thromboses, when they are combined with other genetic, environmental, or local factors.

In conclusion, in APS the 4G allele of the 4G/5G polymorphism of the *PAI-1* gene is associated with increased risk for arterial vascular lesion. This finding may be useful for identifying patients at increased risk for arterial thrombosis and for undertaking a more accurate therapeutic and prophylactic approach.

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COMENTARI:

En aquest primer treball, s'ha determinat en pacients amb síndrome antifosfolipídica primària i secundària, pacients amb lupus eritematós sistèmic amb anticossos antifosfolipídics però sense manifestacions trombòtiques, pacients amb lupus eritematós sistèmic sense anticossos antifosfolipídics i pacients amb trombosis sense factor de risc identificat, la prevalença dels factors genètics causants de trombofília, fent especial atenció al polimorfisme 4G/5G del gen del PAI-1. A més, també s'ha avaluat l'estat d'activació de la fibrinolisi plasmàtica i els marcadors de generació de trombina i plasmina. Finalment, tots aquests paràmetres s'han relacionat amb les manifestacions trombòtiques dels pacients. Cal destacar que aquest és el primer treball en què s'ha estudiat el polimorfisme 4G/5G del gen del PAI-1 en pacients amb síndrome antifosfolipídica.

Com resultats més destacables cal resaltar que no han existit diferències en la prevalença dels principals factors genètics causants de trombofília entre els diferents grups de pacients. Els nivells baixos de proteïna C i de proteïna S lliure trobats en pacients amb síndrome antifosfolipídica s'han relacionat amb la presència de trombosis venoses. Els nivells d'antitrombina van ser normals en els pacients i controls. La mutació heterozigota del factor V Leiden va estar present en un pacient amb síndrome antifosfolipídica primària amb un accident vascular cerebral, en 2 pacients amb síndrome antifosfolipídica secundària (un amb embolisme pulmonar i l'altre amb pèrdues fetals recurrents), en un pacient amb lupus eritematós sense anticossos antifosfolipídics ni trombosis i en 2 controls. La mutació heterocigota G20210A de la protrombina es va objectivar en un pacient amb síndrome antifosfolipídica primària amb trombosi venosa, en un pacient amb síndrome antifosfolipídica secundària amb pèrdues fetals recurrents, en un pacient amb lupus eritematós sense anticossos antifosfolipídics ni trombosis i en un control. Malgrat que els nivells de t-PA antigen han estat més

elevats en els pacients amb síndrome antifosfolipídica, els nivells de tPA activitat no han estat alts, i per tant, l'increment dels primers podria ser explicat, almenys en part, per l'augment del PAI-1 unit al t-PA. Encara que els nivells dels complexes plasmina-$\alpha_2$antiplasmina no han estat diferents entre els grups de pacients, els del marcador de generació de trombina, F1+2, han resultat significativament més alts en els pacients amb síndrome antifosfolipídica i en els que presentaven trombosis sense factor de risc identificat.

Els nivells plasmàtics del PAI-1 han estat més elevats en els pacients i controls homozigots per l'al·lel 4G, si bé aquestes diferències no han estat estadísticament significatives. La distribució del polimorfisme 4G/5G ha resultat similar entre els diferents grups de pacients i els controls i no hem obtingut cap relació significativa amb el perfil d'anticossos antifosfolipídics.

En canvi, quan hem correlacionat els diferents genotips amb les manifestacions clíniques de la síndrome antifosfolipídica (trombosi i pèrdues fetals), hem objectivat que els pacients amb trombosis presentaven una major prevalença del genotip homocigot per l'al·lel 4G respecte als pacients sense trombosi (0,57 versus 0,39; $P < 0,05$) amb un OR de 2,83 (95% IC 1,18-6,76). Aquest fet ha estat secundari a una major freqüència de l'al·lel 4G en els pacients amb trombosi arterial (0,64 versus 0,43; $p < 0,01$), amb un OR per la trombosi arterial en pacients amb síndrome antifosfolipídica amb almenys un al·lel 4G, de 5,96 (95% IC 1,67-21,32).

En conclusió, a la síndrome antifosfolipídica l'al·lel 4G del polimorfisme 4G/5G del gen del PAI-1 està associat a un subgrup de pacients amb un risc augmentat de trombosi arterial.

ARTHRITIS & RHEUMATISM
Vol. 46, No. 12, December 2002, pp 3283–3289
DOI 10.1002/art.10634

Effects of β_2-Glycoprotein I and Monoclonal Anticardiolipin Antibodies in Platelet Interaction With Subendothelium Under Flow Conditions

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Objective. **To evaluate whether the effect of human monoclonal anticardiolipin antibodies (aCL) on platelet interaction with the subendothelium under flow conditions is dependent on β_2-glycoprotein I (β_2GPI).**

Methods. **Three monoclonal IgM aCL with anti-β_2GPI activity (TM1B3, GR1D5, and EY2C9) obtained from patients with antiphospholipid syndrome, a monoclonal aCL with lupus anticoagulant activity but without anti-β_2GPI activity (FRO) obtained from a patient with a splenic lymphoma, and a control monoclonal IgM without aCL activity were used. TM1B3, GR1D5, EY2C9, FRO, and control IgM (30 μg/ml) were added to reconstituted blood containing gel-filtered platelets (200×10^9/liter), factor VIII (100 units/dl), and fibrinogen (1.5 gm/liter). Samples were perfused (wall-shear rate 800 seconds^{-1}), with and without the addition of purified β_2GPI (20 μg/ml), through annular chambers containing collagen-rich denuded vascular segments, and the percentages of surface covered by platelets and by thrombi were evaluated.**

Results. **No differences in the percentages of surface covered by platelets and by thrombi were observed among samples with TM1B3, GR1D5, EY2C9, FRO, and control IgM added when reconstituted blood samples without β_2GPI were used. However, a significant increase in the percentage of surface covered by platelets was observed in the presence of TM1B3, GR1D5, and EY2C9 but not in the presence of FRO when samples containing β_2GPI were used. Increased thrombi formation was induced by TM1B3 and GR1D5 but not by EY2C9 or FRO in samples with added β_2GPI.**

Conclusion. **Monoclonal aCL require anti-β_2GPI activity to promote platelet interaction with the subendothelium under flow conditions.**

The antiphospholipid syndrome (APS) is diagnosed when arterial or venous thrombosis or recurrent miscarriages occur in a person in whom laboratory tests for antiphospholipid antibodies (aPL) (anticardiolipin antibodies [aCL], lupus anticoagulant [LA], or both) are positive (1). This syndrome is considered primary if it is not associated with any other underlying disease (2), or secondary if it appears in association with other autoimmune disorders, mainly systemic lupus erythematosus (SLE) (3).

It is known that aPL are directed against phospholipid-binding proteins expressed on, or bound to, the surface of vascular endothelial cells or platelets. (4). The main protein associated with aCL activity is β_2-glycoprotein I (β_2GPI) bound to phospholipids (5–7). β_2GPI is a highly glycosylated single-chain protein that is present in plasma and avidly binds to negatively charged phospholipids such as cardiolipin, phosphatidylserine, or phosphatidylinositol (8). The physiologic function of β_2GPI is uncertain. A role in lipid metabolism has been suggested based on the interaction of β_2GPI with lipoproteins (9). In addition, this protein exhibits antico-

Supported by Fondo de Investigación Sanitaria grants 99/0148 and 00/1048, and by the Arthritis and Rheumatism Council and Lupus UK. Dr. Espinosa's work was supported by grant FI-00684 Generalitat de Catalunya.

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Submitted for publication April 30, 2002; accepted in revised form August 8, 2002.

agulant properties: it inhibits platelet aggregation induced by adenosine diphosphate (10), intrinsic coagulation pathways (11), the prothrombinase activity of platelets (12), and activation of protein C in the presence of phospholipids (13). However, familial deficiency of β_2GPI is not a risk factor for thrombosis (14).

It has been suggested that β_2GPI may contribute to the pathogenesis of APS-associated thrombosis. In physiologic conditions, β_2GPI may inhibit phospholipid-dependent hemostasis reactions, but β_2GPI is, by itself, only a weak anticoagulant (15). However, aPL may enhance the affinity of β_2GPI to phospholipids, and then β_2GPI may become a real competitor to phospholipid-dependent hemostasis reactions. Therefore, because β_2GPI binds strongly to negatively charged proteins or phospholipids involved in coagulation processes, it is likely that aPL may hamper the β_2GPI-associated coagulation process. However, decreased levels of β_2GPI are not usually observed in APS (16).

In previous studies, we reported that plasma specimens from patients with SLE and aPL (17) or from those with primary APS (18) promoted the interaction of platelets with the collagen-rich subendothelial surface. Furthermore, we demonstrated that monoclonal IgM aCL obtained from patients with APS increased platelet interaction with the subendothelium under flow conditions (19). In the present study, we investigated whether the effect of monoclonal IgM aCL on platelet interaction with a collagen-rich surface under flow conditions is β_2GPI-dependent.

PATIENTS AND METHODS

Reconstituted blood. Blood from normal healthy volunteers was drawn in acid citrate dextrose (ACD-A; National Institutes of Health, Bethesda, MD) (8.5/1.5, volume/volume) by a clean, antecubital venipuncture without venocclusion, using a 19-gauge needle (Becton Dickinson, Rutherford, NH). None of the subjects had taken aspirin or other drugs affecting platelet function in the previous 2 weeks, and informed consent was obtained from all participants.

Blood was processed in the first 2 hours after blood drawing to obtain gel-filtered platelets, as previously reported (20). Briefly, platelet-rich plasma, obtained by centrifugation (3 minutes, 800*g* at 22°C), was pelleted (10 minutes, 2,100*g* at 22°C) after adding an additional one-tenth of ACD-A. Platelet pellet was reconstituted with 1 ml of 0.05*M* Tris HCl, 0.15*M* NaCl, 0.5% bovine serum albumin (BSA) (weight/volume), and 5 m*M* glucose, pH 7.4. Platelets were then gel-filtered through Sepharose 2B (Pharmacia, Uppsala, Sweden) equilibrated with 0.05*M* Tris HCl, 0.15*M* NaCl, and 5 m*M* glucose, pH 7.4, using the same buffer to elute the platelets (20). Erythrocytes from the same donors were washed 5 times in 0.05*M* Tris HCl, 0.15*M* NaCl, 0.5% BSA (w/v) by centrifugation and resuspended in the same buffer.

Reconstituted blood was made by adding gel-filtered platelets (200×10^9/liter, final concentration), purified factor VIII containing von Willebrand multimers (Humate-P; Aventis Behring, Marburg, Germany) (100 units/dl, final concentration), purified fibrinogen (Calbiochem, La Jolla, CA) (1.5 gm/liter, final concentration), $CaCl_2$ (Sigma, St. Louis, MO) (0.14 m*M*, final concentration), and washed erythrocytes (0.45 final hematocrit) to 0.05*M* Tris HCl, 0.15*M* NaCl, 0.5% BSA (w/v), and 5 m*M* glucose, pH 7.4. Dalteparin (Pharmacia & Upjohn, Barcelona, Spain) (20 IU/ml, final concentration) was used as anticoagulant.

Reconstituted blood, with and without the addition of the human monoclonal antibodies studied, and with and without the addition of different concentrations of purified human β_2GPI (Diagnostica Stago, Asnières-sur-Seines, France), was used in perfusion studies. Reconstituted blood samples without additional β_2GPI had levels of β_2GPI under the detection limit when tested by enzyme-linked immunosorbent assay (ELISA) (Diagnostica Stago).

Antibodies. Human monoclonal IgM aCL, with anti-β_2GPI activity (21,22), were obtained from 3 patients with APS (TM1B3 from patient 1, GR1D5 from patient 2, and EY2C9 from patient 3). One patient (patient 1) fulfilled the American College of Rheumatology revised criteria for the classification of SLE (23,24) and had secondary APS. The other 2 patients were diagnosed as having primary APS (25). Two of the patients (patients 1 and 2) had a history of thrombotic events, and 1 presented with only recurrent fetal losses (patient 3). The patients' peripheral B lymphocytes were infected with Epstein-Barr virus and cultured with mouse peritoneal cells. The aCL-producing B cells were then hybridized with ouabain-resistant, non–immunoglobulin-secreting, mouse–human heterohybridoma cell line (SHM-D33) (CRL-1668; American Type Culture Collection, Rockville, MD). Hybridomas were selected by culture in medium containing hypoxanthine, aminopterin, and thymidine. Supernatants were collected after expansion, and the immunoglobulins were purified.

In addition, a human monoclonal IgM aCL (FRO) showing both aCL and LA activity, but not anti-β_2GPI activity (26), was used. This monoclonal antibody was obtained from a patient who had a splenic IgMλ-positive B cell lymphoplasmacytic lymphoma without thrombotic events. Spleen cells from the patient were isolated and cryopreserved immediately after splenectomy. Human heterohybridomas were obtained from these spleen cells. Fusion and screening for immunoglobulin production were performed as previously reported (27,28). Hybridoma supernatants were centrifuged at 12,000 rpm for 10 minutes and filtered (0.45-μm filter) to eliminate possible cell remnants; thereafter, supernatants were dialyzed against 1,000 volume of phosphate buffered saline (PBS) and isolated by gel-filtration column chromatography using Sephacryl S-300 (Amersham Pharmacia Biotech Europe, Barcelona, Spain). Elution fractions were concentrated (Centriprep-30; Amicon, Lexington, MA) and the monoclonal IgMλ further purified by preparative electrophoresis over cellulose acetate blocks (Cellogel; Chemetron, Milan, Italy) (27,29). In its characterization, FRO proved to be a true anti-anionic β_2GPI-independent aPL (26). FRO showed high binding to cardiolipin and phosphatidylserine but failed to bind phosphatidylethanolamine (26).

This binding was similar in both the conventional ELISA and the cofactor-independent ELISA, in which fetal calf serum was replaced by 0.3% gelatin. Furthermore, the addition of 20 μg/ml of human β_2GPI in the cofactor-independent ELISA failed to affect the binding (26).

Finally, a human monoclonal IgM antibody obtained by the same method (21,22) as that used to obtain TM1B3, GR1D5, and EY2C9, but without aCL, LA, or anti-β_2GPI activity, was used as the IgM control.

Perfusion studies and morphometric evaluation. Progressive amounts of human monoclonal aCL TM1B3, GR1D5, EY2C9, and FRO or control IgM (final concentration ranging from 9 to 50 μg/ml) were incubated (15 minutes at 37°C) in tubes containing 20 ml of reconstituted blood, with and without the addition of 20 μg/ml β_2GPI (final concentration). To determine β_2GPI dose-response curves, perfusions were carried out in reconstituted blood incubated (15 minutes at 37°C) with the monoclonal aCL GR1D5 (30 μg/ml) or control IgM (30 μg/ml), with the addition of progressive amounts of human purified β_2GPI (ranging from 0 to 50 μg/ml).

Perfusions were carried out (10 minutes at 37°C) in annular perfusion chambers (30). Enzymatically denuded (with α-chymotrypsin) rabbit aorta segments (31), which were mounted on plastic rods, were exposed to recirculated blood. A hemodialysis blood pump (Minntech Renal Systems, Minneapolis, MN) was used to obtain the appropriate flow to generate a wall shear rate of 800 seconds^{-1}. At the end of the perfusion studies, the segments were rinsed with PBS, pH 7.2, and the rods were removed from the chamber. The segments were sliced off, fixed in glutaraldehyde–formaldehyde solution (2%/3% volume/volume), embedded in JB-4 compound (Polysciences, Warrington, PA), and histologically processed as previously described (32). Three-micron sections were obtained from plastic blocks, stained with toluidine blue, and used for morphometric evaluations.

Morphometry was performed using a manual optical picture analysis system (MOP 20; Kontron, Zurich, Switzerland) connected to a computer with an automated recognition program (32). Studies were designed so that the person performing the morphometric studies was not aware of the experimental design. Platelets interacting with subendothelium were evaluated following criteria previously described (30). Platelets or groups of platelets were classified as follows: contact platelets, which were attached to but not spread on the subendothelium; adhesion platelets, which were spread on subendothelium or form layers <5 μm in height; and large platelet aggregates (or thrombi) that were ≥ 5 μm in height. All of these basic parameters were expressed as a percentage of the total length of the vessel screened. The total platelet-covered surface was calculated by adding these basic parameters (contact platelets + adhesion platelets + thrombi).

Statistical analysis. Results are expressed as the mean ± SD. Morphometric parameters obtained using the different monoclonal antibodies with and without β_2GPI addition were compared by paired *t*-test or Dunnett's test (33). Analysis of variance with repeated measures was used for comparisons between β_2GPI dose-response curves, followed by paired *t*-test. *P* values of 0.05 or less were considered statistically significant.

Table 1. Platelet interaction with the subendothelium as determined by the percentage of total surface covered by platelets*

Antibody, 30 μg/ml	Reconstituted blood	
	Without β_2GPI	With β_2GPI, 20 μg/ml
Control	17.1 ± 5.5	16.4 ± 5.7
TM1B3	19.9 ± 6.7	28.1 ± 5.2†
GR1D5	18.6 ± 5.9	29.7 ± 6.3†
EY2C9	18.7 ± 6.2	27.9 ± 4.6†
FRO	18.8 ± 4.9	20.1 ± 7.3

* Values are the mean ± SD percentage (n = 3 experiments) of total surface covered by platelets. β_2GPI = β_2-glycoprotein I.
† $P < 0.01$ versus control.

RESULTS

Table 1 shows the percentage of total surface covered by platelets for each vessel, observed under the described experimental conditions, using monoclonal aCL with anti-β_2GPI activity (TM1B3, GR1D5, EY2C9), aCL without anti-β_2GPI activity (FRO), and monoclonal IgM (control) (30 μg/ml each). No differences in the percentage of platelet-covered surface in comparison with control IgM were detected when the reconstituted blood samples without the addition of human β_2GPI were incubated with the monoclonal aCL TM1B3, GR1D5, EY2C9, and FRO. A significant increase in the percentage of platelet-covered surface in comparison with the control samples (incubated with control IgM) was observed in the presence of the monoclonal aCL with anti-β_2GPI activity (TM1B3, GR1D5, EY2C9) when samples with β_2GPI (20 μg/ml) were added (Figure 1). However, no increase in the percentage of platelet-covered surface was seen in the reconstituted β_2GPI-supplemented blood samples when they were incubated with the monoclonal FRO and control IgM.

The total surface covered by thrombi (Table 2) followed the same profile as that of total platelet-covered surface. In the reconstituted blood samples without addition of β_2GPI, no increase was seen after incubation with the monoclonal aCL TM1B3, GR1D5, EY2C9, and FRO. In contrast, the total surface covered by thrombi increased in comparison with control samples in the presence of the monoclonal aCL TM1B3 and GR1D5 when reconstituted blood was added to 20 μg/ml of β_2GPI. The monoclonal antibody EY2C9, as well as FRO, did not significantly increase the percentage of total surface covered by thrombi in the presence of reconstituted, β_2GPI-supplemented blood samples.

In the β_2GPI dose-response curves for the mor-

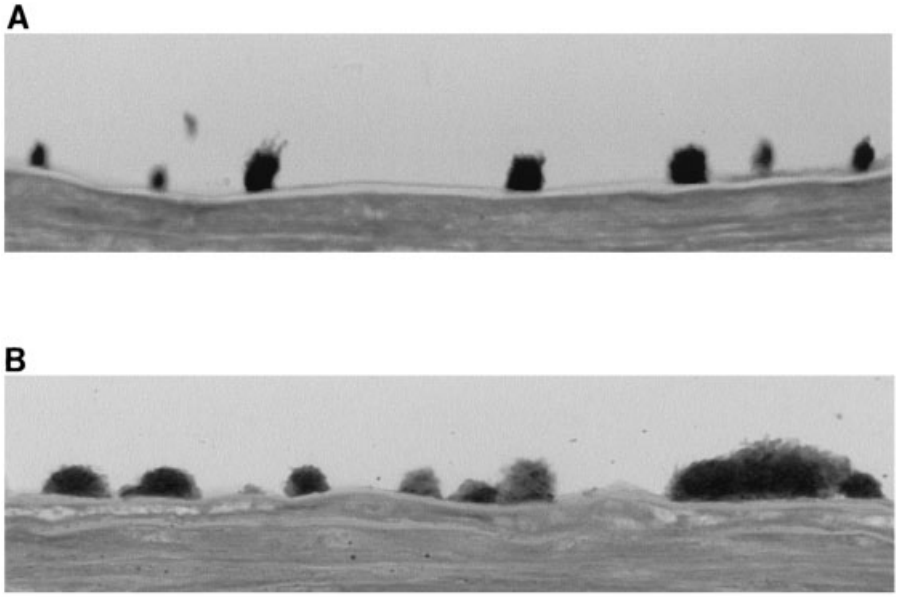

Figure 1. Platelet deposition. Perfusions were performed in reconstituted blood (gel-filtered platelets, washed erythrocytes, purified factor VIII containing von Willebrand multimers, purified fibrinogen) with 30 μg/ml of TM1B3, a human monoclonal anticardiolipin antibody with anti–β_2-glycoprotein I (anti-β_2GPI) activity. Perfusions were performed with and without the addition of β_2GPI in annular chambers containing α-chymotrypsin–denuded rabbit aorta segments (10 minutes, 37°C, wall-shear rate 800 seconds^{-1}). **A,** Effect of TM1B3 in the absence of β_2GPI. **B,** Effect of TM1B3 when 20 μg/ml of purified B_2GPI was added.

phometric parameters, the total platelet-covered surface and total surface covered by thrombi at a fixed concentration of the antibody of 30 μg/ml showed significant increases using GR1D5 ($P < 0.01$) but not using control IgM (Figure 2). The monoclonal antibody GR1D5 increased the total platelet-covered surface and total surface covered by thrombi at β_2GPI concentrations of 5 μg/ml or higher.

Table 2. Platelet interaction with the subendothelium as determined by the total percentage of surface covered by thrombi*

	Reconstituted blood	
Antibody, 30 μg/ml	Without β_2GPI	With β_2GPI, 20 μg/ml
Control	9.1 ± 5.0	9.2 ± 4.6
TM1B3	8.9 ± 4.7	16.7 ± 5.6†
GR1D5	9.0 ± 4.9	17.2 ± 6.3†
EY2C9	8.8 ± 4.8	10.3 ± 5.0
FRO	9.4 ± 6.1	9.8 ± 5.8

* Values are the mean ± SD percentage (n = 3 experiments) of surface covered by thrombi. β_2GPI = β_2-glycoprotein I.
† $P < 0.01$ versus control.

DISCUSSION

In the present study, we evaluated the dependence of β_2GPI on the effect of monoclonal IgM aCL on platelet interaction with collagen-rich subendothelium under flow conditions. This model may reproduce physiologic situations, because lesions causing small denuded arterial surfaces may be produced by pathologic or even physiologic conditions, as, for example, in hemodynamic jets. The use of whole serum or whole Ig fractions to study the role of aPL in the predisposition to thrombosis in APS can yield conflicting results (34). The use of monoclonal aPL, which has well-characterized biologic properties, may remove this problem and provide a more appropriate source of antibodies for investigation of the mechanisms of APS (35).

The 3 monoclonal aCL with anti-β_2GPI activity (TM1B3, GR1D5, and EY2C9) increased the total platelet-covered surface in reconstituted blood, showing a clear β_2GPI dependence, and 2 of them (TM1B3 and GR1D5) increased the total surface covered by thrombi as well. In contrast, the monoclonal FRO, an IgM with aCL and LA activities but without anti-β_2GPI activity,

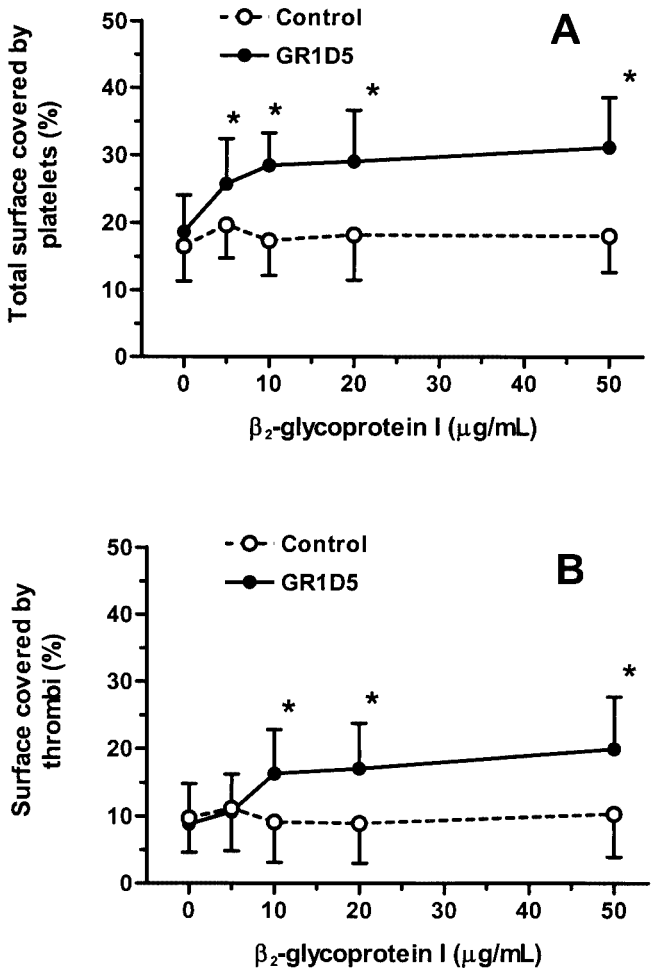

Figure 2. β_2-glycoprotein (β_2GPI) dose-response curves. Perfusions were performed in reconstituted blood incubated (15 minutes at 37°C) with 30 μg/ml of GR1D5, a human monoclonal anticardiolipin antibody with anti-β_2GPI activity, or with 30 μg/ml control IgM, with the addition of progressive amounts (glycoprotein 0–50 μg/ml) of human purified β_2GPI. **A,** Dose-response curves for the morphometric parameter total surface covered by platelets (expressed as percentage of total surface, mean and SD from 3 experiments). **B,** Dose-response curves for the morphometric parameter surface covered by thrombi (large platelet aggregates $\geq$5 μm in height) (mean and SD from 3 experiments). The monoclonal antibody GR1D5 plus β_2GPI at doses of 5 μg/ml or higher increased the total surface covered by platelets and the surface covered by thrombi. $* = P < 0.01$ versus Control.

was not able to increase either the total platelet-covered surface or total surface covered by thrombi, even in the presence of β_2GPI. As recently reported (26), this true anti-anionic monoclonal aPL FRO, which is secreted by hybridoma clones growing in protein-free culture medium, showed, in a β_2GPI-free system, binding to solid-phase cardiolipin and phosphatidylserine and to membrane of phosphatidylserine-expressing cells and to activated platelets, but failed to bind β_2GPI, histone, double- and single-stranded DNA, and human IgG. These findings may help to explain the role of β_2GPI in the thromboses seen in APS.

Platelets may be targets of circulating aPL, thereby having a role in causing antibody-mediated thrombosis (36,37). The interaction of aPL with platelets can occur by at least 3 different mechanisms (38): binding of the Fab terminus to platelet antigens, binding in the form of immune complexes via Fcγ receptor type II (FcγRII), and nonspecific binding. The FcγRII present on platelets (39) has weak affinity for the Fc portion of monomeric IgG but high affinity for the Fc portion of IgG contained in immune complexes or bound to an antigen on the platelet surface (40). The binding of aPL to the platelet surface may be higher on activated or damaged platelets than on resting ones (41–43), and human aCL can bind to activated platelets only in a β_2GPI-dependent manner (42). Antiphospholipid antibodies may potentiate platelet aggregation agonists (44–46), but the presence of β_2GPI was required in a murine model (44).

The mechanisms relating to anti-β_2GPI activity, aPL platelet binding, and platelet activation leading to thrombosis seem to be similar to those proposed for heparin-induced thrombocytopenia and thrombosis (47,48), where the target of heparin-induced antibodies is a complex of heparin and platelet factor 4 that may activate platelets through FcγRII (49,50). The first step in this hypothesis includes previous platelet activation, but it seems unlikely that aPL initiate this activation (51,52). Platelet activation induces the exposure of anionic phospholipids (53), mainly phosphatidylserine, that is located predominantly in the cytoplasmatic portion of the membranes in resting platelets (54), accompanied by shedding of procoagulant microvesicles (55) and an increase in the extent of binding of β_2GPI (56). Our perfusion experiments were performed by reproducing physiologic situations on a collagen-rich subendothelial surface. Collagen increases the amount of phospholipids exposed in platelets by thrombin activation (57,58). In the presence of small disruptions of the endothelium, platelets may be exposed to collagen, leading to their activation and also increasing expression of Fcγ receptor (59). The second step includes aPL binding to the formed β_2GPI–anionic phospholipid complexes on activated platelet membranes. Binding of aPL to β_2GPI in the fluid phase is weak (60), but conformational changes in β_2GPI induced by its binding to negatively charged surfaces have been suggested (61,62).

All of the monoclonal aCL used in the present

experiments belonged to the IgM class. IgM does not activate FcγRII; therefore, these monoclonal IgM aCL with anti-β_2GPI activity could exert their action through complement activation. Although this hypothesis was not investigated in the present study, it may be supported by the increased levels of inactivated terminal membrane attack complex (C5b-9) observed in patients with APS and cerebral ischemia (63), by the decreased levels of serum complement seen in patients with aPL (64), and by the effect of cardiolipin liposomes causing complement activation (65). C5b-9 complex may cause platelet activation (66) and increase the transbilayer migration of phosphatidylserine in the platelet membrane (67). In addition, Holers et al (68) recently demonstrated that in vivo complement activation is required for aPL-induced fetal loss and growth retardation in a murine model of APS in which pregnant mice were injected with human aPL.

In conclusion, we have demonstrated that the platelet interaction with the subendothelium under flow conditions generated by the monoclonal IgM aCL evaluated requires the presence of β_2GPI.

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COMENTARI:

En aquest article hem analitzat el paper patogènic que la β_2GPI juga en el desenvolupament de les trombosis a la síndrome antifosfolipídica. Més concretament, hem estudiat el paper de la β_2GPI en la interacció de les plaquetes amb el subendoteli promoguda per anticossos anticardiolipina monoclonals. Això ho hem portat a terme en condicions de flux utilitzant una càmara de Baumgartner.

L'estudi s'ha portat a terme amb tres anticossos anticardiolipina monoclonals (TM1B3, GR1D5 i EY9C) amb activitat anti-β_2GPI obtinguts de tres pacients amb síndrome antifosfolipídica, un quart anticòs anticardiolipina monoclonal (FRO) però sense activitat anti-β_2GPI, obtingut d'un pacient amb linfoma esplènic sense fenòmens trombòtics, i una immunoglobulina d'isotip IgM (la mateixa que els quatre anticossos monoclonals) sense activitat anticardiolipina ni anti-β_2GPI que s'ha utilitzat com control. A més, s'utilitzaren per a l'estudi, sang reconstituïda i un circuit de flux a on es col·locà un fragment d'artèria de conill amb l'endoteli exposat. La sang reconstituïda juntament amb cada un dels anticossos es va fer circular pel circuit tancat i es va mesurar la superfície de l'endoteli recoberta per agregats i per trombes plaquetars, afegint o no una quantitat determinada de β_2GPI.

Sense l'addició de β_2GPI, no van existir diferències entre les diferents determinacions de la superfície d'endoteli coberta per agregats plaquetars o trombes promoguda pels anticossos anticardiolipina avaluats. En canvi, amb l'addició de β_2GPI, els tres anticossos anticardiolipina amb activitat anti-β_2GPI van ser capaços d'augmentar la superfície d'endoteli coberta per agregats plaquetars. A més, dos d'ells (TM1B3 i GR1D5), procedents de pacients amb síndrome antifosfolipídica i trombosis, també augmentaven la superfície coberta per trombes. El tercer monoclonal amb activitat anti-β_2GPI (EY9C), obtingut d'una pacient amb síndrome antifosfolipídica i exclusivament

patologia obstètrica, no va ser capaç d'augmentar la superfície coberta per trombes. Pel que fa a l'anticòs monoclonal sense activitat anti-β_2GPI (FRO) i a la immunoglobulina d'isotip IgM sense activitat anticardiolipina ni anti-β_2GPI, no van augmentar la superfície de l'endoteli coberta per agregats ni per trombes malgrat l'addició de β_2GPI a la sang reconstituïda.

A la segona part del treball es van determinar les corbes dosi-resposta, utilitzant un dels tres monoclonals (GR1D5), incrementant progressivament la concentració de β_2GPI i mesurant la superfície coberta de l'endoteli. Les corbes resultants van indicar que a partir d'una concentració de β_2GPI de 5 μg/ml, el monoclonal GR1D5 era capaç d'incrementar la interacció plaquetar amb l'endoteli, mesurada tant per la formació d'agregats com de trombes plaquetars.

Per tant, amb aquest segon estudi hem demostrat que el potencial patogènic trombòtic dels anticossos anticardiolipina monoclonals avaluats, mesurat per la interacció de les plaquetes amb el subendoteli en condicions de flux, depèn de la seva activitat anti-β_2GPI. A més, la presència en el medi d'aquesta β_2GPI és essencial per tal que els anticossos analitzats puguin dur a terme aquesta interacció plaquetar.

Un dels aspectes més importants d'aquest treball rau en les possibilitats que permet la metodologia utilitzada. Amb els anticossos monoclonals i la sang reconstituïda aconseguim obviar les variables de confusió que apareixen si es fa servir sang total o plasma dels pacients que, de manera fisiològica contenen β_2GPI. A més, el sistema de perfussió usat permet reproduir situacions fisiològiques, ja que l'endoteli pot lesionar-se en condicions patològiques però també en condicions normals com per exemple en presència de *jets* hemodinàmics.

Vascular Involvement in Behçet's Disease: Relation with Thrombophilic Factors, Coagulation Activation, and Thrombomodulin

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PURPOSE: Thrombosis, usually venous, occurs in 10% to 25% of patients with Behçet's disease, but its pathogenesis is poorly understood. We evaluated parameters of hemostasis and their relation with thrombosis in a series of patients with Behçet's disease.

SUBJECTS AND METHODS: We studied 38 patients with Behçet's disease (13 with venous thrombosis), 38 patients with venous thrombosis without thrombophilia, and 100 control subjects. Levels or presence of protein C, protein S, antithrombin, methylenetetrahydrofolate reductase C677T, factor V Leiden, prothrombin gene G20210A, antiphospholipid antibodies, plasminogen, tissue-type plasminogen activator (tPA), type-1 tPA inhibitor (PAI-1), PAI-1 4G/5G polymorphism, prothrombin fragment 1+2, plasmin/α_2-antiplasmin complexes, thrombomodulin, and activated factors VII and XII were determined.

RESULTS: There were no deficiencies in protein C, protein S, antithrombin, or factor V Leiden in the patients with Behçet's disease, nor was there evidence of most other thrombotic abnormalities. Compared with control subjects, however, the Behçet's disease group had elevated mean (±SD) levels of prothrombin fragment 1+2 (2091 ± 1323 pmol/L vs. 804 ± 398 pmol/L, $P < 0.001$), plasmin/α2-antiplasmin complexes (410 ± 220 μg/L vs. 214 ± 92 μg/L, $P < 0.001$), and thrombomodulin (37 ± 24 ng/mL vs. 27 ± 10 ng/mL, $P < 0.001$). These levels did not differ between patients with or without thrombosis.

CONCLUSION: Thrombophilic factors do not seem to explain most thromboses in Behçet's disease. There is increased thrombin generation, fibrinolysis, and thrombomodulin in Behçet's disease, but these abnormalities are not related to thrombosis. **Am J Med. 2002;112:37–43.** ©2002 by Excerpta Medica, Inc.

Behçet's disease is a systemic vasculitis of unknown etiology, characterized by recurrent oral and genital ulcers and uveitis (1). Cutaneous, articular, neurologic, intestinal, pulmonary, urogenital, or vascular manifestations have also been observed (2). The prevalence of vascular involvement—such as venous or arterial thrombosis and arterial aneurysms—in Behçet's disease is estimated to be about 25% (3).

The pathogenesis of thrombus formation in Behçet's disease is poorly understood. The effects of thrombophilic parameters, such as protein C, protein S, antithrombin deficiencies, antiphospholipid antibodies, and factor V Leiden have been studied (4,5). However, most studies have investigated these factors individually, with conflicting results. Furthermore, only a few studies have analyzed both coagulation and fibrinolysis activation (6,7). Most thrombotic abnormalities have been attributed to the endothelial injury secondary to vasculitis (8,9), but the mechanisms by which endothelial lesions activate the coagulation pathways are not known.

The present study was undertaken to evaluate thrombophilic parameters, thrombin and plasmin generation, and markers of the extrinsic and intrinsic coagulation pathways in patients with Behçet's disease, and to correlate these data with thrombosis.

METHODS

Patients

We studied 38 patients with Behçet's disease (21 men and 17 women). The mean (± SD) age at onset of the disease was 27 ± 12 years (range, 10 to 58). The mean age at diagnosis was 32 ± 13 years (range, 16 to 62); and the mean age at study inclusion was 35 ± 14 years (range, 16 to 67). Follow-up ranged from 1 to 242 months, with a median of 36 months. All patients fulfilled three or more of the International Study Group criteria for the diagnosis of Behçet's disease (10). Blood samples were collected

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Supported by Spanish grants FIS-97/0525, FIS-98/0171, FIS-99/0148, and FIS-00/1048, and grant 1999-FI-00684 CIRIT from Generalitat de Catalunya (1999/2001).

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Manuscript submitted March 14, 2001, and accepted in revised form September 18, 2001.

a median of 15 months (range, 0 to 129) after diagnosis during an inactive phase of the disease.

We also analyzed a group of 38 patients (21 women and 17 men; mean age, 34 ± 15 years) with a first deep venous thrombosis of the lower extremities, including 7 patients with pulmonary embolism who had been consecutively referred for evaluation and in whom causes of thrombophilia were not identified. Thrombophilia was defined as deficiencies in protein C or protein S; antithrombin, plasminogen, or protein C resistance; factor V Leiden mutation; hyperhomocysteinemia; prothrombin gene G20210A mutation; or antiphospholipid antibodies. Obese or pregnant patients, and patients with cancer, were excluded. We also analyzed a control group of 100 healthy subjects (54 women and 46 men; mean age, 41 ± 18 years) without autoimmune disease, bleeding disorders, or thrombosis.

All thrombotic events were assessed clinically and confirmed by objective methods. The diagnosis of deep venous thrombosis was confirmed by Doppler ultrasonographic scans or venography. Pulmonary embolism was diagnosed with ventilation-perfusion lung scan. Cerebrovascular ischemic episodes were confirmed by computed tomographic scanning or magnetic resonance imaging techniques. The study was approved by the Human Experimental Committee of the Hospital Clínic, Barcelona, Spain, and was performed according to the principles of the Declaration of Helsinki. Informed consent was obtained from all the participants.

Sampling

Venous blood samples were drawn from an antecubital venipuncture without venocclusion, in the morning, with the patient sitting and resting. Samples for coagulation and fibrinolysis studies were obtained in tubes containing tripotassium citrate (Becton, Dickinson and Company; Rutherford, New Jersey). Platelet-free plasma was immediately obtained by double centrifugation, first at 2000 g for 10 minutes at 22°C and then at 5000 g for 10 minutes at 4°C. Plasma was aliquoted, snap frozen in a mixture of dry ice/ethanol (1:2, vol:vol), and stored. For tissue-type plasminogen activator (tPA) activity, samples were obtained in strong acidic citrate tubes (Biopool, Umeå, Sweden). For genotype studies, samples were drawn in trisodium ethylene-diamine-tetraacetic acid tubes (Becton, Dickinson and Company), and 100 mL of whole blood was transferred into tubes containing lysis buffer (5 mol/L guanidine thiocyanate, 1.3% [w/v] Triton X-100, and 50 mM Tris-HCl, pH 6.4) and frozen at –70°C. Sera for biochemistry and for antibody studies were drawn in tubes containing no anticoagulants (Becton, Dickinson and Company).

Laboratory Studies

Prothrombin and activated partial thromboplastin time were determined in an automated CA-6000 analyzer (Dade-Behring, Marburg, Germany) using standard reagents (Thromboplastin IS and Actin FSL; Dade-Behring). Fibrinogen was measured by the Clauss technique.

Protein C activity was quantified by a colorimetric assay (Chromogenix, Mölndal, Sweden). Free and total protein S were quantified by enzyme-linked immunosorbent assay (ELISA; Stago, Asnières, France). Antithrombin activity was measured using a chromogenic assay (Dade-Behring). Methylenetetrahydrofolate reductase C677T mutation was determined by polymerase chain reaction (PCR) amplification (11). Activated protein C resistance was determined in coagulative assays (Chromogenix) (12). Factor V Leiden mutation was determined with a nucleic acid sequence–based amplification assay (Organon Teknika, Boxtel, The Netherlands) (13) in subjects with phenotypic activated protein C resistance or with lupus anticoagulant, which may interfere with activated protein C resistance measurements. For the prothrombin gene G20210A mutation, deoxyribonucleic acid (DNA) samples were analyzed by PCR (14).

Serum levels of cholesterol and triglycerides and lipoprotein fractions were determined by enzymatic and colorimetric methods (15). Serum glucose level was measured in an autoanalyzer (Bayer, Tarrytown, New York), and glycosylated hemoglobin was assessed by spectrophotometric chromatography in an ionic exchange microcolumn (Biosystem, Barcelona, Spain).

Lupus anticoagulant was detected using activated partial thromboplastin time, diluted Russell's viper venom time, and tissue thromboplastin inhibition test. Tests were performed following the guidelines of the Subcommittee for the Standardization of Lupus Anticoagulants of the International Society of Thrombosis and Hemostasis (16). Anticardiolipin antibodies were measured using standardized ELISA (Chesire Diagnostics, Chester, United Kingdom).

Plasminogen and tPA activity were evaluated using a chromogenic assay (Chromogenix), and plasma antigen related to type-1 tPA inhibitor (PAI-1) was measured by ELISA (Biopool). For detection of the PAI-1 4G/5G polymorphism, a protocol based on PCR technique and endonuclease digestion was used (17).

The prothrombin fragment 1+2, a thrombin generation marker, was assessed by ELISA (Dade-Behring). Plasma levels of plasmin/α_2-antiplasmin complexes were quantified as plasmin generation marker by ELISA (Dade-Behring).

Activated factor XII was determined by a direct immunoassay (Shield Diagnostics, Dundee, United Kingdom), and activated factor VII was determined by ELISA (American Diagnostica, Greenwich, Connecticut). Plasma levels of thrombomodulin were assessed by ELISA (Diagnostica Stago, Asnières, France).

Table 1. Clinical Characteristics of the 38 Patients with Behçet's Disease

	Number (%)
Mouth ulcers	38 (100)
Genital ulcers	25 (66)
Cutaneous involvement	26 (68)
Erythema nodosum	16 (42)
Pseudofolliculitis	13 (34)
Eye involvement	21 (55)
Anterior uveitis	12 (32)
Retinal vasculitis	6 (16)
Posterior uveitis	6 (16)
Fever	14 (37)
Thrombotic events	14 (37)
Deep venous thrombosis	13 (34)
Stroke	1 (3)
Neurologic manifestations	5 (13)
Central nervous system	4 (11)
Peripheral nervous system	2 (5)
Arterial involvement	3 (8)
Artery aneurysm	1 (3)
Pseudoaneurysm	2 (5)
Gastrointestinal involvement	2 (5)

Statistical Analysis

Continuous results are reported as mean ± SD. Comparisons were made with the chi-squared test or analysis of variance. Homogeneity of variances was confirmed with Snedecor's F test. Patient groups were compared with the control group using Dunnett's procedure (18). When Behçet's disease patients with or without venous thrombosis were compared, the single patient with arterial thrombosis was excluded. The numbers observed of each genotype were compared with those expected for a population in Hardy-Weinberg equilibrium with the chi-squared test. The correlation between thrombomodulin and prothrombin fragments 1+2 levels was evaluated with Pearson's linear regression.

RESULTS

Fourteen patients with Behçet's disease had thrombotic events, including 13 with deep venous thrombosis, one of whom had a pulmonary thromboembolism (Table 1). Two patients had recurrent thrombosis (one deep venous thrombosis and one cerebral venous sinus thrombosis).

Laboratory Studies

Activated partial thromboplastin and prothrombin times, and levels of fibrinogen, were normal in patients and control subjects.

Thrombophilic parameters. No patients with protein C or protein S deficiency were identified. Antithrombin values were normal in all patients. The homozygous methylenetetrahydrofolate reductase C677T mutation was found in 5 Behçet's disease patients (13%), none of whom had thrombotic events, and in 21 control subjects (21%). Activated protein C resistance due to heterozygous factor V Leiden mutation was detected in 2 control subjects but no patients with Behçet's disease. The heterozygous prothrombin gene G20210A mutation was found in 1 patient with Behçet's disease with deep venous thrombosis and in 1 control. Serum levels of cholesterol, triglycerides, and lipoproteins were within the normal range and similar among the groups. Patients and control subjects had normal values of serum glucose and glycosylated hemoglobin.

Antiphospholipid antibody studies. The lupus anticoagulant was positive in 2 Behçet's disease patients (5%), none of whom had thrombotic events. One Behçet's disease patient with venous thrombosis had immunoglobulin (Ig) M class anticardiolipin antibodies. No one in the control group had either the lupus anticoagulant or anticardiolipin antibodies.

Fibrinolysis parameters and polymorphism. Plasminogen and tPA activity were within the normal range in all of the patients and control subjects (Table 2). Plasma levels of PAI-1 antigen, as well as allele (4G/5G) frequencies, did not differ among the groups (Table 2).

Thrombin and plasmin generation markers. Levels of prothrombin fragment 1+2, a thrombin generation marker, was elevated significantly in Behçet's disease patients compared with thrombotic patients without thrombophilia or control subjects (Table 2). Levels of plasmin/α_2-antiplasmin complexes, a plasmin generation marker, were also elevated significantly in patients with Behçet's disease compared with patients with thrombosis without thrombophilia or control subjects (Table 2). Among patients with Behçet's disease, there were no differences in prothrombin fragment 1+2 or plasmin/α_2-antiplasmin complexes when those with and without thrombosis were compared (Table 3).

Activated blood coagulation factors and thrombomodulin. Mean values of activated factor VII and activated factor XII were similar in Behçet's disease patients, patients with thrombosis without thrombophilia, and control subjects (Table 2). There was no difference in activated factor VII or activated factor XII by the presence or absence of thrombosis among patients with Behçet's disease (Table 3).

Thrombomodulin levels were elevated significantly in Behçet's disease patients compared with control subjects ($P < 0.001$, Table 2). Among patients with Behçet's disease, thrombomodulin levels correlated significantly with prothrombin fragment 1+2 levels ($r = 0.40$, $P = 0.01$). No increases in thrombomodulin levels were detected among patients with thrombosis without thrombophilia.

Table 2. Fibrinolysis Parameters, Thrombin and Plasmin Generation Markers, Activated Blood Coagulation Factors, Thrombomodulin, and Type-1 Tissue-type Plasminogen Activator Inhibitor Genotype Distribution in Behçet's Disease Patients and Control Subjects

Measurement (units)	Behçet's Disease (n = 38)	Thrombosis without Thrombophilia (n = 38)	Control Subjects (n = 100)
	Mean ± SD		
Plasminogen (%)	108 ± 16	104 ± 15	105 ± 16
tPA activity (IU/mL)	2 ± 1	3 ± 1	2 ± 1
PAI-1 antigen (ng/mL)	21 ± 12	16 ± 11	20 ± 12
PAI-1 genotype 4G/4G5G/5G	6/22/10	8/19/11	21/52/27
PAI-1 4G allele frequency (%)	45	46	47
Prothrombin fragment 1+2 (pmol/L)	2091 ± 1323*†	1183 ± 521	804 ± 398
Plasmin/α_2-antiplasmin complexes (μg/L)	410 ± 220*†	216 ± 81	214 ± 92
Activated factor XII (ng/mL)	3 ± 1	3 ± 2	3 ± 1
Activated factor VII (ng/mL)	3 ± 1	3 ± 2	3 ± 1
Thrombomodulin (ng/mL)	37 ± 24*†	26 ± 13	27 ± 10

* $P < 0.001$ vs. unexplained venous thrombosis.
† $P < 0.001$ vs. controls.
PAI-1 = type-1 plasminogen activator inhibitor; tPA = tissue-type plasminogen activator.

There was no significant difference in thrombomodulin values in Behçet's disease patients by the presence or absence of thrombosis (Table 3).

DISCUSSION

The pathogenesis of thrombotic events in Behçet's disease is unknown. We found that, compared with healthy control subjects or patients with other causes of thrombosis, those with Behçet's disease have increased thrombin generation and an activated fibrinolytic process.

Like most prior studies (4,8,19,20), we found normal values of protein C, protein S, and antithrombin activity in patients with Behçet's disease. However, two cases of Behçet's disease with venous thrombosis and protein C or protein S deficiency have been described (21,22), and another study reported that free protein S levels and protein S activity were reduced in Behçet's disease (23). Two studies have reported a deficiency in antithrombin activity, unrelated to thrombosis in patients with Behçet's disease (4,24). The prevalence of the methylenetetrahydrofolate reductase C677T mutation in Behçet's disease has been studied in only one series (25); as in the present

Table 3. Fibrinolysis Parameters, Thrombin and Plasmin Generation Markers, Activated Blood Coagulation Factors, Thrombomodulin, and Type-1 Tissue-type Plasminogen Activator Inhibitor Genotype Distribution in Behçet's Disease Patients with or without Venous Thrombosis

Measurement (units)	Behçet's Disease with Thrombosis (n = 13)	Behçet's Disease without Thrombosis (n = 24)
	Mean (± SD)	
Plasminogen (%)	110 ± 10	107 ± 19
tPA activity (IU/mL)	2 ± 1	2 ± 1
PAI-1 antigen (ng/mL)	24 ± 15	19 ± 12
PAI-1 genotype 4G/4G5G/5G	3/6/4	3/15/6
PAI-1 4G allele frequency (%)	46	44
Prothrombin fragment 1+2 (pmol/L)	2454 ± 1487	1913 ± 1210
Plasmin/α_2-antiplasmin complexes (μg/L)	451 ± 220	389 ± 227
Activated factor XII (ng/mL)	3 ± 1	3 ± 1
Activated factor VII (ng/mL)	3 ± 1	3 ± 1
Thrombomodulin (ng/mL)	39 ± 16	36 ± 27

PAI-1 = type-1 plasminogen activator inhibitor; tPA = tissue-type plasminogen activator.

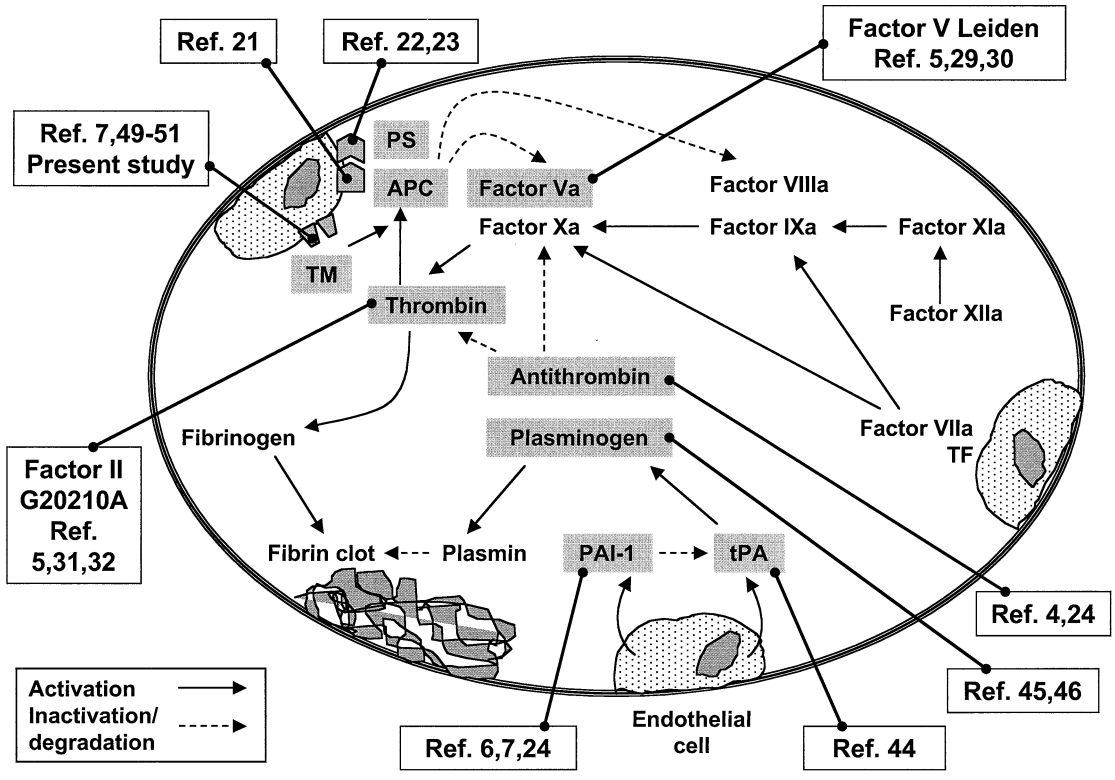

Figure. Model of hemostasis pathways (coagulation and fibrinolysis) showing possible factors involved in the thrombosis in Behçet's disease. APC = activated protein C resistance; factor VIIa = activated factor VII; PAI-1 = type-1 tissue-type plasminogen activator inhibitor; PS = protein S; tPA = tissue-type plasminogen activator; TF = tissue factor; TM = thrombomodulin.

study, no relation was found with thrombosis. The prevalence of factor V Leiden in Behçet's disease varies from 0% to 38% (4,5,26–29). An increased prevalence of factor V Leiden in Behçet's disease patients with venous thrombosis was found in two groups of patients of different ethnic origin (5,29), as well as among patients with retinal occlusive disease (30). We did not detect factor V Leiden in any of our patients with Behçet's disease. The prothrombin G20210A mutation has been studied in two large series, one of which reported no increase in the frequency of the prothrombin gene mutation (25); the other reported a significant relation with thrombosis (5). In addition, 3 patients with Behçet's disease who had recurrent venous thrombosis, arterial thrombosis, or intracardiac thrombosis associated with the prothrombin G20210A mutation have been described (31,32). We observed no differences in the prothrombin gene mutation frequency between patients and control subjects.

The frequency of anticardiolipin antibodies in Behçet's disease has varied between 0% and 47% (4,33–42). Most (4,33–35,37–42), but not all (36), studies have reported no correlation between these antibodies and vascular events. The prevalence of lupus anticoagulant has ranged from 0% to 8% (4,33,43), consistent with our observations.

We detected an activated fibrinolytic process evidenced by increased levels of plasmin/α_2-antiplasmin complexes, as has been found in other series (7,8). Studies of the components of fibrinolysis are contradictory. Tissue-type plasminogen activator antigen values have been reported as normal (6,7,24) or reduced (44), and tPA activity levels as normal (6) or elevated (7). Type-1 tPA inhibitor antigen has been found to be elevated in most studies (6,7,24), with normal (44) or elevated (6,7) activity. No association has been found between these parameters and thrombosis in patients with Behçet's disease (6,7,24,44). These differences could be because of different laboratory methods or disease activity status (24). In our study, all blood samples from patients with Behçet's disease and those with thrombosis without thrombophilia were taken in the inactive phase, perhaps explaining the low or normal values observed.

As in a previous report, we found normal plasminogen concentrations (6). There are two case reports of Japanese patients with Behçet's disease who had recurrent thrombosis associated with hereditary abnormal plasminogen-

emia (45,46). Two studies have reported that levels of prothrombin fragment 1+2, thrombin-antithrombin, and plasmin/α_2-antiplasmin complexes are increased in Behçet's disease patients (7,8). In a single case report, plasmin/α_2-antiplasmin complexes were low, whereas prothrombin fragment 1+2 was increased (47). Plasmin/α_2-antiplasmin complexes were normal in 5 patients with Behçet's disease (48). Increased thrombin generation has been attributed to the activation of coagulation because of endothelial lesions (7,8), leading to factor VII activation. In addition, factor XII can be activated by negatively charged surfaces exposed in injured vessel walls. In our series, levels of activated factors VII and XII were not elevated in patients with Behçet's disease, suggesting that the increased thrombin generation may have been related to disturbances in negative feedback pathways that regulate clotting, such as the anticoagulant protein C system.

Thrombomodulin is essential for protein C activation that is mediated by thrombin in cell surfaces, and we found significantly elevated thrombomodulin levels in patients with Behçet's disease in comparison with patients with thrombosis without thrombophilia and control subjects. Some authors have also reported increased levels of soluble thrombomodulin in Behçet's disease, like most (7,49–51), but not all (52), previous studies.

Soluble thrombomodulin is released in vitro when endothelial cells are damaged (53), and the increased thrombomodulin levels in Behçet's disease may be due to an accelerated release of thrombomodulin from injured endothelial cells. Although soluble thrombomodulin affects blood clotting in vitro (54), its function in vivo is uncertain (55). Increased soluble thrombomodulin levels are mainly due to the release of fragments of degraded forms of thrombomodulin (56). The increased levels of released thrombomodulin fragments in Behçet's disease are probably less efficient than normal thrombomodulin in binding to thrombin and activating protein C. Moreover, thrombomodulin that is released from endothelial cells reduces the level of thrombomodulin expression on endothelial surfaces, and the same stimuli that cause thrombomodulin release may also inhibit thrombomodulin transcription (57,58). These two mechanisms may diminish the ability of patients with Behçet's disease to activate the protein C coagulation inhibitory pathway, thereby leading to thrombosis. This hypothesis may link the effects of coagulopathy and vascular inflammation in Behçet's disease (4) (Figure). In addition, this hypothesis may explain why there is increased thrombin generation without increased markers of coagulation activation.

In summary, we found that Behçet's disease is associated with endothelial cell injury, increased thrombin generation that is independent of the activation of factors VII and XII, and increased fibrinolysis that is unrelated to thrombosis. The tendency for thrombosis in Behçet's disease may be related to abnormalities in the integrity of thrombomodulin or in its surface activity.

ACKNOWLEDGMENT

We thank Carolina Freire and Teresa Farró for their expert technical assistance.

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COMENTARI:

En aquest treball hem avaluat la presència dels factors de risc trombòtic en una població de pacients amb malaltia de Behçet i els hem correlacionat amb les manifestacions trombòtiques de la mateixa malaltia.

Concretament, els malalts analitzats van ser 38 pacients amb malaltia de Behçet (13 d'ells amb trombosi venosa profunda en algun moment de la seva evolució), 38 pacients amb trombosi venosa profunda sense factor de risc trombòtic identificat i 100 individus sans. Cal especificar que les mostres dels pacients amb malaltia de Behçet es van obtenir en fases d'inactivitat clínica de la malaltia. En tots aquests grups vam determinar la prevalença dels factors de risc trombòtic, incloent el polimorfisme 4G/5G del gen del PAI-1, l'estat d'activació de la fibrinolisi plasmàtica, els marcadors de generació de trombina i plasmina, l'estat d'activació de les vies intrínseca i extrínseca de la coagulació (amb la determinació dels factors activats XII i VII) i la lesió de l'endoteli (amb la determinació dels nivells plasmàtics de trombomodulina). Finalment, tots aquests paràmetres es van relacionar amb les manifestacions clíniques trombòtiques dels grups de pacients estudiats.

No vam obtenir diferències en la prevalença dels factors de risc trombòtic entre els pacients amb malaltia de Behçet i el grup control. Concretament, cap pacient va presentar dèficits de proteïna C, proteïna S o la mutació del Factor V Leiden. Un control i un pacient amb malaltia de Behçet i trombosi venosa profunda presentaven la mutació G20210A del gen de la protrombina. La freqüència al·lèlica del polimorfisme 4G/5G també va ser similar entre els dos grups. Dos pacients amb malaltia de Behçet sense fenòmens trombòtics van presentar anticoagulant lúpic i només un pacient amb trombosi venosa tenia anticossos anticardiolipina (d'isotip IgM) positius. Respecte els marcadors de generació de trombina i plasmina, en els pacients amb la malaltia de Behçet, els nivells de F1+2 i dels complexos plasmina/$\alpha_2$antiplasmina van ser

significativament més alts que en el grup control i que en el dels pacients amb trombosis venoses sense factor de risc trombòtic conegut. La mateixa troballa vam obtenir amb els nivells plasmàtics de trombomodulina. L'augment de la fibrinolisi pot ser secundari a l'augment de la generació de trombina, és a dir, seria la resposta que de manera fisiològica i en situació normal té lloc davant d'una activació de la coagulació. En els pacients amb malaltia de Behçet no vam obtenir diferències en els nivells de F1+2, dels complexes plasmina/$\alpha_2$antiplasmina i de trombomodulina entre els pacients amb o sense trombosi. A més, els nivells de trombomodulina es van correlacionar significativament amb els de F1+2. No vam poder establir cap relació entre aquests paràmetres i les manifestacions trombòtiques dels pacients amb malaltia de Behçet. Pel què fa als nivells de factor XII i VII activats van ser similars entre els tres grups de individus estudiats.

Amb els resultats obtinguts podem concloure que a la malaltia de Behçet, malgrat que en algun cas pot coexistir algun factor de risc trombòtic, aquests no expliquen la totalitat de les manifestacions trombòtiques ocorregudes en aquests pacients. El polimorfisme 4G/5G no sembla jugar un paper rellevant en el desenvolupament de les trombosis de la malaltia de Behçet. Per altra part, existeix, inclús en fases d'inactivitat de la malaltia i malgrat el tractament, una persistència de la lesió endotelial i un augment de la generació de trombina amb una fibrinolisi probablement compensadora. Per tant, la tendència trombòtica present en aquests pacients no seria secundària a una hipofibrinolisi. Aquest augment de la generació de trombina és independent de l'activació dels factors XII i VII. Finalment, el grau d'aquestes anormalitats no es relacionen de forma clara amb les manifestacions trombòtiques.

Antiphospholipid Antibodies and Thrombophilic Factors in Giant Cell Arteritis

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Objectives: **To evaluate the prevalence of thrombophilic risk factors known to induce intravascular clotting and to assess their relationship with ischemic manifestations in giant cell arteritis (GCA).**

Methods: **Eighty consecutive patients with established GCA were included: 36 with isolated temporal arteritis (TA), 14 with isolated polymyalgia rheumatica (PMR), and 30 with TA and PMR. Forty-four patients (67%) had ischemic phenomena due to GCA. Twelve patients (15%) had thrombotic events unrelated to GCA (6 strokes, 5 deep venous thrombosis, and 1 myocardial infarction). A control group of 100 age- and sex-matched individuals without autoimmune disease, bleeding disorders, thrombosis, or clinical picture of TA or PMR also was analyzed. All participants were tested for the antiphospholipid antibody (aPL) profile, protein C, protein S, antithrombin activity, factor V Leiden mutation, and prothrombin gene G20210A mutation. We also studied fibrinolysis parameters: plasminogen, tissue-type plasminogen activator (t-PA) antigen, t-PA activity, type-1 plasminogen activator inhibitor (PAI-1) antigen, PAI-1 activity, and the 4G/5G polymorphism of the promoter region of the PAI-1 gene.**

Results: **Eleven patients (18%) tested positive for lupus anticoagulant, 24 (30%) for anticardiolipin antibodies, 9 (11%) for anti–β2-glycoprotein I antibodies, and 29 (36%) for antiprothrombin antibodies. No relationship was found between these autoantibodies and ischemic manifestations. None of the patients had decreased protein C, protein S or antithrombin activity. Two patients and 2 controls were heterozygous for factor V Leiden, and only 1 patient and 2 controls were heterozygous for the prothrombin gene G20210A mutation. No statistically significant correlation was found between any thrombophilic factor and GCA-related or GCA-unrelated ischemic events.**

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Supported by Spanish grants FIS 96/0580 FIS 98/0171 FIS 99/0148 and FIS 00/1048. Gerard Espinosa is a research fellow sponsored by a grant from the Hospital Clínic of Barcelona (1998), and a grant 1999 FI 00684 CIRIT (Generalitat de Catalunya), (1999/2000).

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0049-0172/01/3101-1009$35.00/0
doi:10.1053/sarh.2001.23499

Conclusion: **GCA patients have a high prevalence of aPL that is not related to ischemic manifestations. Moreover, GCA-related or GCA-unrelated ischemic manifestations do not appear to be due to congenital thrombophilic risk factors.**

Semin Arthritis Rheum 31:12-20. Copyright © 2001 by W.B. Saunders Company

INDEX WORDS: Giant cell arteritis; ischemia; thrombosis; antiphospholipid antibodies; anticardiolipin antibodies; thrombophilic risk factors.

GIANT CELL ARTERITIS (GCA) is the most frequent type of vasculitis affecting the elderly (1). The disease course is characterized by arterial ischemia, which may lead to blindness, jaw claudication, myocardial infarction, limb ischemia, or stroke (2). These complications affect only certain patients, for reasons that remain unclear.

There is evidence that thrombosis is a multifactorial disease in which genetic and environmental factors are interrelated. Enhanced intravascular clotting may be due to the presence of factors with procoagulant activity such as antiphospholipid antibodies (aPL). In GCA, moderate serum levels of anticardiolipin antibodies (aCL), mainly of the immunoglobulin (Ig) G class, are frequently detected (3-8), but the correlation of aCL with the occurrence of ischemic complications is a matter of controversy (3,5). A recently introduced enzyme-linked immunosorbent assay (ELISA) named the APhL test (Louisville APL Diagnostics Inc, Louisville, KY) (9) that is apparently more specific for the detection of clinically significant aPL could help clarify this point, but this test has not been used in GCA until now.

In recent years, the deficiency of several factors involved in the regulation of coagulation pathways such as protein C, protein S and antithrombin, factor V Leiden mutation, or the prothrombin gene G20210A mutation have been related to thrombosis (10). In addition, the possible contribution of fibrinolysis to the development of thrombosis in vasculitis has been proposed because of the close interrelation between the fibrinolysis activation pathways and the inflammatory events accompanying autoimmune diseases (11). In this regard, the type 1 plasminogen activator inhibitor (PAI-1) is the major regulator of fibrinolysis and is released by endothelial cells after stimulation by cytokines (12). Decreased fibrinolysis due to increased plasma concentrations of PAI-1 associated with vasculitis in rheumatoid arthritis have been shown (13). Nevertheless, some researchers have found discordant results with normal or increased levels of PAI-1 in patients with GCA (14,15). Although these discrepancies can be partially explained methodologically, they also may be related to genetic factors involving PAI-1 synthesis, particularly to a single base pair guanosine deletion/insertion polymorphism (4G/5G) located in the promoter region of the PAI-1 gene (16,17). The 4G allele association with enhanced PAI-1 expression has been related to myocardial infarction in young patients (16), to the development of coronary artery disease in patients with non–insulin-dependent diabetes mellitus (17) and to the history of arterial thrombosis in patients with the aPL syndrome (APS) (18).

The aims of this study were to investigate all the thrombophilic risk factors in a large, homogeneous, Spanish GCA population and to correlate these factors with the ischemic manifestations related to the disease and to GCA-unrelated thrombotic events.

PATIENTS AND METHODS

Patients

We retrospectively studied 80 consecutive patients with GCA seen in our hospital over a period of 5 years (from January 1995 to January 2000). Thirty-six had isolated temporal arteritis (TA), 14 had isolated polymyalgia rheumatica (PMR), and 30 had TA and PMR. There were 21 men and 59 women, with a mean age of 73.9 years (standard deviation [SD], 7.9 years; range, 50 to 87 years) and a median follow-up of 37.5 months (range, 1 to 159 months). TA was diagnosed according to American College of Rheumatology criteria (19). Sixty-five patients (99%) fulfilled 3 or more of these criteria. PMR was diagnosed according to Bird's criteria (20). Temporal artery biopsy was performed in all TA patients and was positive in 57 of 66 (87%). Temporal artery biopsy was also performed in 10 PMR patients to exclude TA. A total of 108 blood samples were obtained. Of these,

45 were from patients with active disease whose samples were collected before the initiation of steroid treatment. The other 63 had inactive disease, and their samples were collected after a median of 30 months of treatment (range, 18 to 60 months). In 28 patients (16 with isolated TA, 9 with TA and PMR, and 3 with isolated PMR), we studied 2 samples, one during the active phase at disease onset and the other after a median of 30 months of treatment (range, 18 to 48 months).

We also analyzed a control group of 100 sex- and age-matched individuals (74 women, 26 men; mean age, 71.3 years; SD, 6.3 years; range, 55 to 78 years) without autoimmune disease, bleeding disorders, thrombosis, or a clinical picture of TA or PMR. These individuals were recruited among voluntary blood donors (age equal to or lower than 65 years) or from the ophthalmology outpatient clinic attended for cataract unrelated to diabetes mellitus.

The study was approved by the Human Experimental Committee of the Hospital Clínic and was performed according to the principles of the declaration of Helsinki. Informed consent was obtained from all participants.

Clinical Definition

Systemic manifestations were defined by the presence of fever (>38°C) and/or weight loss (≥4 kg). Ischemic manifestations included jaw claudication and/or visual involvement and/or transient or irreversible cerebrovascular attacks. Jaw claudication was defined as pain on chewing that improved when chewing stopped. Visual involvement included one or more of the following: diplopia, transient visual loss, or permanent visual loss. Diplopia was defined as palsy of extrinsic ocular muscles or when it was recalled as a transient symptom by the patient. Transient visual loss (amaurosis fugax) was defined as a temporary loss of sight, followed by recovery to baseline visual acuity without abnormalities on ophthalmologic examination. Permanent visual loss was defined as partial or complete loss of sight in one or both eyes. All these patients were examined by an ophthalmologist. The above-mentioned ischemic events were considered GCA related when they developed concomitantly with disease manifestations in the absence of significant vascular risk factors such as heavy smoking, hypertension, diabetes, or hypercholesterolemia.

GCA-unrelated thrombotic events were assessed clinically and confirmed by objective methods. Diagnosis of deep venous thrombosis was confirmed by Doppler ultrasonographic scans or by venography. Cerebrovascular ischemic episodes were confirmed by computed tomography scanning or by magnetic resonance imaging techniques, and myocardial infarctions were confirmed by electrocardiographic studies and by elevated levels of cardiac enzymes.

Sampling

Venous blood samples were drawn without veno-occlusion in the morning with the patient sitting and resting. Samples for coagulation and fibrinolysis studies were obtained in tubes containing 3.8% trisodium citrate (1:9, vol:vol) (BD Vacutainer 9NC; Becton Dickinson, Rutherford, NJ), and platelet-free plasma was immediately obtained by double centrifugation, first at 2,000 g for 10 minutes at 22°C, and then at 5,000 g for 10 minutes at 4°C. Plasma was aliquoted, snap-frozen in a mixture of dry ice/ethanol (1:2, vol:vol) and stored. For tissue-type plasminogen activator (t-PA), activity samples were obtained in strong acidic citrate tubes (Stabilyte; Biopool, Umeå, Sweden). For genotype studies, samples were drawn in tripotassium EDTA tubes (BD Vacutainer K3E; Becton Dickinson), and 100 μL of whole blood was immediately transferred into tubes containing lysis buffer (5 mol/L guanidine thiocyanate, 1.3% weight/volume [w/v] Triton X-100 [Sigma, St Louis, MO], and 50 mmol/L Tris/HCl; pH 6.4), and frozen at −70°C. Sera were obtained from blood drawn in tubes containing no anticoagulants (BD Vacutainer SST; Becton Dickinson).

HEMOSTASIS STUDIES

General

Prothrombin and activated partial thromboplastin time were determined in an automated Sysmex CA 6000 (Dade Behring, Marburg, Germany) using standard reagents (Thromboplastin IS and Actin FSL; Dade Behring, Marburg, Germany). Fibrinogen was measured by the Clauss' technique (21).

aPL Studies

Lupus anticoagulant (LA) was detected using activated partial thromboplastin time, diluted Rus-

sell's viper venom time, and tissue thromboplastin inhibition test. Tests also were performed in mixtures with control plasmas or phospholipids following the guidelines of the Subcommittee for the Standardization of Lupus Anticoagulants of the International Society of Thrombosis and Hemostasis (22).

aCL levels were measured using a standardized ELISA (23). Results were expressed in IgG and IgM units (GPL and MPL) and reported as negative (<15 units), low positive (16 to 25 units, +), moderate positive (26 to 40 units, ++), and high positive (>40 units, +++).

Detection of anti-β2-glycoprotein I (aβ2GPI) was performed by a standardized previously described ELISA (24) using microtiter plates coated with human β2-glycoprotein I (β2GPI). In each assay, 2 IgG and 2 IgM aβ2GPI-positive sera and 10 negative sera were used as controls. Optical density was measured at 492 nm (OD_{492}), and OD_{492} values higher than 5 SD above the mean of negative controls were considered positive: low positive between 5 and 7 SD, moderate between 7 and 9 SD, and high above 9 SD.

Antiprothrombin antibodies (aPT) were measured with a standardized previously described ELISA (25) using Maxisorp microtiter plates (Nunc, Roskide, Denmark) coated with 10 mg/mL human prothrombin (Prothrombine purifiée; Stago, Asnières, France). In each assay, one positive serum for aPT and 8 negative sera were used as controls. OD_{492} values higher than 5 SD above the mean of negative controls were considered positive: low positive between 5 and 7 SD, moderate between 7 and 9 SD, and high above 9 SD.

aPL were also measured using the APhL ELISA Kit. This test uses a mixture of negatively charged phospholipids and β2GPI instead of cardiolipin alone (9). Tests were performed as specified by the manufacturer. In brief, samples diluted 1/50 were added to the ELISA plate in duplicate wells and incubated 30 minutes at room temperature. After this period, the plates were washed 3 times and then incubated 30 minutes at room temperature with 50 μL/well of anti-IgG or anti-IgM alkaline-phosphatase–labeled antibodies. After a new washing, 50 μL/well of revealing substrate solution was added, and the reaction was stopped after 10 minutes with 100 μL/well of 3.0 mol/L NaOH. Finally, plates were read at 405 nm. The ranges of positivity were estimated following the manufacturer's instructions.

Thrombophilic Risk Factors

Protein C activity was quantified by a colorimetric assay (Coamatic Protein C; Chromogenix, Mölndal, Sweden). Free and total protein S levels were quantified by ELISA with specific monoclonal antibodies (Asserachrom free protein S, Asserachrom total protein S; Stago). Antithrombin activity was measured by using a chromogenic assay (Berichrom Antithrombin III; Dade Behring, Marburg, Germany). Activated protein C resistance was determined in coagulative assays (APC Resistance; Chromogenix), including the direct test and testing after 1:5 dilution in factor V immunodepleted plasma (Clotting Factor-V deficient plasma; Dade Behring). Individuals with phenotypic activated protein C resistance or those with LA, which may interfere with activated protein C resistance measurements, were tested for the factor V Leiden mutation with a nucleic acid sequence–based amplification assay (Factor V Leiden QL NASBA; Organon Teknika, Boxtel, The Netherlands) (26). For the prothrombin gene G20210A mutation, DNA samples were analyzed by polymerase chain reaction as reported by Poort et al (27). For detection of the 4G/5G polymorphism of the promoter region of the PAI-1 gene, a recently described protocol based on polymerase chain reaction technique and endonuclease digestion was used with minor modifications (18,28). In this DNA amplification, a mutated oligonucleotide that inserts a site for the BSiYI enzyme in the amplification product was used. This restriction site enables the identification of the extra G base in 4% agarose gel electrophoresis (Metaphor; FMC Bioproducts, Rockland, ME) visualized under ultraviolet light after staining with ethidium bromide.

Fibrinolysis Parameters

Plasminogen was evaluated with a chromogenic assay (Coamatic Plasminogen; Chromogenix). t-PA antigen was measured by means of an ELISA (Asserachrom t-PA; Stago). t-PA and PAI-1 activities were measured by chromogenic assays (Coaset t-PA, PAI-1; Chromogenix). PAI-1 antigen was measured by an ELISA (Imulyse PAI-1; Biopool, Umeå, Sweden) based on a double antibody principle.

Statistical Analysis

Results are shown as mean ±SD or median and ranges. Comparisons were performed by chi-square test or analysis of variance (ANOVA). The numbers of each genotype observed were compared with those expected for a population in Hardy-Weimberg equilibrium by a chi-square test.

RESULTS

General

Cranial ischemic complications due to GCA were present in 44 patients (67%) (Table 1). The most frequent manifestation was jaw claudication (55%), followed by visual involvement (35%). Stroke was present only in 3% of the GCA patients. The number of ischemic manifestations in patients with ischemic episodes ranged from 1 to 3 (mean, 1.4). All these events occurred when the disease was active. GCA-unrelated thrombosis was present in 12 patients (15%) (6 strokes, 5 deep venous thromboses, and 1 myocardial infarction). Five occurred before the diagnosis of GCA was made (median before diagnosis, 60 months; range, 9 to 658 months). The other 7 occurred during the course of the disease (median after diagnosis, 11 months; range, 3 to 68 months). In all cases, the disease was inactive at the time of GCA-unrelated thrombosis.

aPL Studies

The global prevalence of aPL-positive patients in our series was 39%; 18% had LA and 30% had aCL. A total of 11% of the patients had aβ2GPI, 36% had aPT, and 11% had aPL detected by the APhL test. No differences were found in antibody profile among patients according to the clinical form of presentation (TA, PMR, or TA and PMR) (Table 2). There were no differences in these antibody profiles among the samples obtained in active or inactive phases of the disease. No relationship was found between LA, aCL, aβ2GPI, aPT, and APhL test positivity or any combination of them and ischemic events due to GCA considered together or separately. Similarly, no association was found between GCA-unrelated thrombosis and antibody profiles, considered either singly or grouped together. In reference to the new APhL ELISA, only 9 patients were positive (3 IgG and 6 IgM class), representing 23% of the aPL positive population (considered as LA or aCL positive) but only 11% of the whole series. Only 1 of these 9 patients had aβ2GPI, 2 LA, 7 aCL, and 6 aPT. Five of the APhL-positive test patients had TA, 3 had TA and PMR, and only 1 had isolated PMR. They included 5 men and 4 women whose mean age was 76.2 years (SD, 4.8 years). Four of them suffered jaw claudication, but we were unable to find a relationship between APhL test and ischemic events (GCA-related or GCA-unrelated). All the

Table 1: Cranial Ischemic Events in Patients With TA With/Without PMR

	Total TA Patients (n = 66) n (%)	TA With PMR (n = 30) n (%)	TA Without PMR (n = 36) n (%)
Stroke	2 (3)	0 (0)	2 (6)
Jaw claudication	36 (55)	13 (43)	23 (64)
Visual involvement (occlusive vascular ocular disease)	23 (35)	8 (27)	15 (42)
Unilateral anterior ischemic optic neuropathy	16 (24)	5 (17)	11 (31)
Amaurosis fugax	14 (21)	4 (13)	10 (28)
Visual acuity reduced	7 (11)	3 (10)	4 (11)
Bilateral anterior ischemic optic neuropathy	4 (6)	2 (7)	2 (6)
Ophthalmoplegia	3 (5)	1 (3)	2 (6)
Diplopia	2 (3)	1 (3)	1 (3)
One or more cranial ischemic events	44 (67)	15 (50)	29 (81)

Table 2: Antiphospholipid and Anticofactor Antibody Profile in GCA Patients

	Patients			Samples	
	TA n = 36	TA + PMR n = 30	PMR n = 14	Active Phase n = 43	Inactive Phase n = 65
LA (%)	19.4	13.3	0	13.9	7.9
aCL (%)	33.3	30	21.4	28.8	20.6
IgG	30.5	26.6	21.4	28.8	15.8
IgM	8.3	3.3	0	4.4	4.7
aβ_2GPI (%)	11.1	13.3	7.1	6.6	9.5
IgG	2.7	6.6	7.1	4.4	3.1
IgM	8.3	10	0	4.4	6.3
aPT (%)	33.3	43.3	28.5	35.5	31.7
IgG	27.7	36.6	28.5	28.2	25.4
IgM	11.1	13.3	0	8.8	11.1
APhL (%)	13.8	10	7.1	8.8	9.5
IgG	2.7	6.6	0	6.6	0
IgM	11.1	3.3	7.1	2.2	9.5

controls had negative results on the LA, aβ2GPI, and APhL tests; 1 had aCL (IgM class), and 4 had aPT (2 IgG and 2 IgM).

Thrombophilic Factors

In the study of thrombophilia, no patients were identified with protein C, protein S, or antithrombin deficiency. Two patients were heterozygous for factor V Leiden; one had jaw claudication, and the other had jaw claudication and stroke. Only 1 patient was heterozygous for the prothrombin gene G20210A mutation, and he had jaw claudication. None of these 3 patients with thrombophilic mutations had any GCA-unrelated thrombosis. In the control group, 2 individuals were heterozygous for the factor V Leiden mutation and another 2 were heterozygous for the prothrombin gene G20210A mutation.

Fibrinolysis Parameters

Plasma content of plasminogen, t-PA antigen, and t-PA activity were within the normal ranges in all the patients and controls. PAI-1 antigen levels were significantly elevated in samples obtained in the active phase of the disease (22.4 $\pm$ 9.7 ng/mL) compared with those obtained when the disease was inactive (13.3 $\pm$ 9.3 ng/mL) ($P < .001$). Values of the PAI-1 antigen and activity according to the 4G/5G genotype in patients with GCA tended to be higher in patients with the 4G/4G genotype and lower in those with the 5G/5G genotype, showing a pattern similar to that of the controls, but did not reach statistical significance. The allele frequency of 4G/5G in controls was 0.47/0.53, and its distribution in the patient groups was similar (0.43/0.57). No relationship was found between 4G/5G polymorphism and GCA-related or GCA-unrelated ischemic events.

DISCUSSION

GCA is an inflammatory vasculitis that affects medium and large arteries and is often characterized by ischemic events such as jaw claudication, blindness, stroke, or myocardial infarction (2). These ischemic complications affect only certain patients, and several studies have failed to define a predictive biological factor. In the present study, we have shown that the ischemic manifestations due to GCA-related and GCA-unrelated thrombotic events are not mainly attributable to the presence of thrombophilic factors.

The prevalence of positive aCL in GCA patients in earlier studies is shown in Table 3. Recent studies have shown an increased frequency of aCL in GCA patients, ranging from 8% to 61% (29-33). In the present series we observed a prevalence of aCL of 30%. Although an increased frequency of aCL has been reported in geriatric population (34,35), in the age-matched individuals in our control group the prevalence was 1%—clearly

Table 3: Prevalence of aPL in GCA Patients

Study	Disease (No. of Patients)	LA (%)	aCL (%)	aβ_2GPI (%)
Aguilar et al (37)	PMR (30)	ND	33	ND
	PMR + TA (20)		85	
Font et al (4)	TA (24)	ND	8	ND
Cid et al (3)	TA (40)	ND	7.5	ND
McHugh et al (30)	TA (22; 13 with PMR)	ND	50	ND
Espinoza et al (5)	PMR (30)	ND	26	ND
	PMR + TA (20)		80	
Kerleau et al (6)	TA (20)	ND	50	ND
Liozon et al (8)	TA (86)	ND	50	ND
Chakravarty et al (7)	PMR (64)		17	
	PMR + TA (22)	ND	41	ND
	TA (12)		0	
Liozon et al (38)	PMR (1)			
	PMR + TA (12)	ND	51*	0
	TA (28)			
Meyer et al (39)	PMR (3)		0	
	PMR + TA (16)	ND	13	9*
	TA (3)		67	
Manna et al (29)	PMR + TA (7)	ND	51	ND
	TA (33)		29	
Duhaut et al (33)	PMR (79)		6	
	PMR + TA (82)	ND	21	ND
	TA (105)		29	
Present series	PMR (14)	0	21	7
	PMR + TA (30)	21	30	10
	TA (35)	19	34	11

Abbreviation: ND, not done.

*These percentages correspond to the whole series.

lower than in GCA patients. In addition, aCL in GCA may be associated with ischemic events (5,7,36,37). However, the small sample sizes used in these studies did not prove this relationship. These discrepancies may be partially due to different aCL assay methods, the number of cases and patient selection, or the time of sera collection. Nonetheless, a major shortcoming of the aCL assay is the frequency of false-positive tests, particularly when sera from patients with infectious and autoimmune diseases are tested. In general, the sera obtained from these patients contain low levels of IgM or IgG aCL. False-positive aCL results could result in misdiagnosis and potentially dangerous mistreatment of patients who are not affected by APS.

We tested a new aPL assay (the APhL ELISA) using a mixture of phospholipids instead of cardiolipin alone, in which a lower number of false-positive tests have been reported. In our series, the APhL test showed only 11% of positive patients instead of 30% as observed in standard aCL assay. Both APhL and standard aCL tests have no correlation with ischemic events in GCA patients. Our results confirm the high prevalence of aCL in GCA, but without statistical correlation to ischemic complications.

There are only 2 reports in the literature with regard to aβ2GPI in GCA. In the first, Liozon et al (38) did not find aβ2GPI in their 45 patients, whereas in the second study, Meyer et al (39) reported aβ2GPI in 2 of their 19 patients. In our series, 9 (11%) patients were aβ2GPI positive, but only 1 also showed aCL positivity. No relationship was found between aβ2GPI and the ischemic events in GCA patients.

There are no published data concerning the prevalence of aPT antibodies in GCA patients. A positive correlation between the presence of aPT and the occurrence of vascular events in systemic lupus erythematosus and APS patients has been shown (25,40,41). In the present series, aPT were found in 36% of GCA patients, but we were unable to find a significant correlation between aPT and cranial ischemic events or GCA-unrelated thrombosis.

The origin of all these autoantibodies in GCA is as yet unknown. The high prevalence of some of them (especially aCL and aPT) excludes coincidence. These antibodies could be induced by the exposure of anionic phospholipids at the outer leaflet membrane, after the rearrangement of membrane phospholipids caused by endothelial cell stimulation during inflammation (38). Immunohistochemical studies have shown that the cellular infiltrates in temporal artery lesions of GCA primarily contain macrophages and CD4+ T cells. Weyand et al (42) showed that during this T-cell response, cytokine production (interferon gamma and interleukin 2) play a role in the formation of giant cells in the granulomatous infiltrates and finally in the activation of endothelial cells. One would expect that if aCL are epiphenomenal rather than pathogenic, then aβ2GPI should be absent or have a very low prevalence in this group of patients, as shown in our series. Therefore, in contrast to previous reports (5,7,36,37), aPL would be secondary solely to local inflammation. We suggest that aCL in particular and aPL in general are only markers of endothelial lesions in GCA. On the basis of these results, we have not shown a preponderant role for aPL as a predisposing factor in the development of cranial ischemic events. Therefore, we do not recommend routine screening of patients with GCA for aPL.

There was a significant difference between the plasma concentration of PAI-1 in active and inactive diseases, and plasma levels of PAI-1 antigen and activity are related to the 4G/5G polymorphism of the promoter region of the PAI-1 gene. However, we could not find any relationship between this genotype and the presence of ischemic manifestations.

In conclusion, this study confirms the high prevalence of aPL—mainly aCL and aPT but not aβ_2GPI—in GCA patients. Despite this high prevalence, no relationship between antibody profile and GCA-related or GCA-unrelated ischemic manifestations was found. Moreover, the ischemic manifestations in GCA patients are not due to congenital thrombophilic risk factors and are not related to disturbances in fibrinolysis parameters.

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COMENTARI:

En aquest treball hem avaluat la presència dels factors genètics i adquirits causants de trombofília en una població de pacients amb arteritis de cèl·lules gegants i els hem correlacionat amb les manifestacions isquèmiques relacionades o no amb la malaltia. En concret, hem estudiat 36 pacients amb arteritis de Horton aïllada, 14 amb polimiàlgia reumàtica aïllada i 30 amb les dues entitats. En tots ells, s'ha determinat la prevalença dels principals factors genètics i adquirits causants de trombofília, incloent el polimorfisme 4G/5G del gen del PAI-1 i fent especial esment als anticossos antifosfolipídics i anticofactor (anti-β_2GPI i antiprotrombina). Respecte a aquests, hem utilitzat per a la seva determinació, a més dels mètodes d'enzimimmunoassaig (ELISA) estandarditzats al nostre laboratori, un nou mètode del mateix tipus (APhL ELISA Kit®) que, en lloc de cardiolipina sola, usa una barreja de fosfolípids aniònics amb β_2GPI. Això li confereix en aquest nou ELISA una major sensibilitat i especificitat, disminuint el nombre de falsos positius. Finalment, hem analitzat en els mateixos pacients els diferents paràmetres de la fibrinolisi plasmàtica i també els hem intentat relacionar amb les manifestacions isquèmiques relacionades o no amb la malaltia. Respecte als pacients, hem identificat les manifestacions isquèmiques relacionades amb la malaltia com aquelles en forma de claudicació mandibular, afectació visual o accidents isquèmics cerebrals incloent els transitoris que tenen lloc en fases d'activitat de la malaltia. Pel contrari, els fenòmens trombòtics no relacionats amb l'arteritis de cèl·lules gegants i, per tant, no secundaris a aquesta, els hem definit com aquells que tenen lloc en fases d'inactivitat de la malaltia.

Quaranta quatre (67%) pacients van presentar fenòmens isquèmics relacionats amb la malaltia i només 12 (15%) van patir algun fenomen trombòtic no relacionat amb aquella (concretament, 6 accidents isquèmics cerebrals, 5 trombosis venoses profundes i un infart agut de miocardi). Cap dels pacients va presentar dèficit de proteïna C, proteïna S

o antitrombina. Dos pacients van ser heterozigots pel factor V Leiden i un per la mutació G20210A de la protrombina. De totes maneres, no vam trobar cap relació estadísticament significativa entre aquestes troballes i les manifestacions isquèmiques relacionades o no amb la malaltia. La freqüència al·lèlica de 4G/5G fou similar en els controls i en el grup de pacients. A més, no vam poder establir cap relació entre aquest polimorfisme i les manifestacions isquèmiques relacionades o no amb la malaltia.

Respecte als paràmetres de la fibrinolisi, els nivells plasmàtics de plasminogen, t-PA antigen i l'activitat de t-PA van ser normals. Els nivells de PAI-1 antigen van ser més alts en les mostres obtingudes durant la fase activa de la malaltia. Els valors de PAI-1 antigen i d'activitat de PAI-1 van ser més alts en els pacients amb genotip 4G/4G i més baixos en els pacients homozigots per l'al·lel 5G, però sense significació estadística.

Pel què fa als anticossos antifosfolipídics, 11 (18%) pacients tenien anticoagulant lúpic positiu, 24 (30%) anticossos anticardiolipina, 9 (11%) anti-β_2GPI i 29 (36%) anticossos antiprotrombina. Quan vam testar els pacients amb el nou mètode d'ELISA, només 9 (11%) pacients presentaven anticossos antifosfolipídics. No vam obtenir diferències significatives en el perfil d'autoanticossos i els pacients d'acord amb la seva forma de presentació (arteritis o polimiàlgia reumàtica aïllades o les dues juntes). Finalment, tampoc vam trobar diferències entre el perfil d'aquests autoanticossos, ja sigui de forma aïllada o conjunta, i les manifestacions isquèmiques dependents o no de la malaltia.

En conclusió, les manifestacions isquèmiques relacionades o no amb aquesta entitat no són degudes als factors genètics causants de trombofília ni estan relacionades amb alteracions dels paràmetres de la fibrinolisi plasmàtica. Els pacients amb arteritis de cèl·lules gegants presenten una elevada prevalença d'anticossos antifosfolipídics, si bé aquests no es relacionen amb les manifestacions isquèmiques degudes a la malaltia.

La caracterització d'un determinat perfil de risc trombòtic o isquèmic pot ser molt important en entitats com la síndrome antifosfolipídica, la malaltia de Behçet i l'arteritis de Horton. L'estudi en aquest camp podria identificar pacients amb un risc augmentat de patir fenòmens trombòtics i, per tant, establir mesures més agressives tant de tractament com de profilaxi primària o secundària.

Les nostres investigacions han anat dirigides a estudiar les característiques dels pacients afectes de síndrome antifosfolipídica, malaltia de Behçet i arteritis de cèl·lules gegants. Concretament, hem determinat en aquestes tres entitats els principals factors genètics i adquirits de trombofília i l'estat de la fibrinolisi plasmàtica i els hem relacionat amb les manifestacions trombòtiques i isquèmiques. Per altra part, i en el cas de la síndrome antifosfolipídica, no ens hem interessat només per les característiques dels pacients sinó també per les dels anticossos anticardiolipina, específicament la seva dependència de la β_2GPI en la interacció plaquetar amb el subendoteli.

En el camp de la síndrome antifosfolipídica, hem demostrat que la presència de l'al·lel 4G del polimorfisme 4G/5G del gen del PAI-1 s'associa amb l'existència de trombosis arterials. És a dir, aquest polimorfisme pot ser un factor de risc addicional pel desenvolupament de trombosis arterials en pacients amb síndrome antifosfolipídica. El mecanisme que explicaria aquesta associació seria precisament la inhibició de la fibrinolisi plasmàtica a partir de l'augment de les concentracions plasmàtiques de PAI-1 que presentarien els pacients portadors d'almenys un al·lel 4G. Recordem que això és degut a que la deleció d'una guanina (al·lel 4G) en el lloc d'unió de les proteïnes reguladores de la transcripció fa que aquest no sigui reconegut per la proteïna repressora d'aquesta transcripció (123) (Figura 4). En absència d'unió de la proteïna repressora (4G) la transcripció de PAI-1 augmenta. La manca de correlació entre els nivells plasmàtics de PAI-1 i el polimorfisme 4G/5G del gen del PAI-1 que existeix en el primer dels treballs pot ser deguda a dos factors. En primer lloc, cal tenir en compte que

les nostres investigacions s'han portat a terme en condicions basals. Això explicaria que els nivells plasmàtics de PAI-1 hagin estat similars entre els grups de pacients i els controls i entre els diferents genotips del polimorfisme 4G/5G. És a dir, aquests resultats no reflecteixen els nivells de PAI-1 en el moment agut de la trombosi. En aquest sentit, els estudis experimentals han demostrat que la presència de l'al·lel 4G incrementa la transcripció de PAI-1 només en condicions d'estimulació, però no en condicions basals (121), el que suggereix que els individus amb el genotip 4G podrien tenir alterada la resposta de PAI-1 però només durant la fase aguda de la trombosi. En segon lloc, la presència de l'al·lel 4G, en condicions d'activació, pot donar lloc a un augment local de la transcripció de PAI-1, sobretot en el lloc de lesió endotelial vascular. S'ha demostrat que el ARN missatger del PAI-1 pot expressar-se de manera local en lesions arterioscleròtiques (205). És a dir, un increment local de la transcripció de PAI-1 no ha de reflectir-se necessàriament en els seus nivells plasmàtics. D'aquest treball es desprèn que en l'estudi inicial d'aquests pacients potser estaria indicada la determinació del polimorfisme 4G/5G del gen del PAI-1, ja que la presència de l'al·lel 4G identificaria un subgrup de pacients amb síndrome antifosfolipídica amb un risc elevat de trombosi arterial i definiria millor la intensitat i duració de la profilaxi i del tractament anticoagulant en aquests pacients.

Amb la intenció de definir millor un determinat perfil trombòtic, també hem analitzat en la síndrome antifosfolipídica les característiques dels anticossos antifosfolipídics i la relació amb el seu potencial trombòtic. És a dir, hem intentat determinar quines característiques específiques dels anticossos antifosfolipídics són les responsables de la seva patogenicitat. En aquest camp, hem pogut demostrar que els anticossos anticardiolipina monoclonals avaluats requereixen d'una activitat anti-β_2GPI per tal de promoure la interacció plaquetar amb el subendoteli i que aquesta interacció plaquetar requereix, a més, de la presència en el medi de β_2GPI. Això ve a confirmar la hipòtesi

que els anticossos anticardiolipina patogènics serien només aquells que posseissin activitat anti-β_2GPI, a diferència dels que apareixen en el decurs d'algunes infeccions, com la lues o la síndrome d'immunodeficiència adquirida que no la presenten (159). Aquests resultats recolzen, per altra part, l'afirmació feta per alguns autors (206) en el sentit del benefici de la determinació dels anticossos anti-β_2GPI en casos d'individus amb anticossos antifosfolipídics que no han patit mai fenòmens trombòtics (els anomenats portadors assimptomàtics) o en aquells amb antecedents trombòtics però títols baixos d'anticossos anticardiolipina i anticoagulant lúpic negatiu . La positivitat dels anticossos anti-β_2GPI podria ajudar a prendre la decisió d'iniciar un tractament profilàctic.

En contraposició a aquestes dades, en els malalts afectes de vasculitis, i més concretament malaltia de Behçet i arteritis de cèl·lules gegants, no hem pogut demostrar que alteracions de la fibrinolisi plasmàtica es relacionin amb l'aparició de fenòmens trombòtics. A la malaltia de Behçet hem demostrat l'existència d'un increment de la generació de trombina i d'una activació de la fibrinolisi plasmàtica, en forma d'elevació dels complexes plasmina-$\alpha_2$antiplasmina, malgrat que creiem que això podria ser bàsicament degut a la resposta fisiològica davant l'augment de generació de trombina. A més, no hem detectat que el polimorfisme 4G/5G del gen del PAI-1 estigui relacionat amb les manifestacions clíniques de la malaltia de Behçet en general, ni amb els fenòmens trombòtics en particular. D'altra banda, l'augment dels nivells plasmàtics de trombomodulina és indicativa de l'existència d'una lesió endotelial, present inclús en fases d'inactivitat de la malaltia. És a dir, malgrat un tractament adequat, la malaltia manté de forma permanent un cert grau d'activitat biològica. Això explicaria que es pogués produir afectació vascular en fases d'aparent inactivitat clínica. Tal com hem comentat amb anterioritat, a la malaltia de Behçet també existeix un augment en la generació de trombina que és independent de l'activació de la via intrínseca i extrínseca

de la coagulació, com ho demostren els nivells plasmàtics normals de factor XII activat i VII activat. Aquests dades ens han portat a plantejar la hipòtesi que els fenòmens trombòtics a la malaltia de Behçet podrien ser deguts a anomalies en algun dels sistemes anticoagulants naturals. La trombina en presència de trombomodulina unida a les cèl·lules endotelials, activa la proteïna C, el principal sistema anticoagulant natural, que per la seva banda, inactiva els factors V activat i VIII activat (30). L'augment de trombomodulina que hem descrit en els pacients amb malaltia de Behçet pot provenir de la cèl·lula endotelial lesionada. Malgrat que s'ha demostrat *in vitro* que la trombomodulina soluble pot actuar a la coagulació (207), la seva funció *in vivo* és desconeguda (208). A més, l'augment dels nivells plasmàtics de trombomodulina soluble són deguts principalment a l'alliberació de fragments o formes degradades de la trombomodulina de la superfície endotelial (209), i aquests fragments de trombomodulina són probablement menys eficients que la trombomodulina normal. Per altra part, s'ha descrit que la trombomodulina alliberada redueix l'expressió de trombomodulina a la superfície endotelial, de manera que el mateix estímul que origina l'alliberació de trombomodulina pot inhibir la seva transcripció (210,211). Aquests dos mecanismes podrien disminuir l'activació de la proteïna C a la malaltia de Behçet, creant una situació protrombòtica. A més, explicarien el perquè hem detectat un increment de la generació de trombina sense un augment dels marcadors d'activació de la coagulació. Aquesta hipòtesi ha de ser corroborada per més estudis en el futur. Per tant, malgrat que en alguns casos de malaltia de Behçet els fenòmens trombòtics puguin ser deguts a la coexistència d'alguns dels factors genètics i adquirits de trombofília avaluats, aquests no expliquen la majoria de les trombosis que tenen lloc en aquesta entitat.

Finalment, a l'arteritis de Horton només hem detectat un augment dels nivells plasmàtics de PAI-1 en les fases d'activitat de la malaltia però sense relació amb

fenòmens isquèmics. Per tant, el PAI-1 sembla que es comporta en aquesta malaltia com un reactant de fase aguda sense, probablement, significació patogènica. En aquesta entitat, hem observat una elevada prevalença d'anticossos antifosfolipídics, sobretot d'anticossos anticardiolipina i antiprotrombina però no d'anti-β_2GPI. Malgrat aquesta elevada prevalença, no hem trobat relació amb l'aparició de fenòmens isquèmics. Això estaria en consonància amb els resultats del nostre segon treball, que suggereix que la patogenicitat dels anticossos anticardiolipina dependria de la seva activitat anti-β_2GPI. Existeix la possibilitat que l'origen d'aquests anticossos sigui secundari a la lesió vasculítica que afecta l'endoteli (201). Aquesta provocaria que els fosfolípids de membrana quedessin exposats directament a la llum del vas, podent provocar el desenvolupament d'anticossos que no tindrien potencial patogènic. En aquest cas, la presència d'anticossos antifosfolipídics en els pacients amb arteritis de cèl·lules gegants seria un epifenomen i no tindria valor en l'avaluació del perfil isquèmic d'aquests pacients. Per altra part, les manifestacions isquèmiques durant el curs de l'arteritis de cèl·lules gegants, no semblen ser degudes a la coexistència dels factors genètics causants de trombofília avaluats.

De l'estudi d'aquestes tres entitats, podem concloure que a la síndrome antifosfolipídica la presència de l'al·lel 4G del polimorfisme 4G/5G del gen del PAI-1 identifica un subgrup de pacients amb un risc augmentat de trombosi arterial, i que l'activitat anti-β_2GPI confereix als anticossos anticardiolipina el seu potencial patogènic en la interacció plaquetar amb el subendoteli. Per contra, a la malaltia de Behçet i a l'arteritis de cèl·lules gegants no hem pogut establir un perfil específic per identificar els pacients amb un risc augmentat de patir fenòmens trombòtics o isquèmics. Aquestes diferències en els mecanismes patogènics entre la síndrome antifosfolipídica i les vasculitis avaluades semblen tenir la seva traducció en les característiques histopatològiques d'aquestes tres entitats. En l'arteritis de cèl·lules gegants, els estudis histoquímics han

posat en evidència la presència d'infiltrats cel·lulars formats majoritàriament per macròfags i limfòcits T CD4 (212), que, durant la fase aguda són responsables de la producció de mediadors inflamatoris com la interleuquina-1β, interleuquina-6, *transforming growth factor-β*, interleuquina-2, i interferó γ (23). Alguns d'aquests mediadors com la interleuquina-1β i el *transforming growth factor-β* estimulen la secreció de PAI-1 *in vivo* (118), el que explicaria l'augment dels nivells plasmàtics d'aquest durant la fase aguda de la malaltia. Els macròfags, a més, són els encarregats de produir els factors de creixement derivats de les plaquetes (213). La correlació clínica ha demostrat que els fenòmens isquèmics en l'arteritis de cèl·lules gegants tenen lloc en pacients amb nivells elevats d'aquests factors, mentre que en casos d'arteritis de cèl·lules gegants amb una mínima producció d'aquests factors de creixement, l'oclusió vascular no es detecta a la biòpsia i el risc de complicacions isquèmiques és baix (212). Per la seva banda, a la malaltia de Behçet, l'infiltrat objectivat a les mostres histològiques està compost per cèl·lules mononuclears activades i neutròfils. Com en el cas anterior, també existeix un patró de mediadors inflamatoris de tipus Th1 (214) que a més s'ha relacionat amb l'activitat de la malaltia (215). L'estat d'hipercoagulabilitat que s'ha descrit en aquesta entitat podria ser degut, en part, a l'activació de les cèl·lules endotelials i de les plaquetes (216). Finalment i en contraposició a aquestes dades, a la síndrome antifosfolipídica no es formen infiltrats inflamatoris a la paret del vas i el fenomen trombòtic sembla ser primari. És a dir, sense existir resposta inflamatòria, es produeix directament trombosi vascular. A més, si bé s'han descrit alteracions a nivell d'alguna citocina, fonamentalment interleuquina-3 en relació a les manifestacions obstètriques (217), no s'ha pogut demostrar una sobreexpressió d'un determinat perfil de citocines proinflamatòries. Aquesta heterogeneïtat pot raure en el fet que mentre a la síndrome antifosfolipídica, els anticossos antifosfolipídics tenen un paper patogènic

clau, a la malaltia de Behçet i a l'arteritis de cèl·lules gegants en particular, no s'ha descrit cap anticòs amb un paper patogènic preponderant.

Tot això comporta que el tractament d'aquestes entitats sigui diferent; en el cas de la síndrome antifosfolipídica es persegueix minimitzar l'efecte dels anticossos antifosfolipídics amb el tractament anticoagulant o antiagregant, mentre que a les vasculitis, mitjançant la corticoteràpia i els immunosupressors es pretén disminuir aquesta sobreexpressió Th1 de citoquines proinflamatòries.

8.A. Conclusions del Primer Estudi

1. L'al·lel 4G del polimorfisme 4G/5G del gen del PAI-1 s'associa amb la presència de trombosis arterials en els pacients amb síndrome antifosfolipídica.

2. Els altres factors genètics de trombofília avaluats no semblen tenir un paper fonamental en la patogènia de la majoria dels fenòmens trombòtics en els pacients amb síndrome antifosfolipídica.

3. En els pacients amb síndrome antifosfolipídica, existeix en situació basal un increment de la generació de trombina amb uns marcadors d'activació de la fibrinolisi dins dels valors normals.

8.B. Conclusions del Segon Estudi

1. Els anticossos anticardiolipina monoclonals avaluats han de tenir activitat anti-β_2GPI per tal d'incrementar la interacció de les plaquetes amb el subendoteli en un sistema de perfussió en condicions de flux.

2. L'increment de la interacció plaquetar amb el subendoteli promoguda pels anticossos anticardiolipina monoclonals en condicions de flux requereix de la presència de β_2GPI.

8.C. Conclusions del Tercer Estudi

1. La majoria de les trombosis que tenen lloc a la malaltia de Behçet no poden ser explicades pels factors genètics i adquirits causants de trombofília avaluats.

2. A la malaltia de Behçet es detecten, inclús en fases d'inactivitat, una lesió endotelial determinada per l'augment dels nivells de trombomodulina, juntament amb un increment de la generació de trombina i d'activació de la fibrinolisi.

3. L'augment de la generació de trombina objectivat a la malaltia de Behçet no està relacionat amb els marcadors de l'activació de la via intrínseca i extrínseca de la coagulació.

8.D. Conclusions del Quart Estudi

1. En els pacients amb arteritis de cèl·lules gegants, la prevalença d'anticossos anticardiolipina i antiprotrombina és alta però no així la d'anti-β_2GPI.

2. No hem observat relació entre la presència dels anticossos antifosfolipídics i l'aparició de fenòmens isquèmics o trombòtics, relacionats o no amb la malaltia.

3. La prevalença dels factors genètics causants de trombofília avaluats no es relaciona amb l'existència de les manifestacions isquèmiques o trombòtiques en el decurs de l'arteritis de cèl·lules gegants.

8.E. Conclusió Global

A la síndrome antifosfolipídica, a diferència del què succeeix en els malalts afectes de malaltia de Behçet i arteritis de cèl·lules gegants, les alteracions de la fibrinolisi plasmàtica, determinades genèticament, podrien jugar un paper rellevant en el desenvolupament dels fenòmens trombòtics.

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