Análisis de la fibrinolisis como nuevo mecanismo patogénico trombótico en la patología gestacional precoz y avanzada. Relación con los anticuerpos antifosfolipídicos

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ANÁLISIS DE LA FIBRINOLISIS COMO NUEVO MECANISMO PATOGÉNICO TROMBÓTICO EN LA PATOLOGÍA GESTACIONAL PRECOZ Y AVANZADA. RELACIÓN CON LOS ANTICUERPOS ANTIFOSFOLIPÍDICOS

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Memoria presentada por María Ángeles Martínez Zamora para aspirar al grado de Doctor en Medicina por la Universitat de Barcelona, bajo la dirección del Profesor Juan Balasch Cortina, Catedrático de Obstetricia y Ginecología de la Universitat de Barcelona y Director del Institut Clínic de Ginecologia, Obstetrícia i Neonatología del Hospital Clínic de Barcelona, el Doctor Francisco Carmona Herrera, Profesor titular de Obstetricia y Ginecología de la Universitat de Barcelona y Jefe de Servicio de Ginecología del Hospital Clínic de Barcelona, y la Doctora Dolors Tàssies Penella, Consultora del Servicio de Hemoterapia y Hemostasia del Hospital Clínic de Barcelona. Universitat de Barcelona.

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HACEN CONSTAR: Que Dª María Ángeles Martínez Zamora ha realizado bajo nuestra dirección el trabajo titulado "Análisis de la fibrinolisis como nuevo mecanismo patogénico trombótico en la patología gestacional precoz y avanzada. Relación con los anticuerpos antifosfolipídicos" para aspirar al grado de Doctor en Medicina y que dicho trabajo está en condiciones de ser defendido por la aspirante a partir del día de la fecha.

Lo que hacemos constar a los efectos oportunos en Barcelona a 20 de septiembre de 2011.

Fdo.: Prof. J. Balasch. Dr. F. Carmona. Dra. D. Tàssies.

A mis padres

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PRESENTACIÓN

La presente Tesis Doctoral se estructura siguiendo las directrices de la normativa para la presentación de tesis doctorales como un compendio de publicaciones, aprobada por la Divisió de Ciències de la Salut de la Universistat de Barcelona en octubre del 1999 y asumida con modificaciones mínimas por la Comissió de Doctorat de la Facultat de Medicina el 19 de abril de 2006.

Los estudios que conforman esta Tesis Doctoral pertenecen a una misma línea de investigación. Los resultados obtenidos gracias a la realización de estos estudios, han aportado información relevante y novedosa sobre el tema y han sido recogidos en 5 artículos originales, publicados en diversas revistas de amplia difusión internacional.

PUBLICACIONES INTERNACIONALES QUE COMPONEN EL CUERPO DOCTRINAL DE ESTA TESIS DOCTORAL, AYUDAS Y PREMIOS SURGIDOS COMO CONSECUENCIA DE LA LABOR INVESTIGADORA DESARROLLADA

PUBLICACIONES

1. **"Reduced plasma fibrinolytic potencial in patients with recurrent implantation failure alter IVF and embryo transfer".**

   M.Angeles Martínez-Zamora, Montserrat Creus, Dolors Tassies, Juan Carlos Reverter, Salvadora Civico, Francisco Carmona, Juan Balasch.

   Hum Reprod 2011;26:510-516.

   Factor de impacto: 4.357

   Cuartil: 1º Obstetrics & Gynecology

2. **"Thrombin activatable fibrinolysis inhibitor and clot lysis time in women with recurrent miscarriage associated with the antiphospholipid syndrome".**

   M.Angeles Martínez-Zamora, Montserrat Creus, Dolors Tassies, Albert Bove, Juan Carlos Reverter, Francisco Carmona, Juan Balasch

   Fertil Steril 2010;94:2437-2440.

   Factor de impacto: 3.122

   Cuartil: 1º Obstetrics & Gynecology

3. **“Thrombin activatable fibrinolysis inhibitor and clot lysis time in pregnant patients with antiphospholipid syndrome: relationship with pregnancy outcome and thrombosis”.**

M.Angeles Martínez-Zamora, Dolors Tassies, Francisco Carmona, Gerard Espinosa, Ricard Cervera, Juan Carlos Reverter, Juan Balasch

Am J Reprod Immunol 2009;62:381-389.

Factor de impacto: 2.451

Cuartil: 3º Reproductive Biology

4. **“Clot lysis time and thrombin activatable fibrinolysis inhibitor in severe preclampsia with or without associated antiphospholipid antibodies”.**

M.Angeles Martínez-Zamora, Dolors Tassies, Francisco Carmona, Gerard Espinosa, Ricard Cervera, Juan Carlos Reverter, Juan Balasch

J Reprod Immunol 2010;86:133-140.

Factor de impacto: 2.204

Cuartil: 3º Reproductive Biology

5. **“Risk for thromboembolic events after recurrent miscarriage in antiphospholipid syndrome: a case-control study”.**

M.Angeles Martínez-Zamora, Sara Peralta, Montserrat Creus, Dolors Tassies, Juan Carlos Reverter, Gerard Espinosa, Ricard Cervera, Francisco Carmona, Juan Balasch

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PREMIOS

Premio Fin de Residencia Emili Letang concedido por el Hospital Clínic en el año 2005 para el trabajo de investigación: “ Análisis de la fibrinolisis como nuevo mecanismo patogénico en el Síndrome Antifosfolípidico Obstétrico. Relación con los resultados perinatales”.

ÍNDICE

ABREVIATURAS UTILIZADAS EN ESTA TESIS DOCTORAL

AAC: Anticuerpos anticardiolipina.

AAF: Anticuerpos antifosfolipídicos.

AL: Anticoagulante lúpico.

AR: Aborto de repetición.

CLT: Tiempo de lisis del coágulo.

FIR: Fallo de implantación repetido.

FIV: Fecundación *in vitro.*

LES: Lupus eritematoso sistémico.

PAI-I: Inhibidor del activador del plasminógeno tipo I.

PC: Proteína C.

PCa: Proteína C activada

PDF: productos de degradación de la fibrina.

PG: plasminógeno.

PN: plasmina.

TAFI: Inhibidor de la fibrinolisis activable por la trombina.

TAFIa: Inhibidor de la fibrinolisis activable por la trombina activado.

t-PA: Activador tisular del plasminógeno.

SAF: Síndrome antifosfolipídico.

1. INTRODUCCIÓN. PLANTEAMIENTO GENERAL DEL PROBLEMA Y JUSTIFICACIÓN DE LA TESIS

Durante la gestación normal tienen lugar cambios en la hemostasia que conllevan un estado de hipercoagulabilidad. Los responsables de esta hipercoagulabilidad son un aumento de los niveles de factores de coagulación y cambios en el sistema fibrinolítico (Brenner, 2004; O´Riordan, 2003). Estos cambios se producen mayoritariamente para evitar una hemorragia en el momento del parto. Pero esta situación de hipercoagulabilidad puede desencadenar alteraciones de la hemostasia durante la gestación que se asocian a complicaciones gestacionales tanto precoces (aborto de repetición, fracaso implantatorio tras fecundación in vitro) como tardías (patología gestacional avanzada asociada a trombosis placentaria como por ejemplo preeclampsia y retraso de crecimiento intrauterino), así como a un aumento de riesgo trombótico (Roberts and Cooper, 2001). Además, es conocido que las gestantes con trombofilias hereditarias o adquiridas tienen un riesgo mayor de presentar complicaciones obstétricas y tienen un peor pronóstico obstétrico (Pabinger, 2009). El paradigma de estas trombofilias, que claramente aporta un mayor riesgo de complicaciones obstétricas y de trombosis, es el síndrome antifosfolipídico (Carp, 2004; Wu and Stephenson, 2006; Miyakis et al., 2006).

Es decir, que nos encontramos frente a una situación habitual, el embarazo, en la que se producen cambios fisiológicos que inducen un estado de hipercoagulabilidad, estado que a su vez puede ser el origen de complicaciones tanto en la gestación inicial como avanzada en casos de desequilibrio hemostático o cuando inciden ciertos factores sobreañadi-

dos. Es en este contexto en el que se desarrolla el trabajo realizado en esta tesis doctoral.

1.1 CAMBIOS FISIOLÓGICOS DE LA HEMOSTASIA DURANTE LA GESTACIÓN

El proceso de la hemostasia es un equilibrio dinámico entre los sistemas de coagulación y fibrinolisis. Durante la gestación normal se producen importantes cambios en la hemostasia de forma que predomina el efecto procoagulante. La gestación normal se asocia a cambios en todos los componentes de la hemostasia, incluyendo un aumento de las concentraciones de la mayoría de factores de la coagulación, un descenso de la concentración de algunos anticoagulantes naturales y el descenso de la actividad fibrinolítica (Rai, 2003; Brenner, 2004; O´Riordan, 2003). En resumen, durante la gestación normal se produce un aumento marcado de la actividad procoagulante en sangre materna compuesto por la elevación de los factores VII, X, VIII, fibrinógeno y factor de Von Willebrand, que llega a su máximo al término de la gestación. Estos cambios se asocian a un incremento de los marcadores de la activación de la coagulación como son los fragmentos de protrombina (F1+2) y de los complejos trombina-antitrombina. También se produce un descenso de los anticoagulantes fisiológicos, concretamente una importante reducción de la actividad de la proteína S y una resistencia a la proteína C activada. También la actividad fibrinolítica global se altera durante la gestación pero regresa a la normalidad rápidamente en el posparto inmediato. Esto se debe, en gran medi-

da, al derivado placentario del inhibidor del activador del plasminógeno tipo 2 (PAI-2), que está presente en grandes cantidades durante la gestación. De la misma manera, el dímero D, que es un marcador específico de activación de fibrinolisis, y deriva de la rotura de los polímeros de fibrina producida por la plasmina, aumenta a medida que la gestación progresa.

Estos cambios tienen lugar durante la gestación como parte de una compleja adaptación fisiológica que permitirá el correcto funcionamiento de la circulación materna y fetal en la interfase uteroplacentaria durante el embarazo y que, al mismo tiempo, asegurará el control de la hemorragia placentaria en el momento del alumbramiento placentario (Brenner, 2004; O´Riordan, 2003). El alumbramiento placentario sucede de forma rápida, y el flujo de sangre materna, de aproximadamente 700 mL/minuto en el lecho placentario debe ser interrumpido rápidamente mediante el efecto combinado de la compresión extravascular miometrial y la oclusión trombótica de los vasos placentarios maternos (Brenner, 2004; O´Riordan, 2003).

La activación del sistema de coagulación en la circulación uteroplacentaria puede predisponer al depósito anormal de fibrina. En la circulación sistémica esta activación de la coagulación se manifiesta clínicamente como un aumento de 4 a 10 veces del riesgo trombótico durante la gestación y el posparto, de forma que los eventos tromboembólicos venosos se presentan en una de cada 1000 gestaciones (Greer, 2000) y constituyen la causa más frecuente de muerte materna durante la gestación y el periodo

postparto (Eldor, 2001). La trombosis uteroplacentaria parece ser un rasgo común a muchas complicaciones obstétricas precoces y tardías frecuentes de la gestación humana, como por ejemplo, abortos de repetición, retraso de crecimiento intrauterino o, como paradigma, la preeclampsia.

Asimismo, durante el estado fisiológico de hipercoagulabilidad de la gestación, las trombofilias congénitas o adquiridas constituyen un factor de riesgo sobreañadido que puede incrementar los eventos tromboembólicos durante el embarazo y llevar al desarrollo de complicaciones obstétricas graves secundarias a trombosis placentaria como abortos de repetición, preeclampsia, retraso de crecimiento intrauterino o desprendimiento de placenta. Una inadecuada perfusión placentaria, que constituye un rasgo característico de la preeclampsia, incluye anormalidades del árbol vascular velloso placentario asociado a disfunción endotelial, hipercoagulabilidad y trombosis de las arterias espirales. La preeclampsia es una alteración placentaria primaria que parece que se inicia en el embarazo temprano por falta de invasión trofoblástica de las arterial espirales maternas, dando lugar a un estado de hipoperfusión de la circulación uteroplacentaria agravado por oclusiones vasculares (Said and Dekker, 2003; Roberts and Cooper, 2001). Las trombofilias genéticas se encuentran presentes hasta en el 30% de las pacientes que presentan este tipo de complicaciones obstétricas (Buchholz and Thaler, 2003; Kupferminc et al., 1999; Pabinger, 2009; Sarig et al., 2002). Ya en el momento de la implantación, se requiere un fino equilibrio entre coagulación y fibrinolisis para asegurar una correcta polimerización de la fibrina y una estabilización de lecho placentario

basal, así como para evitar el depósito excesivo de fibrina en los vasos placentarios y en el espacio intravelloso. No obstante, de los diferentes tipos de trombofilias, el síndrome antifosfolípidico, que se expone a continuación, es el que aporta un mayor riesgo de trombosis y complicaciones obstétricas.

En resumen pues, la hipercoagulabilidad durante el embarazo supone un cambio fisiológico defensivo frente a la hemorragia posparto, pero constituye en algunas mujeres el sustrato abonado para el desarrollo de complicaciones trombóticas ya en el embarazo inicial (aborto de repetición, fallo implantatorio repetido tras fecundación in vitro) o avanzado (preeclampsia y/o retraso de crecimiento intrauterino esencialmente).

1.2 SÍNDROME ANTIFOSFOLIPÍDICO OBSTÉTRICO

El síndrome antifosfolipídico (SAF) es una enfermedad autoinmune en la que se producen complicaciones obstétricas y/o trombosis en presencia de anticuerpos antifosfolipídicos (AAF). El SAF se puede presentar aislado, como un SAF primario, o asociado a otras enfermedades autoinmunes (SAF secundario), típicamente al lupus eritematoso sistémico (LES). La presencia de AAF en pacientes LES es de aproximadamente el 37% (Pierangeli et al., 2008; Wu and Stephenson, 2006). El LES también se asocia a malos resultados obstétricos, especialmente en aquellas pacientes que presentan AAF. Entre las

complicaciones maternas y fetales en pacientes con SAF se encuentran la hipertensión gestacional, preeclampsia, eclampsia, desprendimiento de placenta, retraso de crecimiento intrauterino, oligoamnios, parto prematuro, aborto y muerte fetal y trombosis materna (Carp, 2004; Wu and Stephenson, 2006).

Los AAF son un grupo heterogéneo de autoanticuerpos adquiridos de forma espontánea, de isotipos IgG e IgM, que se caracterizan por ir dirigidos contra estructuras fosfolipídicas de las membranas celulares. Desde el punto de vista clínico los dos AAF de mayor interés son el anticoagulante lúpico (AL) y los anticuerpos anticardiolipina (AAC). Los anticuerpos antifosfolipídico se encuentran en aproximadamente el 5% de la población general en edad reproductiva pero su sola presencia no implica necesariamente un mal pronóstico gestacional en pacientes que no cumplen criterios clínicos (Wu and Stepherson, 2006; Pierangeli et al., 2008). Los criterios diagnósticos del SAF se definieron en 1998 y se revisaron en 2006 (Miyakis et al., 2006). Se necesitan tanto criterios clínicos como de laboratorio para diagnosticar el SAF (Tabla 1).

Tabla 1. Clasificación revisada de los criterios diagnósticos de SAF.

El síndrome antifosfolipídico está presente si al menos se cumple uno de los siguientes criterios clínicos y uno de los criterios de laboratorio:

- *Criterios clínicos*:

1. Trombosis vascular:

Uno o más episodios clínicos de trombosis arterial, venosa o de pequeños vasos, en cualquier tejido o órgano. La trombosis debe ser confirmada mediante criterios objetivos validados (p. ej. hallazgos inequívocos mediante pruebas de imagen o diagnóstico histopatológico). La confirmación histopatológica debe mostrar la presencia de trombosis en ausencia de inflamación de la pared vascular.

2. Morbilidad obstétrica:

(a) Una o más muertes fetales inexplicadas con un feto morfológicamente normal (comprobado por estudio ecográfico o inspección directa) en la semana 10 o más allá de la semana 10 de gestación, o

(b) Uno o más partos prematuros de un neonato morfológicamente normal antes de la semana 34 de gestación debido a: (i) eclampsia o preeclampsia severa definidas según definiciones estándar, o (ii) signos de insuficiencia placentaria (mediante registro cardiotocográfico sospechoso de hipoxemia o estudio Doppler sospechoso de hipoxemia fetal o oligoamnios o peso fetal postnatal inferior al percentil 10 para edad gestacional), o

(c) Tres o más abortos consecutivos de causa desconocida antes de la semana 10 de gestación, habiendo excluido alteraciones anatómicas u hormonales maternas o alternaciones cromosómicas paternas o maternas.

- *Criterios de laboratorio*:

1. Anticoagulante lúpico presente en plasma, en dos o más ocasiones separados por, al menos, 12 semanas, detectado según las guías de la International Society on Trombosis and Haemostasis.

2. Anticuerpos anticardiolipina de los isotipos IgG y/o IgM en suero o plasma, presentes a títulos medios o altos (p. ej. > 40 GPL o MPL, o > al percentil 99) en dos o más ocasiones, separadas por al menos 12 semanas, determinados por métodos de ELISA estándar.

3. Anticuerpos anti β_2 glicoproteina I de los isotipos IgG y/o IgM en suero plasma en títulos > al percentil 99, presentes en dos o más ocasiones, separadas por al menos 12 semanas, determinadas por métodos de ELISA estándar, según los protocolos recomendados.

La presencia de AAF en la sangre se ha asociado a cuadros patológicos de diversa índole que implican a diferentes especialidades médicas tales como por ej. la medicina interna (trombosis arteriales o venosas), la cardiología (cardiopatía isquémica), la hematología (trombocitopenia y trombosis), la dermatología (lívedo reticularis), y la obstetricia-ginecología (pérdidas embrionarias o fetales de repetición).

Las pérdidas embriofetales repetidas constituyen la forma de presentación clínica más frecuente del SAF habiéndose descrito tasas del 50-75% de gestaciones malogradas en pacientes con lupus y pudiendo llegar hasta el 90% en el caso del SAF primario (Greaves et al., 2000; Balasch et al., 2002). Sin embargo, en el momento actual, se sabe que la patología asociada a la presencia de los AAF en medicina reproductiva implica algo más que el aborto en la gestación precoz o la muerte fetal intraútero en el embarazo avanzado. Así, hoy sabemos que la incidencia de AAF en pacientes con fracasos repetidos de implantación embrionaria tras fecundación in vitro (FIV) es similar a la observada en pacientes con abortos de repetición (Balasch et al., 1996). Además, conocemos que hasta un 70% de las fecundaciones naturales no alcanzan la viabilidad y la mayoría se pierden en forma de abortos subclínicos o “abortos ocultos” (Li et al., 2002) y el fracaso repetido de la implantación de los embriones en el útero tras la FIV representa por tanto una situación similar al aborto de repetición oculto. Por todo ello, las manifestaciones clínicas del denominado “SAF obstétrico-ginecológico” se han expandido bajo el concepto de “síndrome de fracaso reproductivo autoinmune”

(Balasch et al., 2009). Por otra parte, hoy está bien establecido que además de la muerte fetal in útero, la presencia de los AAF circulantes se asocia a patología gestacional avanzada, especialmente la preeclampsia grave y el retraso de crecimiento intrauterino (Carp, 2004).

El fracaso reproductivo vinculado al SAF se ha explicado esencialmente en base a la existencia de trombosis en la circulación útero-placentaria con la consiguiente inadecuada perfusión sanguínea que lleva a una placentación anómala en el embarazo inicial y al infarto placentario en fases posteriores. El retraso del crecimiento intrauterino, la preeclampsia y las pérdidas embrio-fetales, procesos todos ellos comunes a las gestaciones asociadas a la presencia de los AAF, constituyen un grupo de trastornos con una patología similar en el lecho del embarazo, y el resultado del embarazo depende de la severidad de esta patología (Levine, 2002). Las opciones terapéuticas en estos casos incluyen la aspirina a bajas dosis, heparina, corticoides e inmunoglobulinas. En nuestro departamento tratamos estas pacientes según un protocolo bien establecido que incluye diferentes combinaciones de todos estos fármacos. A pesar de ello, nuestros resultados y los de otros autores indican que aún en las pacientes tratadas, se mantiene una tasa de perdida gestacional del 15%, presentando las gestaciones evolutivas una alta incidencia (cercana al 20%) de preeclampsia, retraso de crecimiento intrauterino y parto pretérmino (Balasch et al., 1993; Balasch et al., 2002; Carmona et al., 2001).

Este fracaso relativo en el tratamiento del SAF obstétrico-ginecológico puede explicarse en parte porque el mecanismo fisiopatológico exacto subyacente a aquellas trombosis e infartos placentarios no ha sido completamente aclarado y si bien se han propuesto diferentes posibles mecanismos de acción de los AAF (interferencia en la formación y desarrollo del trofoblasto, producción de lesiones en el endotelio vascular de la placenta, interferencia con la actividad anticoagulante de las proteínas C y S, alteración de la secreción de tromboxano y/o prostaciclina, disminución de la expresión de proteínas placentarias con actividad anticoagulante como la anexina V o la trombomodulina, disminución de los niveles de interleukina-3, y otros [Caruso et al., 1999; Abrahams, 2009]), ninguno de ellos ha demostrado tener una validez universal.

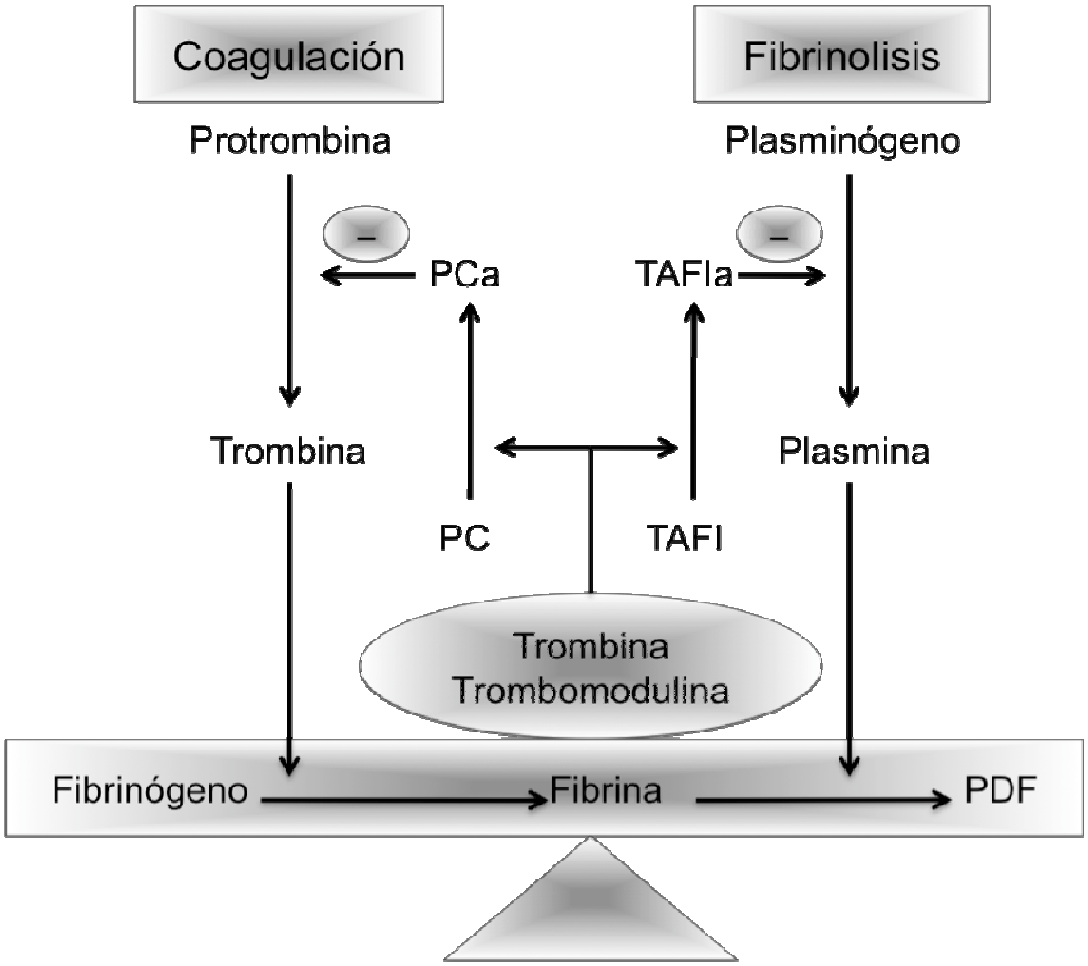
Tal como se expone a continuación, avances recientes en los conocimientos sobre los mecanismos que regulan el proceso de la fibrinolisis podrían contribuir a arrojar nueva luz tanto sobre la patogenia del SAF obstétrico-ginecológico como del fracaso reproductivo asociado a problemas trombóticos independientes de la presencia de AAF circulantes.

1.3 NUEVOS CONCEPTOS EN LOS MECANISMOS PATOGÉNICOS TROMBÓTICOS DE LA PATOLOGÍA GESTACIONAL PRECOZ Y AVANZADA. ALTERACIONES DE LA FIBRINOLISIS

Tradicionalmente se ha considerado que los sistemas de la coagulación y de la fibrinolisis son entidades más o menos separadas e independientes. Sin embargo, estudios recientes indican que son procesos estrechamente relacionados (Nesheim, 2003; Bouma and Mosnier, 2004). Cuando la actividad de los sistemas de la coagulación y de la fibrinolisis está adecuadamente regulada de manera que la formación del coágulo de fibrina y su eliminación están debidamente equilibradas, el sistema vascular queda protegido de una pérdida hemática catastrófica en caso de lesión de los vasos, mientras que se asegura la fluidez necesaria del contenido intravascular. En cambio, si existe algún desequilibrio en este sentido existe el riesgo de hemorragia o trombosis. Se requiere por tanto un delicado balance entre la coagulación y la fibrinolisis que determine la estabilidad justa y necesaria del coágulo de fibrina. De esta manera, el constante depósito y eliminación de fibrina por un lado protege al organismo de la hemorragia y por otro mantiene la fluidez de la circulación sanguínea. El depósito de fibrina tiene lugar tras la activación de la cascada de coagulación, cuando la conversión de protrombina a trombina cataliza la conversión de fibrinógeno soluble a fibrina (Figura 1). La destrucción de fibrina sucede cuando se activa el sistema fibrinolítico. Entonces, el plasminógeno se convierte en la enzima plasmina que cataliza la digestión de la fibrina formada, dando lugar a los productos de degradación de la fibrina. La coagulación y

fibrinolisis están reguladas por diferentes proteínas plasmáticas y de la superfície celular. Un componente importante en esta regulación es la trombomodulina, que es una proteína de membrana integral que se encuentra en la célula endotelial. La trombomodulina se une a la trombina y cambia su sustrato específico de forma que ya no reconoce al fibrinógeno como sustrato. Entonces cataliza la conversión del zimógeno proteína C en la enzima anticoagulante proteína C activada. Esta enzima inactiva los factores Va y VIIIa de la coagulación y de esta manera inhibe la formación de trombina. Al mismo tiempo, la trombina, especialmente cuando se encuentra unida a la trombomodulina, cataliza la activación del zimógeno TAFI (Inhibidor de la fibrinolisis activable por la trombina o “Thrombin-activatable fibrinolysis inhibitor”) a TAFI activado (TAFIa) que tiene una acción antifibrinolítica. Esta vía definida por la trombina, la trombomodulina, y el TAFI, crea, pues, una conexión entre las vías de la coagulación y la fibrinolisis, de forma que la activación de la primera inhibe la activación de la última (Figura 1). Por tanto, queda demostrado (Nesheim, 2003; Bouma and Mosnier, 2004) que el denominado TAFI juega un papel esencial como nexo de unión en la interfaz entre coagulación y fibrinolisis y por tanto se le confiere un importante valor potencial como regulador de la hemostasia, si bien las investigaciones al respecto son aún escasas (Nesheim, 2003; Bouma and Mosnier, 2004).

Figura 1. Equilibrio entre la formación y degradación de fibrina. (PCa = proteína C activada; PC = proteína C; TAFI = Inhibidor de la fibrinolisis activable por la trombina; TAFIa = Inhibidor de la fibrinolisis activable por la trombina activado; PDF = productos de degradación de la fibrina).



De acuerdo con datos de nuestro grupo y de otros autores está bien establecido el papel destacado que juegan las alteraciones de la fibrinolisis en la génesis de la trombosis en pacientes no gestantes (Tassies et al., 2000; Reiner et al., 2001; Leurs and Hendriks, 2005; Zorio et al., 2008). Sin embargo,

en lo que se refiere al papel de la fibrinolisis en la gestación normal o complicada, existe información escasa, en parte contradictoria y por lo general basada en metodología no comparable (Catov et al., 2008; Sartori et al., 2007; Fabbro et al., 2003; Sotiriadis et al., 2007). Por otra parte, ninguno de los estudios previos incluye pacientes con patología gestacional precoz ni la investigación del TAFI. Además, tal como demuestran estudios experimentales recientes, las alteraciones de la fibrinolisis parece que juegan un papel en los mecanismos patogénicos de los AAF (Forastiero et al, 2008; Curnow et al., 2007; Lu et al., 2005; Espinosa et al., 2003; Pierangeli et al., 2008).

Lo cierto es que en la bibliografía (Nesheim, 2003; Bouma and Mosnier, 2004) se destaca la necesidad de investigar el papel del estado de la fibrinolisis en las situaciones de trombosis y en este sentido se confiere un gran valor potencial a dos aspectos: 1) la investigación de tests globales del potencial fibrinolítico tales como el tiempo de lisis del coágulo (“clot lysis time, CLT”) (Lisman et al., 2001); y 2) el papel que pueda jugar en este sentido el TAFI, un inhibidor de la fibrinolisis activado por la trombina y que constituye uno de los principales inhibidores naturales del proceso fibrinolítico (Mosnier et al., 1998). La aplicación conjunta de estos dos métodos permite tanto un estudio directo de la lisis del coágulo como la influencia potencial de uno de sus principales mediadores en el proceso.

1.3.1 TESTS GLOBALES DEL POTENCIAL FIBRINOLÍTICO. TIEMPO DE LISIS DEL COÁGULO (CLT)

Con el fin de analizar *in vitro* la actividad fibrinolítica del plasma se diseñaron algunos tests, como el tiempo de lisis de las euglobulinas o el tiempo de lisis en sangre total diluida. Estas pruebas han demostrado una gran variabilidad y mucha dependencia de características técnicas por lo que los estudios realizados con las mismas no han sido concluyentes (Prins and Hirsh, 1991). Por esta razón y para superar las dificultades técnicas se ha diseñado el CLT que se basa en la inducción de la formación de un coágulo por factor tisular y la medición del tiempo que tarda en lisarse tras la adición de activador tisular del plasminógeno (t-PA). El test del CLT, diseñado originalmente por Lisman et al. en 2001, está influenciado por niveles plasmáticos de proteínas claves en el sistema fibrinolítico, como por ejemplo el inhibidor del activador del plasminógeno tipo I (PAI-I), el TAFI, la α2-antiplasmina y el plasminógeno, pero también está influenciado por niveles de diversos factores de coagulación, en especial el factor II (protrombina) (Lisman et al., 2001; Meltzer et al., 2010). Así pues, los resultados del CLT reflejan el potencial global fibrinolítico del plasma. El descenso del potencial fibrinolítico, es decir un alargamiento del CLT, indica una capacidad fibrinolítica menor y se ha asociado a un riesgo incrementado de trombosis venosa y arterial (Lisman et al., 2005; Meltzer et al., 2009). El CLT se ha utilizado también para el estudio de la fibrinolisis en patologías como la hemofilia y en pacientes tratados con heparina (Lisman et al., 2003), habiéndose demostrado en estas situaciones la existencia de un estado hiper-

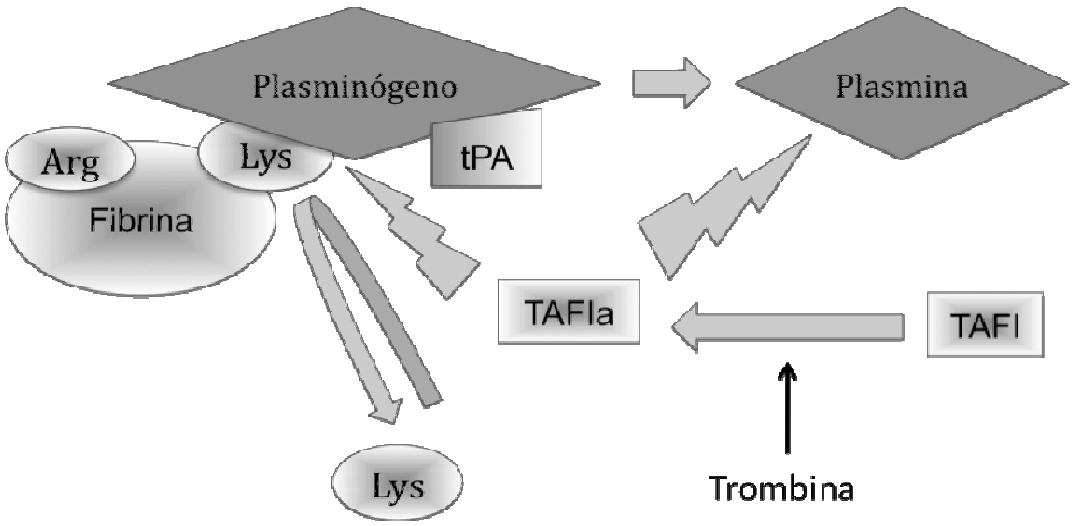
fibrinolítico que se ha asociado a una disminución de la activación del TAFI. El test inicialmente diseñado por Lisman et al (2001) ha sido posteriormente perfeccionado (Lisman et al., 2005), para lograr una valoración más precisa del potencial fibrinolítico global.

Finalmente, desde la perspectiva del presente proyecto, hay que destacar que hasta el momento se han realizado escasos estudios del CLT en pacientes gestantes normales (Goldenberg et al., 2005; Curnow et al., 2007; Mousa et al., 2004), y que no existen datos acerca de pacientes con SAF obstétrico, o en pacientes con complicaciones gestacionales trombóticas sin AAF, por lo que se desconoce el potencial fibrinolítico global del plasma en estos grupos de riesgo trombótico.

1.3.2 TAFI (THROMBIN-ACTIVATABLE FIBRINOLYSIS INHIBITOR)

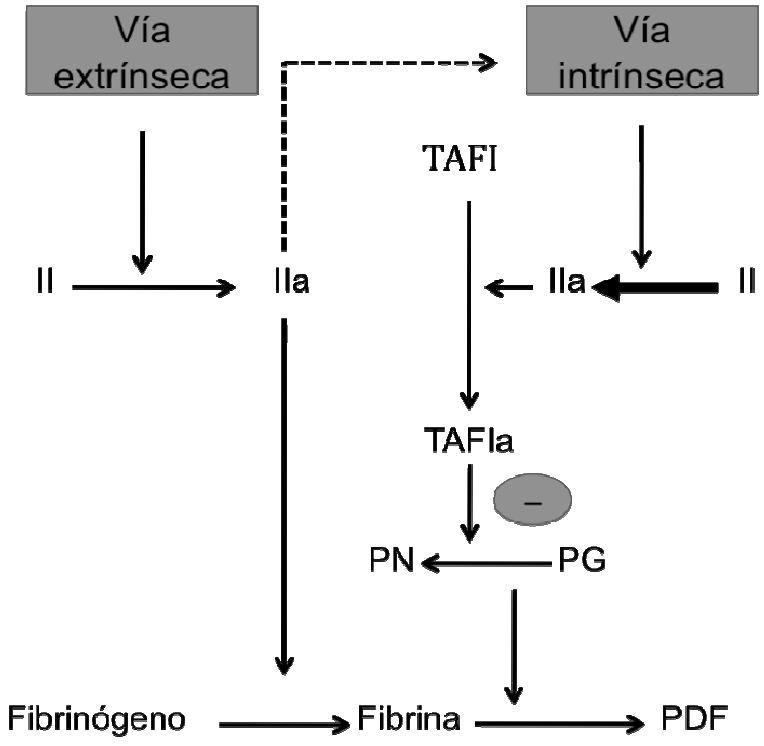
El TAFI es un potente inhibidor natural de la fibrinolisis identificado recientemente (Nesheim, 2003; Bouma and Mosnier, 2004). Se sintetiza en el hígado y circula como un zimógeno que puede ser convertido en TAFI activado (TAFIa). El TAFIa actúa eliminando los residuos lisina carboxi-terminales de la fibrina parcialmente degradada, eliminando así los sitios de unión del plasminógeno y, por tanto, inhibiendo la generación de plasmina (Figura 2). El TAFI puede ser activado por niveles relativamente altos de trombina o, de una forma más eficiente, por el complejo trombina-trombomodulina.

Figura 2. Activación de TAFI y su acción sobre la fibrinolisis (tPA = activador tisular del plasminógeno; Arg = arginina; Lys = lisina; TAFI: Inhibidor de la fibrinolisis activable por la trombina; TAFIa = Inhibidor de la fibrinolisis activable por la trombina activado).



Tras la formación del coágulo de fibrina, la trombina generada activa la vía intrínseca de la coagulación a través de la retroalimentación del factor XI, generando una mayor cantidad de trombina la cual resulta en la activación del TAFI y, por tanto, en la inhibición de la fibrinolisis (Figura 3) (Bouma and Mosnier, 2006; Boffa and Koschinsky, 2007; Mosnier and Bouma, 2006). Así pues, el TAFI actúa como un importante nexo entre la coagulación y la fibrinolisis y la activación del TAFI, que finalmente conlleva la inhibición de la fibrinolisis, resulta, cuando es excesiva, en un riesgo trombótico aumentado.

Figura 3. Inhibición de la fibrinolisis mediada por la vía intrínseca de coagulación (PN = plasmina; PG = plasminógeno; PDF = productos de degradación de la fibrina).



El TAFI presenta una marcada variabilidad interindividual de sus niveles plasmáticos (Chetaille et al., 2000) que están en gran parte determinados genéticamente (Henry et al., 2001; Boffa and Koschinsky, 2007). El gen del TAFI está localizado en el cromosoma 13q14.11, tiene un tamaño de 48 kb, contiene 11 exones y 10 intrones y su región promotora carece de la secuencia de consenso TAATA, de manera que la transcripción se inicia en diferentes lugares del gen. Se han descrito varios polimorfismos en el gen del TAFI y parece que muchos de los polimorfismos del TAFI están en desequilibrio de ligamiento entre ellos. En concreto, dos de ellos, el C+1542G en la región 3'-UTR y el A+505G (ó Ala147Thr) en la región codificante del gen son los que determinan en gran medida los niveles plasmáticos de TAFI circulante (Henry et al., 2001). Ambos polimorfismos son responsables de más del 60-70% de la variabilidad interindividual en los niveles plasmáticos de esta proteína (Henry et al., 2001) siendo escaso el impacto de los factores ambientales (Juhan-Vague et al., 2000). La frecuencia alélica para el polimorfismo C+1542G es de 0,70/0,30 y para el polimorfismo A+505G es 0,76/0,24. (Henry et al., 2001). No obstante, aunque se conoce que los niveles de TAFI son escasamente influenciados por los factores de riesgo cardiovascular tradicionales parece que existen diferentes reguladores de los niveles de TAFI independientes de los polimorfismos (Boffa and Koschinsky, 2007). De este modo parece que los niveles de glucocorticoides, hormonas sexuales, tratamientos hormonales, enfermedades inflamatorias o la gestación, pueden elevar los niveles de TAFI (Boffa and Koschinsky, 2007).

La importancia clínica del TAFI no está todavía bien establecida, aunque se ha demostrado que un aumento de los niveles plasmáticos de TAFI conlleva un estado hipofibrinolítico que se ha identificado como factor de riesgo en trombosis venosas (Van Tilburg et al., 2000; Eichinger et al., 2004) y podría estar relacionado también con un aumento del riesgo de enfermedad tromboembólica arterial (Zorio et al., 2003; Silveira et al., 2000).

Recientemente se ha demostrado un incremento progresivo de TAFI durante la gestación hasta aproximadamente la semana 20. A partir de entonces los niveles de TAFI aumentan moderadamente hasta el final del embarazo, para descender rápidamente tras el parto (Watanabe et al., 2004; Chetaille et al., 2000; Chabloz et al., 2001, Mousa et al., 2004). Se han realizado investigaciones que intentan correlacionar los niveles de TAFI (Antovic et al., 2002; Alacacioglu et al., 2004) y sus variantes alélicas (De Maat et al., 2004) en dos situaciones patológicas obstétricas asociadas a estados trombóticos placentarios como son la preeclampsia y el retraso de crecimiento intrauterino. Los resultados de estos estudios son, sin embargo, en parte contradictorios. Por otra parte, tampoco existen datos en la literatura sobre los niveles de TAFI en las gestaciones con complicaciones obstétricas en el embarazo avanzado en pacientes con SAF, ni sobre el papel que el genotipo del TAFI y sus niveles plasmáticos puedan tener como sustrato del aborto de repetición o del fracaso implantatorio repetido.

Por tanto, todo lo anterior avala el interés de investigar las alteraciones en la fibrinolisis mediante un test global como el CLT y mediante el análisis del TAFI como posibles nuevos mecanismos patogénicos implicados en la patología trombótica gestacional precoz y/o avanzada.

1.4 ANTICUERPOS ANTIFOSFOLIPÍDICOS, FRACASO REPRODUCTIVO Y RIESGO TROMBÓTICO

El síndrome antifosfolipídico (SAF) es una patología multisistémica autoinmune asociada a trombosis vascular (arterial o venosa) y/o morbilidad obstétrica en presencia de anticuerpos antifosfolipídicos (AAF) (Wilson et al., 1999). Por ello, las pacientes portadoras de AAF se considera que presentan un riesgo elevado de complicaciones obstétricas y eventos trombóticos (Empson et al., 2002). El SAF puede aparecer en hasta un 20% de mujeres con abortos de repetición sin otra clínica trombótica u obstétrica asociada. Por otro lado, literatura científica reciente muestra un riesgo elevado de trombosis y un estado protrombótico basal en pacientes con antecedentes de abortos de repetición (Rai, 2003; Smith et al., 2003).

En la actualidad, es conocido que los AAF pueden persistir durante años en determinados pacientes sin presentar complicaciones asociadas. Pero otros pacientes presentaran trombosis y/o complicaciones obstétricas años después de ser portadores de AAF. Hoy en día no se disponen de conocimientos que permitan estratificar el riesgo en estos pacientes portadores de AAF y valorar la necesidad de tratamiento profiláctico (Khamashta, 2001; Meroni, 2008).

Para explicar el hecho de que los eventos trombóticos suceden sólo ocasionalmente en pacientes portadores de AAF de forma persistente se ha propuesto una hipótesis en “dos tiempos” (“two-hit hypothesis"). En un “primer tiempo” se produciría una situación pro-trombótica por la presencia de AAF,

que requeriría la actuación de un factor adicional en un “segundo tiempo” (gestación, inmovilización, cirugía, etc.) para que se desencadenara la trombosis (Meroni and Shoenfeld, 2008; Shoenfeld et al., 2008). Recientemente, dos comunicaciones en la literatura (una de ellas en forma de carta al editor y la otro como comunicación breve) han sugerido, por primera vez, la alta incidencia de trombosis vasculares en pacientes con SAF que previamente sólo habían presentado complicaciones obstétricas como pérdidas gestacionales (Erkan et al., 2001; Tincani et al., 2002). Uno de estos estudios, no obstante, se trata de un estudio retrospectivo que incluyó 65 pacientes, de las que el 50% recibieron aspirina a dosis bajas (Erkan et al., 2001), mientras que la segunda publicación fue un estudio de cohortes con 52 mujeres tratadas con aspirina a dosis bajas (Tincani et al., 2002). Estos estudios no concuerdan con los resultados de un estudio caso-control publicado recientemente (Quenby et al., 2005), que usó cuestionarios para valorar el riesgo trombótico en pacientes con abortos de repetición que concluyó que tanto las abortadoras idiopáticas como en el contexto de un SAF presentan un riesgo a largo plazo similar de trombosis.

Por tanto, la evidencia existente hasta el momento, aunque escasa y en parte controvertida, avala el interés de dilucidar si las pacientes con abortos de repetición, asociados o no a los anticuerpos antifosfolipídicos, tienen un mayor riesgo de trombosis arterial o venosa a largo plazo no relacionado con la gestación.

De lo expuesto hasta aquí se comprende la hipótesis de trabajo y se desprenden la intencionalidad y objetivos de esta Tesis Doctoral tal como se exponen a continuación.

2. HIPÓTESIS DE TRABAJO

1. Existe patología gestacional de origen trombótico tanto en el embarazo precoz (abortos de repetición y fracaso implantatorio tras fecundación in vitro) como avanzado (preeclampsia y retraso de crecimiento intrauterino, entre otros), cuyo nexo común es con frecuencia la existencia de trombofilias hereditarias o adquiridas. El paradigma de trombofilia y patología gestacional trombótica es el síndrome antifosfolipídico (SAF) que configura tanto el denominado “SAF obstétrico-ginecológico” como el “síndrome de fracaso reproductivo autoinmune”.

2. El fracaso reproductivo o las complicaciones obstétricas asociados a los anticuerpos antifosfolipídicos (AAF) se atribuyen esencialmente a la existencia de problemas trombóticos en la circulación útero-placentaria con la consiguiente inadecuada perfusión sanguínea que lleva a una placentación anómala en el embarazo inicial y al infarto placentario en fases posteriores del mismo.

3. A pesar de los tratamientos anticoagulantes y/o inmunosupresores aplicados en las pacientes con problemas reproductivos afectas de un SAF, las pérdidas embriofetales en las gestaciones tratadas siguen siendo de un 15%-20% y los embarazos evolutivos presentan una elevada incidencia (~20%) de complicaciones obstétricas, esencialmente en forma de preeclampsia y/o retraso de crecimiento intrauterino. Esto puede explicarse porque desconocemos en parte los mecanismos patogénicos exactos en el SAF.

4. Conocimientos recientes sobre los mecanismos reguladores de los sistemas de la coagulación y la fibrinolisis avalan el interés y la necesidad de investigar el papel de las alteraciones de la fibrinolisis en las diferentes situaciones de trombosis. En este sentido cabe destacar que: a) el embarazo es un estado protrombótico, y b) la hipofibrinolisis asociada a la gestación en si misma o, en mayor medida, cuando coexiste con otros factores protrombóticos, como son los AAF, pueden implicar un aumento significativo del riesgo de trombosis.

5. Estudios recientes aunque observacionales, retrospectivos, no controlados y basados en un escaso número de casos, han sugerido que las pacientes con abortos de repetición asociado a AAF presentan un riesgo aumentado de sufrir eventos trombóticos, pero sin que exista evidencia definitiva al respecto.

En base a lo anterior, nuestra ***hipótesis de trabajo*** es que, al igual que se ha descrito en patología trombótica en el individuo no gestante, la investigación del potencial fibrinolítico global del plasma y de la regulación de la fibrinolisis puede contribuir a arrojar luz sobre la patogenia de patología gestacional precoz y avanzada de sustrato trombótico vinculado o no a la presencia de AAF. Por otro lado, es posible que el haber presentado abortos de repetición asociados o no a los AAF, constituya un sustrato predisponente a la trombosis arterial o venosa a largo plazo.

3. OBJETIVOS

De forma general, investigar el posible papel de alteraciones de la fibrinolisis como una nueva forma de mecanismo patogénico de la patología trombótica en la gestación inicial y avanzada tanto en pacientes con síndrome antifosfolipídico (SAF) como sin él, basándonos para ello en recientes avances en los conocimientos sobre el proceso de la hemostasia que permiten investigar in vitro tanto el potencial fibrinolítico global del plasma (CLT) como la acción de los principales inhibidores naturales de la fibrinolisis (concretamente el TAFI antígeno [TAFI antígeno se abrevia como TAFI en el resto del texto]). Este objetivo general se concreta en los objetivos específicos siguientes:

1. Determinar el CLT, los niveles plasmáticos de TAFI, y los polimorfismos genéticos de esta proteína en pacientes con pérdidas gestacionales precoces (aborto de repetición y fracaso implantatorio repetido tras fecundación in vitro) **(Estudios 1 y 2).**

2. Investigar si lo anterior, es decir, CLT y niveles plasmáticos y polimorfismos de TAFI, presentan diferencias según se trate de pacientes con abortos de repetición, asociados o no, a la presencia de anticuerpos antifosfolipídicos **(Estudio 2).**

3. Valorar el CLT, los niveles plasmáticos de TAFI, y los polimorfismos de TAFI, a lo largo la gestación tanto en gestantes normales como en pacientes con SAF primario **(Estudio 3).**

4. Correlacionar los resultados obtenidos en cuanto a los niveles de TAFI, sus polimorfismos genéticos y el CLT en las mujeres gestantes, con o sin SAF, con los resultados perinatales y el riesgo de trombosis **(Estudio 3).**

5. Comparar los resultados del CLT, los niveles plasmáticos de TAFI, y los polimorfismos de TAFI en gestantes complicadas con preeclampsia severa, como paradigma de complicación obstétrica de origen trombótico placentario en: a) una cohorte de pacientes con SAF primario; y b) en una cohorte de pacientes sin SAF ni otras trombofilias **(Estudio 4).**

6. Investigar el riesgo de trombosis arterial o venosa a largo plazo no relacionado con la gestación en pacientes con abortos de repetición, asociados o no a los anticuerpos antifosfolipídicos **(Estudio 5).**

4. INVESTIGACIONES REALIZADAS, MÉTODOS Y RESULTADOS

La descripción de las pacientes así como la metodología utilizada en las investigaciones llevadas a cabo para el logro de los objetivos planteados antes, se encuentran detalladamente expuestas en las secciones de “Material y Métodos” de cada uno de los cinco artículos que constituyen el cuerpo doctrinal de la presente Tesis Doctoral.

Dichos artículos se incluyen a continuación tal como han sido publicados en la literatura científica (páginas 34 a 72).

ESTUDIO 1

“Disminución del potencial fibrinolítico del plasma en pacientes con fallo implantatorio repetido tras FIV y transferencia embrionaria”.

“Reduced plasma fibrinolytic potencial in patients with recurrent implantation failure alter IVF and embryo transfer”.

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human reproduction

ORIGINAL ARTICLE *Early pregnancy*

Reduced plasma fibrinolytic potential in patients with recurrent implantation failure after IVF and embryo transfer

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BACKGROUND: Recurrent implantation failure (RIF) following embryo transfer (ET) is a major continuing problem in IVF. Women with haemostatic defects may be at increased risk of miscarriage and preclinical pregnancy loss. The fibrinolytic system is considered, at present, the key to new thrombotic pathogenic mechanisms. Patients with unexplained recurrent miscarriage have an impairment of fibrinolysis, as demonstrated by prolonged clot lysis time (CLT) in association with increased plasma levels of thrombin-activatable fibrinolysis inhibitor (TAFI). In this study, we investigated fibrinolytic potential in patients with RIF.

METHODS: Three groups of patients were studied: 30 women with RIF (RIF group), 60 patients undergoing a first successful IVF–ET cycle (IVF group) and 60 healthy fertile women (FER group). Plasma CLT was measured using a global fibrinolysis assay. TAFI antigen plasma levels and polymorphisms in the TAFI gene (+505A/G and +1542C/G) were analysed using enzyme-linked immunosorbent assay and allele-specific PCR, respectively.

RESULTS: CLT was significantly longer ($P < 0.0001$ and $P < 0.0009$, respectively) and TAFI antigen levels were significantly higher (both $P < 0.0001$) in the RIF versus the IVF and FER groups. A direct relationship between CLT and TAFI antigen levels ($r = 0.40$; $P = 0.001$) was detected in the whole study population. There were no differences in distribution of TAFI polymorphisms between groups.

CONCLUSIONS: Patients with RIF have reduced plasma fibrinolytic potential, as shown by a prolonged CLT, and this may be explained, at least in part, by increased TAFI antigen levels.

Key words: clot lysis time / IVF / recurrent implantation failure / recurrent reproductive failure / thrombin-activatable fibrinolysis inhibitor

Introduction

Despite recent advances in reproductive medicine, human reproductive failure remains a surprisingly frequent event. Early embryonic mortality in humans is very high and it has been postulated that the largest single cause of failed pregnancy is an error of implantation (Bulleti *et al.*, 1996; Bischof *et al.*, 2006; Christiansen *et al.*, 2006). The rate of spontaneous miscarriage may be as high as 60–80% if one takes into account those miscarriages occurring within the first months of conception, which usually go undetected by patients (Bulleti *et al.*, 1996; Choudhury and Knapp, 2000).

Miscarriages affect 15% of women, primarily in the first trimester, and while most are sporadic and non-recurrent, there is a subset comprising 2–5% of couples that suffers recurrent miscarriage (RM) (Clark *et al.*, 2001). These repetitive losses suggest the presence of a specific cause, and much work has been carried out to try to identify the underlying mechanisms. However, the current medical literature suggests that causes are identified in only ~50% of patients (ACOG, 2002; Li *et al.*, 2002). Thus, recurrent pregnancy loss is a vexing problem facing many couples and doctors.

Similarly, implantation failure following embryo transfer (ET) is a major continuing problem in IVF. Thus, it has been disappointing that only ~20% of transferred human embryos resulting from IVF implant in the uterus despite the selection of apparently normal embryos for transfer [International Committee for Monitoring Assisted Reproductive Technology (ICMART, 2009); Nyboe Andersen *et al.*, 2009]. There are some couples who failed to conceive with IVF treatment despite repeated transfers of good-quality

embryos, and these couples are described as having recurrent implantation failure (RIF).

Remarkably, as recently stressed, it is possible that RM and RIF may, in some situations, represent different manifestations of the same pathogenic spectrum and in fact it has been documented that some abnormalities are associated with both implantation failure and miscarriage (Christiansen *et al*., 2006; Stern and Chamley, 2006). Thus, it is postulated that women with haemostatic defects may be at an increased risk of miscarriage and preclinical pregnancy loss, and research aiming to clarify thrombophilic causes of RM and RIF is encouraged (Christiansen *et al*., 2006; Stern and Chamley, 2006).

At present, the fibrinolytic system is considered the key to new thrombotic pathogenic mechanisms (Zorio *et al*., 2008). Routine laboratory assays of fibrinolytic factors provide static data and therefore have been of limited diagnostic use (He *et al*., 1999). In contrast, global coagulation assays provide a source of information that assesses changes over time in the balance of the fibrinolytic system (Curnow, 2006; Wichers *et al*., 2009). Clot lysis time (CLT) is a global test to evaluate fibrinolysis (Lisman *et al*., 2005; Wichers *et al*., 2009). Impaired fibrinolysis increases CLT, and elevated thrombin-activatable fibrinolysis inhibitor (TAFI) antigen levels may contribute to this in part (Wang, 1998).

TAFI is a procarboxypeptidase B-like proenzyme, which is a main inhibitor of fibrinolysis and a known contributing factor in the development of thrombotic events (Bouma and Meijers, 2003). Several polymorphisms have been identified in the TAFI gene (Franco, 2001; Henry, 2001). Among TAFI polymorphisms, +505A/G and +1542C/G (Henry, 2001) have been the most thoroughly studied, and associations have been reported between these polymorphisms and plasma concentrations of TAFI (Franco, 2001; Henry, 2001). Both TAFI levels and TAFI polymorphisms have been related to arterial and venous thrombosis. Plasma hypofibrinolysis, explained by elevated plasma levels of TAFI and plasminogen activator inhibitor-1 (PAI-1), has recently been reported as a cause of venous thrombosis (Meltzer *et al*., 2010).

Very recently, we have reported for the first time that patients with unexplained RM have an impairment of fibrinolysis, as demonstrated by a prolonged CLT, which can at least be partly explained by higher TAFI antigen levels (Martínez-Zamora *et al*., 2010). Therefore, this study was undertaken to investigate CLT, TAFI antigen plasma levels and TAFI polymorphisms in patients with unexplained RIF. The CLT, a global fibrinolysis assay, is considered to be a better method for detecting the risk of venous and arterial thrombosis than traditional laboratory testing of fibrinolytic factors (Curnow *et al*., 2006; Meltzer *et al*., 2009; Wichers *et al*., 2009).

Materials and Methods

Study populations and design

This study was a retrospective analytic investigation of frozen blood samples prospectively collected from 150 women at the Hospital Clínic of Barcelona. A total of 90 infertile patients undergoing IVF treatment at the Assisted Reproduction Unit of our hospital and 60 healthy women fulfilling inclusion criteria reported below were included. All the women involved gave informed consent to participate in the present study, which was approved by the Ethics Committee of our hospital.

Three groups of patients were considered. The study group consisted of 30 women diagnosed as having RIF (RIF group) on the basis of the following criteria. All women have had ≥3 failed ET attempts (range: 3–6) replacing ≥1 high-quality (grades I and II) fresh or frozen-thawed embryos each. All patients underwent at least two fresh ET. Embryos were classified, as we previously reported (Creus *et al*., 2003; Puerto *et al*., 2003), as follows: grade 1: perfectly symmetrical with no fragmentation; grade 2: perfectly symmetrical with slight fragmentation (<20% fragmentation of the total embryonic volume); grade 3: uneven blastomeres with no fragmentation; grade 4: uneven blastomeres with gross fragmentation (>20% fragments). Embryos of grades 1 or 2 were considered high quality and they were at the 4-cell or 8-cell stage on Day 2 or 3 post-fertilization, respectively. For statistical comparison purposes and to quantify objectively the embryo quality, embryos of grades 1–4 were scored 2.5, 2, 1.5 and 1, respectively, as previously reported (Puerto *et al*., 2003). For the final analysis of results, the embryo score per ET was considered as the mean value of the scores given to each of the transferred embryos. All the ETs were performed by senior physicians with ultrasonographic guidance. A difficult ET was defined as previously reported (Puerto *et al*., 2003).

Patients in the RIF group were screened and found to be unaffected by systemic diseases, diabetes mellitus, thyroid dysfunction, polycystic ovary disease, thrombophilia (plasma levels of protein S and C, plasminogen and tissue plasminogen activator (t-PA), factor V Leiden and prothrombin G20210A mutations, acquired protein C resistance and antiphospholipid antibodies) and endometrial and cervical infection. The RIF group also showed no abnormalities in parental chromosome assessment, and uterine and endometrial morphology.

Controls included two groups of women. The IVF group ($n = 60$) comprised the nearest patient undergoing a first successful IVF–ET cycle before and after each ET defining a patient as an RIF (i.e. the closest IVF–ET cycles resulting in live birth and being in temporal relationship with the third failed ET for each patient in group RIF). A fertile control group (FER group) included 60 healthy women who had at least one child born at term and no history of infertility or miscarriage. The FER group was recruited from women who requested surgical sterilization at our hospital. No patient had taken medications known to affect plasma CLT for ≥6 months before the study.

To the best of our knowledge, this study is the first to investigate CLT in patients with RIF. The sample size was based on our previous study, which showed an impairment in fibrinolysis, as demonstrated by prolonged CLT, in patients with unexplained first-trimester RM compared with fertile controls (Martínez-Zamora *et al*., 2010). The sample size required to provide power of 80% to detect at least a similar magnitude of difference between groups using a case–control study design (1:2) was calculated to be ≥15 cases and ≥30 controls, using a two-tailed analysis with a detection limit of 5% of avoiding a type-I error in hypothesis testing. As it is the policy of our laboratory to store blood samples from IVF cycles carried out during the previous 12–14 months, at the time of designing this study, appropriate frozen blood samples were available for 30 patients fulfilling the criteria of RIF provided above. Thus, 60 subjects were included in both control groups (IVF and FER).

Blood collection

In our IVF programme, women have blood samples routinely drawn during the early follicular phase of their cycle within 3 months of the IVF attempt. For the specific purpose of this study, the time point of blood sampling was the same in fertile controls. Thus, in all subjects included in the current investigation, blood samples were obtained on menstrual cycle days 2–4, between 0800 and 1000 h after overnight fasting from food, liquids and cigarettes (only water was allowed). Venous blood samples

for coagulation and fibrinolysis studies were collected in tubes containing 3.8% trisodium citrate (1/9 volume/volume; Becton Dickinson, Rutherford, NJ, USA), and platelet-free plasma was immediately obtained by double centrifugation, first at 2000*g* for 10 min at 22°C, and then at 5000*g* for 10 min at 4°C. Plasma was aliquoted, snap-frozen in a mixture of dry ice/ethanol (1/2 volume/volume) and stored. 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 mM Tris–HCl, pH 6.4] and frozen at −80°C.

Fibrinolysis parameters

TAFI antigen levels were determined by an enzyme-linked immunosorbent assay that is known to detect all the isoforms of TAFI (Gils, 2003) (Asserachrom TAFI, Diagnostica Stago, Asnieres, France).

The CLT, which is the lysis of a thrombin-induced fibrin clot by exogenous t-PA, was studied by monitoring changes in turbidity during clot formation and subsequent lysis. Plasma CLT was measured as previously described, with modifications (Lisman, 2002). Briefly, 75 μl of a mixture containing thrombin (0.2 U/ml), 40ng/ml of t-PA (Actilyse, Boehringer Ingelheim, Germany), 12.5 mM $CaCl_2$ and HEPES buffer (25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, 3 mM $CaCl_2$, 0.1% bovine serum albumin, pH 7.4) was added to 75 μl of citrated platelet poor plasma. After thorough mixing, turbidity at 405 nm was measured in time (minutes) at 37°C in a Multiskan Ascent (Thermolab Systems). Changes in optical density at 405 nm were monitored every 3 min. CLT was defined as the time from the midpoint of the clear to maximum turbid transition, which characterizes clot formation, to the midpoint of the maximum turbidity to clear transition, which represents clot lysis. Samples were tested in duplicate.

Genetic analyses

For detection of TAFI polymorphisms, genomic DNA was extracted from 100 μl of whole blood by a silica gel column method (QIAamp DNA Blood Mini Kit, Qiagen GmbH, Hilden, Germany).

TAFI +505A/G and +1542C/G polymorphisms were determined by allele-specific PCR as previously reported (Henry, 2001) with minor modifications. For the +1542C/G polymorphism, the following primers were used: forward primer: 5′-CCA GCA AGA CCA AAT CA-3′; reverse primer: 5′-ATT ACC GTG GAG CAA AC-3′; C allele (reverse) primer: 5′-AGT CAA ACG TCG AAA CT-3′. G allele (reverse) primer: 5′-AGT CAA ACG TCG AAA GT-3′. PCR was carried out in 50 μl samples, with 40 cycles at 95°C for 60 s, 55°C for 60 s and 72°C for 60 s. PCR products were separated by electrophoresis in a 2.5% agarose gel and visualized under UV light after staining with ethidium bromide. The expected size of the products was a common 408-bp band and a 238-bp band specific for the C or the G allele.

For the +505A/G polymorphism, the following primers were used: a allele primer: 5′-GTT TCT GGA AAA GAA CAA A-3′, G allele primer: 5′-GTT TCT GGA AAA GAA CAA G-3′, common reverse primer: 5′-ATG GCC TAT GAA CCA CAA GC-3′. PCR was carried out in 50-μl samples, with 40 cycles at 95°C for 60 s., 58°C for 60 s and 72°C for 60 s. PCR products were analysed by electrophoresis in a 4% agarose gel. The expected size of the products was 105 bp.

Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences software, Release 15.0 for Windows (SPSS, Chicago, IL, USA). Comparison of quantitative variables was performed using analysis of variance with Bonferroni's *post hoc* analysis. Comparison of qualitative variables was carried out using the χ^2 test. Correlation between quantitative variables was assessed by the Pearson's test. Statistical significance was defined as $P < 0.05$. Results are presented as mean ± SD or median and range.

Results

Clinical characteristics of subjects

Clinical characteristics of the three groups studied are presented in Table I. There were no differences among groups for age, BMI and smoking habit. Also, causes and duration of infertility, the day of ET, the number and quality of embryos replaced, and the number of difficult ETs were similar in the RIF and IVF groups.

CLT, TAFI antigen levels and TAFI polymorphisms

CLT was significantly longer in the RIF group compared with the IVF and FER groups and, similarly, TAFI antigen levels were significantly higher in the study group when compared with both control groups (Fig. 1). A significant direct relationship was found between CLT values and TAFI antigen levels ($r = 0.40$; $P = 0.001$) in the whole study population (Fig. 2).

Allele distribution of TAFI polymorphisms did not differ among the groups (Table II). Plasma TAFI antigen levels were significantly higher in patients with RIF when compared with IVF and FER groups,

Table I Clinical characteristics of the three groups of patients analysed.

Parameter	RIF group ($n = 30$)	IVF group ($n = 60$)	FER group ($n = 60$)	*P*
Age (years)	34.1 ± 2.3	33.8 ± 3.1	32.1 ± 3.9	NS
BMI (kg/m^2)	23.9 ± 4.2	24.3 ± 2.7	24.1 ± 3.4	NS
Smoker	12 (40)	28 (47)	20 (33)	NS
Duration of infertility (years)	4.9 ± 2.1	4.2 ± 1.9		NS
Infertility factor				
Male factor	6 (20)	12 (19)		NS
Tubal factor	13 (43)	28 (47)		
Unexplained	5 (17)	10 (17)		
Endometriosis	6 (20)	10 (17)		
Day of ET				
+2	23 (76.6)	48 (80)		NS
+3	7 (23.4)	12 (20)		
No. of embryos per replacement	2.5 ± 0.5	2.3 ± 0.4		NS
High-quality embryos replaced	1.6 ± 0.2	1.5 ± 0.2		NS
Embryo score/ replacement	2.1 ± 0.2	2.2 ± 0.3		NS
Difficult ET	2 (6.7)	2 (5)		NS

RIF group: patients with recurrent implantation failure; IVF group: infertile patients achieving a live birth on the first IVF/ET attempt; FER group: fertile controls. Values are mean ± SD or *n* (%).

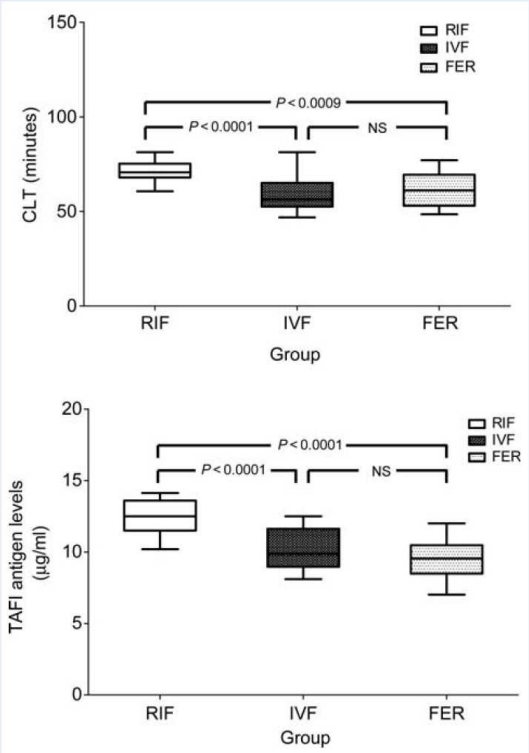

Figure 1 Box plot showing CLT and TAFI antigen levels in RIF patients (RIF group, $n = 30$), infertile patients achieving a live birth with their first IVF/ET attempt (IVF group, $n = 60$) and fertile controls (FER group, $n = 60$). Each box represents the middle 50% of the data (25–75% range). The central horizontal line represents the median. Vertical lines represent the 10–90% range of data, as indicated by the small horizontal lines. Statistical comparisons between groups are indicated (NS, not significant).

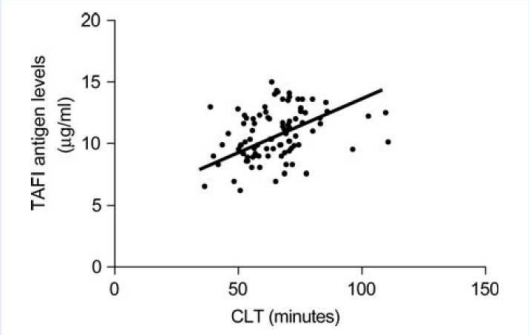

Figure 2 Relationship between CLT values and TAFI antigen levels ($r = 0.40$; $P = 0.001$) in the whole study population ($n = 150$).

Table II **Allele distribution of TAFI polymorphisms in the three groups of patients studied.**

Group	TAFI + 505A/G			TAFI + 1542C/G		
	AA	GA	GG	GG	CG	CC
RIF ($n = 30$)	3 (10)	15 (50)	12 (40)	2 (7)	15 (50)	13 (43)
IVF ($n = 60$)	6 (10)	25 (42)	29(48)	6 (10)	31 (52)	23 (38)
FER ($n = 60$)	7 (12)	26 (43)	27 (45)	4 (7)	30 (50)	26 (43)

Values are *n* (%).

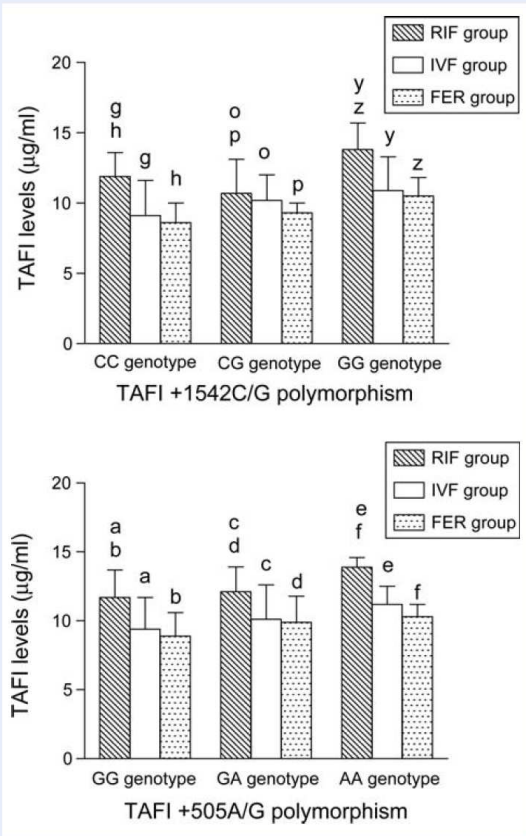

Figure 3 TAFI antigen levels (mean ± SD) according to the genotypes of two TAFI polymorphisms (+1542C/G and +505A/G) in the three groups of patients. Results with common superscripts were significantly different ($^{a-h, p, y, z}P < 0.0001$; $P = 0.001$) (TAFI antigen levels were significantly higher in genotypes +1542 G/G and +505 A/A compared with the respective patient group with the other genotypes, $P < 0.001$).

irrespective of polymorphisms investigated (Fig. 3). As expected, TAFI antigen levels were significantly higher in women with +1542 G/G and +505 A/A versus other genotypes (Franco, 2001; Henry, 2001) (Fig. 3).

Discussion

The haemostatic pathways are intimately involved in ovulation, implantation and placentation and thus, the hypothesis has been developed that many cases of recurrent reproductive failure (i.e. RM and RIF) are caused by a defective maternal haemostatic response leading to thrombosis of the uteroplacental vasculature (Rai, 2003; Christiansen et al., 2006; Stern and Chamley, 2006). The precise mechanism by which thrombophilia may affect RIF, however, is as yet undetermined. Women with a history of RM are in a prothombic state even outside pregnancy and coagulation changes precede pregnancy loss in pregnant women with a history of RM (Regan and Rai, 2002; Rai, 2003; Sebire et al., 2003). While the mechanisms by which thrombophilic factors impact on the frequency of RM are thought to be mainly related to clotting in placental vessels, the methods involved in RIF appear to involve the effects of hypofibrinolysis on trophoblast migration (Coulam et al., 2006; Coulam and Jeyendran, 2009).

The potential implication of fibrinolytic defects in recurrent reproductive failure was first suggested in the early 1990s, but research in this field has only recently undergone a systematic review (Sotiriadis et al., 2007). The fibrinolytic system includes a broad spectrum of proteolytic enzymes with physiological and pathophysiological functions in several processes such as haemostatic balance, tissue remodelling, tumour invasion, angiogenesis and reproduction (Zorio et al., 2008). As early as implantation, the fibrinolytic system participates in the regulation of early human trophoblastic migration and invasion (Coulam et al., 2006; Sotiriadis et al., 2007). Trophoblastic invasion during implantation involves extracellular matrix (ECM) degradation, which is facilitated by matrix metalloproteinases. Expression of metalloproteinases at the implantation site is stimulated by the serine protease plasmin (Coulam et al., 2006; Sotiriadis et al., 2007). Plasmin promotes trophoblastic invasion also by directly degrading certain components of the ECM of decidua (Sotiriadis et al., 2007). Therefore, fibrinolytic abnormalities are likely to result in decreased trophoblast invasion and implantation failure.

Plasmin is the main enzyme of the plasminogen activator system and it is responsible for the degradation of fibrin into soluble degradation products. The activation of plasminogen into plasmin is mediated by two types of activators, urokinase-type plasminogen activator and tissue-type plasminogen activator, and the activity of both types is regulated by specific PAIs. Plasmin can be inhibited by specific plasma inhibitors (mainly α_2-antiplasmin and also α_2-macroglobulin). Fibrinolysis is also reduced by TAFI, which acts as an inhibitor of tissue-type plasminogen activator (Zorio et al., 2008).

According to the above evidence, there is a biological basis for a causative role of fibrinolytic disorders in recurrent reproductive failure. The current investigation is the first report showing increased TAFI antigen levels in patients with RIF, and this was associated with an impairment of fibrinolysis, as demonstrated by an increased CLT. Therefore, our results are in agreement with previous studies suggesting that hypofibrinolysis may be a parameter involved in early reproductive failure, either in the form of RM or RIF (Gris et al., 1997; Coulam et al., 2006; Sotiriadis et al., 2007; Martínez-Zamora et al., 2010). This notwithstanding, it should be stressed that different determinants of CLT exist. Thus, a recent study (Meltzer et al., 2010) showed that PAI-1 levels were the main determinants of CLT, followed by TAFI and other fibrinolytic factors: a clear increase in CLT with increasing plasma levels of PAI-1, TAFI, α2-antiplasmin and decreasing levels of plasminogen was found. In simple regression analyses, all fibrinolytic factors except plasminogen were associated with CLT, the strongest association being found with PAI-1, which, as an independent factor, explained 40% of the variation in CLT followed by TAFI levels, while including all fibrinolytic factors in a multiple regression model increased the explained variance to 53% (Meltzer et al., 2010). In addition, as stressed by the authors themselves (Meltzer et al., 2010), while the role of PAI-1 in venous thrombosis is controversial, TAFI (which defines the molecular connection between the coagulation and fibrinolytic cascades) is clearly an independent risk factor for venous thrombosis. Finally, studies using a clot lysis assay and a model of thrombus lysis (Mutch et al., 2007) have shown a substantial and complementary, but approximately equal, role for PAI-1 and TAFI in the regulation of thrombus lysis. Therefore, although PAI-1 levels were not measured in the current investigation, the above evidence and the direct relationship found between CLT values and TAFI antigen levels in our study allow us to conclude that the impairment in fibrinolysis observed in RIF patients may be explained, at least in part, by increased TAFI antigen levels.

A feature of the present investigation is that we included only patients who have had at least three failed ETs in which reasonably high-quality embryos were transferred. Including patients after only two failed attempts may represent a bias (El-Toukhy and Taranissi, 2006), while an increasing number of authors consider that inability to produce good-quality embryos is itself an important predisposing factor for RIF (Ola and Li, 2006). Also, the case–control study design used (where each case was sampled with two controls at the date when the corresponding case was entered into the IVF programme) helps us to preclude any bias owing to possible changes in IVF laboratory techniques. In addition, women were only recruited for this study if the screening investigations reported above excluded a possible contributing factor for their recurrent reproductive failure (Quenby et al., 2009). This notwithstanding, it could be argued that embryonic aneuploidy, even in couples with normal karyotype, may be a cause of RIF (Margalioth et al., 2006) and preimplantation genetic screening (PGS) was not performed in our study. However, at present, it is widely accepted that there is no evidence that routine PGS is beneficial for patients with RIF (Practice Committee of Society for Assisted Reproductive Technology and The Practice Committee of American Society for Reproductive Medicine, 2008; ACOG, 2009; Harper et al., 2010). Finally, if fibrinolysis was critical to implantation it could be postulated that correction of the supposed defect should reduce miscarriage rates. Recent RCTs showed no benefit of aspirin or heparin in the maintenance of pregnancy in women with idiopathic reproductive failure (Laskin et al., 2009; Clark et al., 2010; Kaandorp et al., 2010). However, it has been previously reported that heparin is unable to stimulate fibrinolysis through a TAFI-dependent mechanism (Colucci et al., 2002) while chronic high-dosing (650 mg every 12 h) with aspirin is necessary to increase susceptibility of fibrin clots to lysis in human subjects (Bjornsson et al., 1989). In contrast, in the RCTs the patients received low-dose (75–80 mg daily) aspirin treatment. In addition, while we have previously reported that aspirin treatment is an independent and significant prognostic factor associated with favourable outcome in patients with fetal losses related to thrombophilia only when used before conception (≥1 month before attempting conception)

(Carmona *et al.*, 2001), in the RCTs (Laskin *et al.*, 2009; Clark *et al.*, 2010; Kaandorp *et al.*, 2010) patients started treatment usually once pregnancy was confirmed by ultrasonography at 6–7 weeks' gestation.

In conclusion, patients with RIF have a reduced plasma fibrinolytic potential, as shown by a prolonged CLT. Analysis of CLT is considered as a better approach to detecting the risk of thrombosis than traditional laboratory testing of fibrinolytic factors. The prolonged CLT may be explained, at least in part, by increased TAFI antigen levels.

Authors' roles

M.A.M.-Z. participated in study design and execution, interpretation of data, manuscript drafting and critical discussion. M.C. has contributed to study execution, analysis and interpretation of data and critical revision. D.T. and J.C.R. have contributed to study design and execution, and analysis and interpretation of data. S.C. participated in study execution, analysis of data and critical revision. F.C. has contributed to study design, interpretation of data, critical revision and provided statistical analysis. J.B. developed the idea for the paper, formulated the study design, participated in the analysis and interpretation of data and wrote the manuscript. All authors have approved the final version of the manuscript. The authors declare no financial or commercial interests involved in this study.

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ESTUDIO 2

“Inhibidor de la fibrinolisis activable por la trombina y tiempo de lisis del coágulo en pacientes abortadoras de repetición con síndrome antifosfolipídico”.

“Thrombin activatable fibrinolysis inhibitor and clot lysis time in women with recurrent miscarriage associated with the antiphospholipid syndrome”.

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(Páginas 43 a 46)

Thrombin activatable fibrinolysis inhibitor and clot lysis time in women with recurrent miscarriage associated with the antiphospholipid syndrome

Antiphospholipid syndrome patients with recurrent miscarriage have an impairment in fibrinolysis demonstrated by prolonged clot lysis time (CLT) that cannot be attributed to differences in thrombin activatable fibrinolysis inhibitor (TAFI) antigen levels. Patients with unexplained recurrent miscarriage have an impairment in fibrinolysis demonstrated by increased CLT, that can be at least partly explained by higher TAFI antigen levels. (Fertil Steril® 2010;94:2437–40. ©2010 by American Society for Reproductive Medicine.)

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Normal pregnancy is associated with changes in all aspects of hemostasis, including an increase in concentrations of most clotting factors, a decrease in the concentrations of some of the natural anticoagulants, and a reduction in overall fibrinolytic activity (1, 2). These changes help to maintain placental function during pregnancy and to meet the hemostatic challenge of delivery but may predispose the woman to thrombosis and placental vascular complications (1, 2). Thus, evidence has been accumulating to suggest that some cases of recurrent miscarriage (RM) are the result of an exaggerated hemostatic response during pregnancy, resulting in thrombosis of the uteroplacental vasculature and subsequent fetal loss (3, 4). Although thrombophilias are debated as a cause of early RM, in the recent literature both acquired and genetic thrombotic conditions are considered to be associated with increased RM (3–5).

The antiphospholipid syndrome (APS) is a common acquired prothrombotic condition characterized by vascular thrombosis and pregnancy morbidity in association with persistently positive circulating antiphospholipid (aPL) (6). We previously reported that these antibodies are detected in 10% of patients with RM (7) and they have emerged as one of the most common causes of this reproductive complication (8). A number of mechanisms by which aPL may promote thrombotic events have been proposed, most of which involve the disturbance of the coagulation pathway and the cells that control them (9, 10). Reduced fibrinolytic activity has been described in APS patients and may be responsible for thrombotic events (11, 12).

Thrombin activatable fibrinolysis inhibitor (TAFI) is a main inhibitor of fibrinolysis and a known contributing factor in the development of thrombotic events (13). Several polymorphisms have been identified in the TAFI gene (14, 15). Among TAFI polymorphisms, +505A/G and +1542C/G (15) have been the most thoroughly studied, and strong associations have been reported between these polymorphisms and plasma concentrations of TAFI (14, 15). Both TAFI levels and TAFI polymorphisms have been related to arterial and venous thrombosis. Routine laboratory assays of fibrinolytic factors provide static data and therefore have been of limited diagnostic use. Overall coagulation assays provide a source of information that assesses changes over time in the balance of the fibrinolytic system (16). Impaired fibrinolysis increases clot lysis time (CLT) and elevated TAFI antigen levels may contribute to this in part (17).

A few studies have investigated TAFI levels (18, 19), TAFI polymorphisms (20), and overall fibrinolytic capacity (21, 22) in patients with unexplained RM, but to the best of our knowledge

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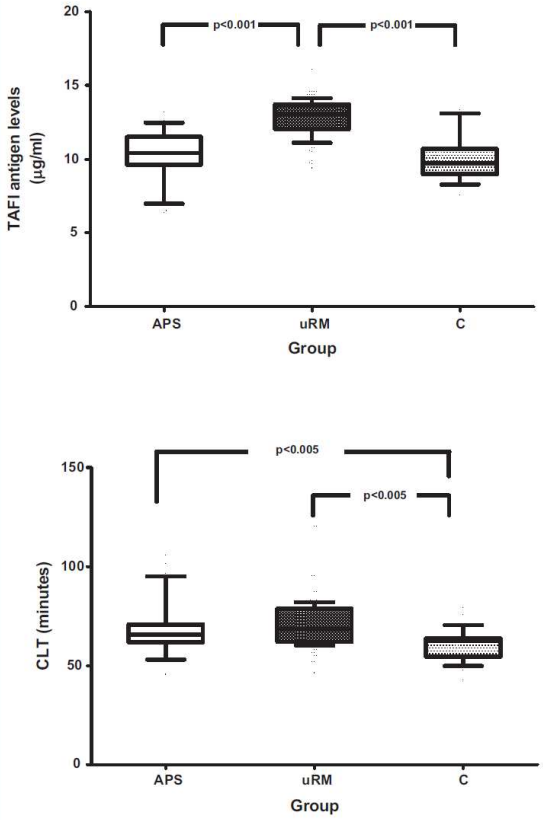
FIGURE 1

Box plot showing thrombin activatable fibrinolysis inhibitor (TAFI) antigen levels determined by an enzyme-linked immunosorbent assay (ELISA) technique and clot lysis time (CLT) in antiphospholipid syndrome patients with ≥3 recurrent miscarriages (APS group), patients with ≥3 unexplained recurrent miscarriages (uRM group), and fertile controls (group C). Each box represents the middle 50% of the data (25% to 75% range). The central horizontal line represents the median. Vertical lines represent the 10% to 90% range of data, as indicated by the small horizontal lines. Statistical differences between groups are indicated.



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there has been no previous report on TAFI or CLT in patients with aPL as the cause of their RM. This case-control study is a retrospective analysis of prospectively collected material to analyze the association between TAFI plasma antigen levels, TAFI polymorphisms, and CLT in APS patients with ≥3 RM (APS group). To this end, two control groups of patients were used: patients with ≥3 unexplained RM (uRM group) and fertile controls (group C).

A total of 246 women were prospectively recruited at the Department of Gynecology at the Hospital Clínic of Barcelona from September 2003 to October 2007. All the women included gave informed consent to participate in the study, which was approved by the ethics committee of our hospital. The study group consisted of 63 women aged 27 to 39 years (mean ± standard deviation [SD], 33.4 ± 4.1 years) who had been previously diagnosed with primary APS and had ≥3 consecutive first trimester spontaneous abortions (APS group). The diagnosis of APS was based on the international consensus statement of the classification criteria for definite APS (6). The uRM patients who tested negative for the lupus anticoagulant and anticardiolipin antibodies were checked for the presence of β2 glycoprotein-I antibodies and all were seronegative.

The first control group included 119 women aged 27 to 38 years (mean ± SD, 34.8 ± 4.1 years) with ≥3 consecutive first trimester spontaneous abortions of unknown etiology (uRM group). Nine patients in the APS group (14%) and 22 patients in the uRM group (18%) had previous successful pregnancies before RM. All patients were routinely screened according to a diagnostic work-up previously reported elsewhere (7, 23) and were found to be normal for known causes of RM (except aPL in patients in the APS group). Fifty-two additional patients were excluded from participation in this study because any abnormality (apart from aPL) in the diagnostic workup for RM was found.

The second control group was composed of 64 women (group C) aged 25 to 37 years (mean ± SD, 32.7 ± 3.9 years) who were selected on the basis of clinical health, having at least one healthy child after an uneventful pregnancy, and having no history of pregnancy loss. Controls were frequency matched in 5-year age groups to patients. No other characteristics were considered in this respect.

No patient had taken medications known to affect plasma CLT for ≥8 weeks before the study. Blood samples for coagulation and fibrinolysis studies and genotype studies were obtained, stored, and evaluated as previously reported elsewhere (15, 24–28).

Statistical analysis was performed with the SPSS 15.0 for Windows (SPSS, Chicago, IL). Comparison of quantitative variables was performed using analysis of variance (ANOVA) with Bonferroni's post hoc analysis. Comparison of qualitative variables was carried out using the chi-square test. $P<.05$ was considered statistically significant. Results are presented as mean ± SD.

The mean age, number of previous pregnancy losses, and mean gestational age at pregnancy loss were similar in the three groups studied (data not shown). As expected, women in the APS and uRM groups had more previous pregnancies compared with group C (3.9 ± 4, 4.3 ± 17, and 1.4 ± 3, respectively), but no differences were observed between the APS and uRM groups in this respect.

Figure 1 shows TAFI antigen levels and CLT values in the three groups studied. The TAFI antigen levels were statistically significantly higher in the uRM group compared with the other two groups. The CLT was statistically significantly longer in the APS and uRM groups compared with group C. The allele distribution of TAFI polymorphisms was similar within the three groups studied; the TAFI antigen levels were found to be as expected considering each allele (14, 15), and were higher in genotypes +1542 GG and +505 AA (data not shown).

Although the association of aPL antibodies with pregnancy loss and pregnancy morbidity is widely accepted, its mechanisms remain unknown (28). Impaired fibrinolysis has been one of

pathogenic mechanisms proposed (27, 29). Previous work in recent literature has focused on fibrinolytic defects and RM (18–22, 30). Remarkably, there are no eligible studies for TAFI, and data on other factors have either failed to show an association or were quite limited (30). High TAFI levels in RM patients without aPL have been proposed as a protective factor against miscarriage (18, 19). This is in contrast with the results of our study in which higher TAFI antigen levels were observed in the uRM group.

Discrepancies between the previous reports and our study may be accounted for in several ways. As noted by the investigators themselves, those studies were retrospective, the clinical characteristics of the patients were ill-defined, and systematic research for other causes of RM was not performed; thus, selection bias may have been introduced. Only two previous studies have investigated the overall fibrinolytic capacity of RM patients without aPL, and both reports concluded that impaired fibrinolysis could be a risk factor for thrombosis in future untreated pregnancies (21, 22). This is in keeping with our study, in which longer CLT in the uRM group was found.

Genetic factors may explain between 20% and 62% of the variation in TAFI antigen levels, but the relationships among TAFI polymorphisms, TAFI antigen levels, and the risk of thrombotic complications are not well understood (21, 22). We found no differences between groups in the distribution of alleles of the two investigated. This is in contrast with the report by Masini et al. (20) that concluded that some single nucleotide polymorphisms could be associated with a reduced risk of RM. However, as stressed by the investigators (20), further investigation is needed to also evaluate TAFI antigen levels and CLT in patients with RM to better understand the actual role of TAFI antigen levels in RM. We examined this issue and, in addition, included only patients with $\geq$3 miscarriages of unknown etiology whereas Masini et al. (20) included patients with $\geq$2 abortions and as many as 45% of patients had at least one definite cause of RM detected in the diagnostic work-up.

Our results show increased TAFI antigen levels in uRM patients compared with fertile controls, thereby adding further evidence to support the previous work favoring a role of TAFI antigen levels in RM (20). Notwithstanding, our study found that increased CLT was associated with higher TAFI antigen levels in the uRM but not the APS group. Similarly, we have reported impaired fibrinolysis to be associated with normal TAFI antigen levels in APS pregnant patients with late obstetric complications (27). These findings can be explained on the basis that the contribution of TAFI antigen levels to CLT is limited, despite an association between these two parameters (31). On the other hand, it should be emphasized that the pathogenesis of pregnancy complications in APS is multifactorial (10–12), and that disruption of fibrinolysis is just one of multiple pathophysiologic mechanisms for thrombotic events in pregnant patients.

Our study has two possible limitations. The one is that the sample size was decided arbitrarily but in keeping with a recent study investigating TAFI polymorphisms in RM patients without the APS (20). However, our study included a sufficient number of patients to avoid chance findings (type 1 error) and to avoid missing the detection of a true difference (type 2 error) when comparing CLT in APS and uRM groups with controls, as well as when TAFI antigen levels were compared between the APS versus uRM groups and the uRM versus C groups. On the other hand, considering the differences in CLT values and TAFI antigen levels observed in our study, a sample size of 436 and 914 patients per group would be necessary to provide 80% statistical power for avoiding a type 2 error, and a 5% chance of making a type 1 error when comparing CLT values between APS and uRM groups and TAFI antigen levels between APS and C groups, respectively.

In addition, a common drawback to most studies on potential etiologic factors in uRM patients is that the most common cause of miscarriage is aneuploidy. The study of products of conception is mandatory to rule out the possibility that patients had aneuploidy pregnancies. Cytogenetic analysis of miscarriages was not available for the specific purposes of our investigation. However, the rationale for abortus tissue karyotyping is that if the abortus is aneuploid the physician may conclude that a maternal cause of pregnancy loss is excluded. Moreover, an abnormal abortus karyotype is a legitimate explanation for the loss that may provide a source of comfort to the couple. However, as stressed by the American College of Obstetricians and Gynecologists (32), no published evidence supports these hypotheses, and definite recommendations for routinely obtaining abortus karyotypes cannot be made. In addition, maternally rather than fetally caused RM is primarily observed in young women ($\leq$35 years) (33), and as many as 81% and 87% of the women in our APS and uRM groups, respectively, were younger than 35 years.

Patients with RM associated with APS and uRM patients have an impairment in fibrinolysis demonstrated by prolonged CLT. These fibrinolytic changes can be partially attributed to differences in TAFI antigen levels in uRM patients but not in APS patients. To the best of our knowledge, this has not been previously reported. Therefore, further studies are warranted to confirm or refute our findings.

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ESTUDIO 3

“Inhibidor de la fibrinolisis activable por la trombina y tiempo de lisis del coágulo en gestantes con síndrome antifosfolipídico: relación con el resultado gestacional y con las trombosis”.

“Thrombin activatable fibrinolysis inhibitor and clot lysis time in pregnant patients with antiphospholipid syndrome: relationship with pregnancy outcome and thrombosis”.

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(Páginas 48 a 56)

ORIGINAL ARTICLE

Thrombin Activatable Fibrinolysis Inhibitor and Clot Lysis Time in Pregnant Patients with Antiphospholipid Syndrome: Relationship with Pregnancy Outcome and Thrombosis

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Keywords
Antiphospholipid syndrome, clot lysis time, pregnancy, thrombin activatable fibrinolysis inhibitor

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Problem
Antiphospholipid syndrome (APS) pregnancies are associated with thrombotic obstetric complications, despite treatment. This study evaluated Thrombin Activatable Fibrinolysis Inhibitor (TAFI) levels, TAFI gene polymorphisms and Clot Lysis Time (CLT) in pregnant patients with APS in relation to pregnancy outcome and thrombosis.

Method of study
Group 1 consisted of 67 pregnant patients with APS. Group 2 included 66 pregnant patients with uneventful term pregnancies and delivery. Patients were sampled during each trimester and at baseline. TAFI antigen and CLT and two polymorphisms of the TAFI gene, Ala147Thr and +1542C/G, were determined.

Results
Significantly prolonged CLT was found at baseline in Group 1. Allele distribution of the TAFI gene polymorphisms was similar in both groups. Basal TAFI and CLT in patients with APS having an adverse or a good obstetrical outcome were similar. Comparison of TAFI and CLT baseline levels in patients with APS with or without previous thrombosis showed no statistical differences.

Conclusion
Patients with APS have impairment in fibrinolysis evidenced by prolonged CLT at baseline. TAFI and CLT do not seem to be useful as markers of obstetric outcome or risk of thrombosis in patients with APS.

Introduction

The association of antiphospholipid (aPL) antibodies with pregnancy complications has increased the frequency with which antiphospholipid syndrome (APS) is diagnosed and has generated substantial interest in elucidating its pathophysiology. Nowadays, although the association of aPL antibodies with pregnancy loss and pregnancy morbidity is clear, its mechanism remains unknown.[1] Defining this mechanism is crucial to find optimal treatment regimens as many treated pregnant patients have thrombotic-related complications such as pre-eclampsia (PE), uteroplacental insufficiency, intrauterine fetal

growth restriction (IUFGR), fetal loss and pre-term birth despite treatment.[2,3]

Recent insight into aPL antibody-mediated thrombosis has proposed a number of pathogenic mechanisms involving cell-mediated events on monocytes, endothelial cells, platelets, inhibition of anticoagulant reactions and impaired fibrinolysis.[4,5] Referring to this last mechanism, it seems that aPL antibodies may disturb fibrinolysis and thereby contribute to vascular thrombotic complications.[4–6] A recent study published by our group involving pregnant patients with APS, showed impaired factor XIIa-dependent activation of fibrinolysis as a mechanism related to late pregnancy complications in patients with APS,[7] and other recent studies have described abnormal fibrinolytic states in patients with aPL antibodies.[8–10]

Thrombin Activatable Fibrinolysis Inhibitor (TAFI) is a procarboxypeptidase B-like proenzyme, which is a main inhibitor of fibrinolysis and a known contributing factor in the development of thrombotic events.[11] Activated TAFI down-regulates fibrinolysis by removing C-terminal lysine residues from fibrin, thereby reducing adsorption of tissue plasminogen activator (tPA) and plasminogen to fibrin, and decreasing plasmin formation.[12] The main physiological activator of TAFI is thrombin/thrombomodulin complex.[13] Several polymorphisms have been identified in the TAFI gene.[14–17] Among TAFI polymorphisms, Ala147Thr (505A/G), and +1542C/G[16] have been the most thoroughly studied, and strong associations have been reported between these polymorphisms and plasma concentrations of TAFI.[17,18] Both TAFI levels and TAFI polymorphisms have been related to arterial and venous thrombosis.

Routine laboratory assays of fibrinolytic factors provide static data and therefore have been of limited diagnostic use.[19] Global coagulation assays may provide an alternative source of information that assesses changes over time in the balance of the fibrinolytic system.[20,21] Clot lysis time (CLT) is a global test to evaluate fibrinolysis. Impaired fibrinolysis slows CLT and TAFI may contribute to this in part.[12]

To our knowledge, there are no previous reports on TAFI and/or CLT in pregnant patients with APS. Several groups have studied TAFI and CLT in patients with other autoimmune diseases with a thromboembolic tendency such as Behçet disease[22,23] or systemic lupus erythematosus,[24] and only a few reports have described TAFI or CLT in patients with aPL but without APS.[20,24,25] A few studies have investigated TAFI levels and CLT in normal[26–31] and complicated pregnancies with PE and IUFGR with contradictory results.[32–34] Moreover, only one previous study investigated TAFI polymorphisms as a genetic risk factor of PE showing no association.[35]

The aim of this study was to describe TAFI levels, TAFI polymorphisms and CLT in pregnant patients with APS and analyze them according to pregnancy outcome and previous thrombosis.

Materials and methods

Patients

A total of 151 pregnant women were prospectively recruited at the Hospital Clínic of Barcelona from July 2003 to September 2006. All the women involved gave informed consent to participate in this study, which was approved by the Ethics Committee of our hospital. Group 1 consisted of 75 pregnant patients that had been previously diagnosed with primary APS. The diagnosis of APS was based on the International consensus statement of the classification criteria for definite antiphospholipid syndrome,[36] and it was confirmed that patients that had been diagnosed before the consensus was published, fulfilled all the updated international consensus criteria. These patients were managed as having high-risk pregnancies and treated with low-dose aspirin alone ($n = 44$) or associated with low-molecular weight heparin ($n = 31$), according to the protocol that our group has previously described.[2,37] Briefly, our standard treatment for APS consists of low-dose aspirin (100 mg/day) started whenever possible from 1 month before attempting conception and maintained throughout the pregnancy. Low-molecular-weight heparin is used in combination with aspirin in cases of previous vascular thrombotic history or after treatment failure of aspirin alone in a previous pregnancy. Group 2 ($n = 76$) comprised the next consecutive pregnant patients having a normal term pregnancy and delivery after each patient in Group 1 was recruited. As inclusion criteria, all patients had to carry their pregnancies beyond 26 weeks of gestation, thus reaching fetal viability.[38] Exclusion criteria were a history of venous thrombosis (except in patients in group 1), known thrombophilia (except APS in patients in group 1), hypertension or other significant medical diseases. Receiving drugs, except for iron supplementation during gestation, was also considered as exclusion criteria. At recruitment, patients were checked for the procoagulant

factor V Leiden or prothrombin G20210A genetic variations, the lupus anticoagulant and anticardiolipin antibodies, and the levels of protein C and protein S.

A total of 18 patients were excluded. There were eight spontaneous abortions during the first trimester in Group 1. In Group 2, there were eight spontaneous abortions during the first trimester, one patient was found to have the G20210A prothrombin genetic variation and another patient had the factor V Leiden genetic variation. After excluding these patients, a total of 133 pregnant women were included in the study. Thus, there were 67 patients in Group 1 and 66 patients in group 2 for the final analysis of results.

Poor obstetric outcome was defined as maternal thrombosis, and/or maternal death, and/or fetal death beyond 26 weeks gestation, and/or severe preeclampsia below 32 weeks gestation, and/or severe intrauterine fetal growth restriction below 32 weeks gestation, and/or pre-term delivery below 32 weeks gestation and/or severe fetal distress. Pregnancy complications were defined as previously reported.[2,39–41]

Blood Collection

Four blood samples were obtained in both groups: at each trimester of gestation (between weeks 6 to 10, 18 to 20, and 28 to 32) and at 4–6 months after delivery.

Venous blood samples for coagulation and fibrinolysis studies were obtained in tubes containing 3.8% trisodium citrate (1/9 v/v; Becton Dickinson, Rutherford, NJ, USA), and platelet-free plasma was immediately obtained by double centrifugation, first at $2000 \times g$ for 10 min at 22°C, and then at $5000 \times g$ for 10 min at 4°C. Plasma was aliquoted, snap-frozen in a mixture of dry ice/ethanol (1/2 v/v), and stored. For genotype studies, samples were drawn in trisodium ethylene diamine tetra-acetic acid (EDTA) tubes (Becton Dickinson), and 100 µL of whole blood was immediately transferred into tubes containing lysis buffer [5 M guanidine thiocyanate, 1.3% (wt/v) Triton X-100, and 50 mM Tris HCl, pH 6.4] and frozen at −80°C.

Antiphospholipid Antibody Testing

Lupus anticoagulant (LA) was detected using activated partial thromboplastin time, diluted Russell's viper venom time, and 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.[42] The anticardiolipin antibodies (aCLs) were determined by enzyme-linked immunosorbent assay (ELISA) (Cheshire Diagnostics, Cheshire, UK).

Fibrinolysis Parameters

Thrombin Activatable Fibrinolysis Inhibitor (TAFI) antigen levels were determined by an ELISA known to detect all the isoforms of TAFI[43] (Asserachrom TAFI, Diagnostica Stago, Asnieres, France).

The CLT, which is the lysis of a thrombin-induced fibrin clot by exogenous tPA, was studied by monitoring changes in turbidity during clot formation and subsequent lysis. Plasma clot lysis time was done as previously described with modifications.[44] Briefly, 75 µL of a mixture containing thrombin (0.2 U/mL), 40 ng/mL tPA (Actilyse, Boehringer Ingelheim, Germany), 12.5 mM $CaCl_2$ and HEPES buffer (25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, 3 mM $CaCl_2$, 0.1% bovine serum albumin, pH 7.4) was added to 75 µL of citrated platelet poor plasma. After thorough mixing, turbidity at 405 nm was measured in time (min) at 37°C in a Multiskan Ascent (Thermolab Systems, Waltham, MA, USA). Changes in optical density at 405 nm were monitored every 3 min. CLT was defined as the time from the midpoint of the clear to maximum turbid transition, which characterizes clot formation, to the midpoint of the maximum turbidity to clear transition, which represents clot lysis. Samples were tested in duplicate.

Genetic Analyses

For detection of polymorphisms genomic DNA was extracted from 100 µL of whole blood by a silica gel column method (QIAamp DNA blood mini kit; Qiagen GmbH, Hilden, Germany).

TAFI Ala147Thr and +1542C/G polymorphisms were determined by allele-specific polymerase chain reaction (PCR) as previosly reported[16] with minor modifications. For the +1542C/G polymorphism the following primers were used: Forward primer: 5′-CCA GCA AGA CCA AAT CA-3′. Reverse primer: 5′-ATT ACC GTG GAG CAA AC-3′. C allele (reverse) primer: 5′-AGT CAA ACG TCG AAA CT-3′. G allele (reverse) primer: 5′-AGT CAA ACG TCG AAA GT-3′.

PCR was carried out on 50 µL volume samples, with 40 cycles at 95°C for 60 s, 55°C for 60 s, and 72°C for 60 s. Detection was made in 2.5% agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide. The expected size of the products was a common 408 bp band and a 238 bp band specific for the C or the G allele.

For Ala147Thr polymorphism the following primers were used: A allele primer: 5′-GTT TCT GGA AAA GAA CAA A-3′, G allele primer: 5′-GTT TCT GGA AAA GAA CAA G-3′, common reverse primer 5′-ATG GCC TAT GAA CCA CAA GC-3′. PCR was carried out on 50 µL volume samples, with 40 cycles at 95°C for 60 s., 58°C for 60 s., and 72°C for 60 s. Detection was made in 4% agarose gel electrophoresis. The expected size of the products was 105 bp.

Statistics

Statistical analysis was performed with the Statistical Package for the Social Sciences software, Release 15.0 for Windows (SPSS, Chicago, IL, USA). Comparison of quantitative variables was performed using the independent or paired-samples *t*-test or ANOVA as appropriate. Comparison of qualitative variables was carried out using the chi-squared test. Statistical significance was defined as $P < 0.05$.

Results

The clinical characteristics of both groups are shown in Table I. As expected, patients in Group 1 had more of previous abortions and fetal deaths compared with Group 2. There were more cesarean sections and a lower neonatal birth weight in Group 1. Previous neonatal death and neonatal mortality was higher in Group 1, although the difference did not reach statistical significance.

Seventeen patients of Group 1 had poor obstetric outcome: There were five gestational thromboses (two arterial and three venous thromboses); five severe PE; two severe IUFGR (one neonatal death); four severe PE and severe IUFGR (two neonatal deaths); one antenatal death.

Fig. 1 shows TAFI levels and CLT values throughout the study period in both groups. There was a progressive increase in TAFI levels and CLT from the first to the third trimester, dropping to normality after delivery. TAFI levels and CLT showed no statistical differences between groups during pregnancy (First trimester TAFI levels group 1/group 2: 10.5 ± 1.9 µg/mL/10.3 ± 1.6 µg/mL; CLT group 1/group 2: 68.4 ± 15.9 min/66.9 ± 9.4 min; Second trimester TAFI levels group 1/group 2: 11.7 ± 1.9 µg/mL/ 11.8 ± 1.9 µg/mL; CLT group 1/group 2: 78.5 ±

Table I Clinical Characteristics and Pregnancy Outcome

Parameter	Group 1(n = 67)	Group 2 (n = 66)	P
Age (years)[a]	33.9 ± 4.4	32.8 ± 3.8	NS
Previous pregnancies[b]	157(2.45;0–7)	48(0.74;0–5)	<0.05
Previous abortions <10 weeks[b]	99(1.55;0–6)	22(0.33;0–3)	<0.05
Previous fetal deaths >10 weeks[b]	19(0.3;0–2)	1(0.02;0–1)	<0.05
Previous neonatal death[b]	8(0.3;0–1)	0(0;0–0)	NS
Gestational age at delivery (weeks)[a]	37.3 ± 3.5	39.1 ± 1.8	NS
Neonatal birth weight (g)[a]	2800 ± 807	3275 ± 432	<0.05
Cesarean section[c]	34 (50.7)	9 (13.6)	<0.05
Pre-term delivery[c]	13 (19.4)	7 (10.6)	NS
Neonatal mortality[c]	3 (4.5)	0	NS
Gestational age at first trimester blood drawn (weeks)[a]	8.7 ± 1.4	9.2 ± 1.2	NS
Gestational age at second trimester blood drawn (weeks)[a]	19.0 ± 1.6	19.3 ± 1.7	NS
Gestational age at third trimester blood drawn (weeks)[a]	30.4 ± 0.9	29.6 ± 1.2	NS
Post-partum blood drawn (weeks)[a]	21.1 ± 3.6	19.7 ± 2.7	NS

[a]Values are mean ± S.D.
[b]Values are *n* (mean; range).
[c]Values are *n* (%).
NS ($P > 0.05$; no statistical differences).

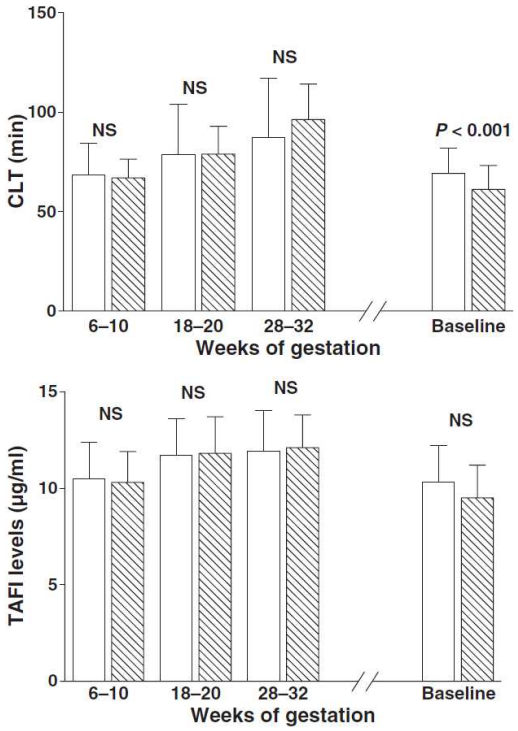

Fig. 1 Clot lysis time (CLT) and thrombin activatable fibrinolysis inhibitor (TAFI) levels during the three trimesters of pregnancy and at baseline in patient (Group 1, open bars) and control (Group 2, dashed bars) groups. Statistical comparisons between groups are indicated with superscripts (NS: non- significant).

Table II Distribution of Thrombin Activatable Fibrinolysis Inhibitor (TAFI) Polymorphisms in Both Groups of Patients Studied

	TAFI Ala147Thr			TAFI C + 1542G		
Group	Ala/Ala	Ala/Thr	Thr/Thr	GG	CG	CC
Group 1	32	30	5	4	25	38
Group 2	29	32	5	6	24	36

25.4 min/78.9 ± 14 min; Third trimester TAFI levels group 1/group 2: 11.9 ± 2.1 µg/mL/12.1 ± 1.7 µg/mL; CLT group 1/group 2: 87.3 ± 29.6 min/96.3 ± 17.8 min). CLT was statistically higher in group 1 at baseline [CLT group 1/group 2: 69.2 ± 12.8 min/61.1 ± 12.1.0 min ($P < 0.001$)]. These differences were not observed in TAFI levels at baseline (TAFI antigen levels group 1/group 2: 10.3 ± 1.9 µg/mL/9.5 ± 1.7 µg/mL).

The allele distribution of TAFI polymorphisms was similar in both groups (Table II) and TAFI levels were found to be as expected considering each allele, being higher in genotypes +1542 CC and 147 AA or GA (Table III).

The comparison of TAFI and CLT baseline levels in patients with APS and previous thrombosis ($n = 23$) [during this pregnancy ($n = 5$) and/or before this pregnancy ($n = 22$)] and patients with APS without previous thrombosis ($n = 44$) showed no statistical differences [TAFI previous thrombosis: 9.6 ± 2.2 µg/mL; TAFI no previous thrombosis 10.7 ± 1.7 µg/mL ($P = 0.1$); CLT previous thrombosis: 63.6 ± 12.8 min; CLT no previous thrombosis: 71.8 ± 12.3 min ($P = 0.08$)].

The comparison of TAFI levels and CLT in APS patients with complicated or uneventful pregnancies did not show any differences during pregnancy or baseline (data not shown). The allele distribution of the TAFI polymorphisms was similar in APS patients with complicated or uneventful pregnancies (data not shown).

Discussion

This study shows that patients with APS have an impairment in fibrinolysis demonstrated by a prolonged baseline CLT compared with controls. These fibrinolysis changes were not related to differences in TAFI levels. TAFI levels and prolonged CLT were not related to obstetric outcome or previous history of thrombosis in patients with APS. This is, to our knowledge, the first study that has analyzed TAFI antigen levels and polymorphisms and CLT in pregnant patients with APS and has searched for the possible relationship with pregnancy outcome and the risk of thrombosis.

This study confirms and expands our previous work by providing evidence supporting an impairment in fibrinolysis in patients with APS.[7] We demonstrated that the impaired factor XIIa-dependent activation of fibrinolysis seems to be a key mechanism related to late-pregnancy complications in patients with the APS, showing an alteration of the intrinsic fibrinolysis pathway.[7] In this study, we aimed to investigate the capacity of fibrinolysis activated by the tPA pathway and its regulation by TAFI, the so-called extrinsic fibrinolysis pathway. We found a prolonged CLT in patients with APS at baseline demonstrating a disturbed fibrinolysis that may contribute to vascular complications. This study

Table III Thrombin Activatable Fibrinolysis Inhibitor (TAFI) Levels[a] According to the Polymorphism in Both Groups of Patients Studied During the Pregnancy and at Baseline

		TAFI Ala147Thr polymorphism				TAFI C + 1542G polymorphism			
Sampling time	Group	Ala/Ala	Ala/Thr	Thr/Thr	*P*	CC	CG	GG	*P*
1st trimester	Patients	9.4 ± 1.8	11.2 ± 1.5	11.9 ± 1.8	<0.05	7.1 ± 1.3	8.6 ± 2.3	9.8 ± 2.7	<0.05
	Controls	9.1 ± 1.3	11.2 ± 1.3	11.2 ± 1.8	<0.05	7.7 ± 0.9	7.9 ± 1.3	10.2 ± 0.7	<0.01
2nd trimester	Patients	11.0 ± 2.0	12.0 ± 1.4	13.6 ± 0.9	<0.05	8.6 ± 2.3	12.0 ± 2.0	11.9 ± 1.6	<0.01
	Controls	10.6 ± 1.9	12.6 ± 1.4	13.6 ± 0.5	<0.05	7.9 ± 1.3	11.4 ± 1.6	12.6 ± 1.3	<0.01
3rd trimester	Patients	11.2 ± 2.1	12.6 ± 1.8	14.7 ± 0.8	<0.01	9.8 ± 2.7	12.0 ± 1.4	12.2 ± 2.2	<0.05
	Controls	10.9 ± 0.9	12.7 ± 1.6	15.2 ± 0.6	<0.01	10.2 ± 0.7	11.4 ± 1.3	12.7 ± 1.8	<0.01
Baseline	Patients	10.0 ± 2.0	10.5 ± 1.7	12.1 ± 1.2	<0.05	8.8 ± 2.6	10.3 ± 1.3	10.6 ± 2.2	<0.05
	Controls	8.6 ± 1.3	10.5 ± 1.5	11.6 ± 0.6	<0.01	7.4 ± 1.3	9.0 ± 0.7	10.5 ± 1.6	<0.01

[a]Values are mean ± S.D. in μg/mL.

did not show an association between pregnancy complications and higher TAFI and/or prolonged CLT. This may be attributable to several factors. First, pathogenesis of obstetric complications is multifactorial,[4–6,45] and disruption of fibrinolysis is just one of the proposed pathophysiological mechanisms for APS obstetric thrombotic complications. Second, in this study, complications were heterogeneous, from severe PE and/or severe IUFGR to maternal thrombosis, with a low number of pregnancy complications in the APS group. Finally, studies investigating TAFI levels and CLT during pregnancy in patients with thrombotic complications such as PE and/or IUFGR,[30,31] have shown contradictory results. Unfortunately, however, in these studies,[30,31] baseline TAFI and CLT values were not provided.

The role of TAFI in thrombotic manifestations and autoimmune diseases different from APS has been investigated in several studies with contradictory results. TAFI has been studied in systemic lupus erythematosus (SLE) with and without aPL, and Behçet disease (BD) without aPL, as both diseases are considered vasculitis associated with a thromboembolic tendency. Similar to our study, the TAFI antigen was not statistically different between SLE patients and healthy controls, or between SLE patients with or without either a history of a thrombotic event or the presence of lupus anticoagulant or both.[24] A study including patients with BD found significantly elevated TAFI antigen levels in patients compared with controls but found similar TAFI antigen levels for BD subgroups with or without thrombosis.[22] Contrarily, in another study[23] BD patients with thrombosis had significantly increased levels of TAFI activity in comparison with patients without thrombosis but no differences were found in TAFI antigen levels.

Global fibrinolytic potential tests have been investigated in several settings. Patients with aPL (only LA) have been previously examined with the overall hemostatic potential assay,[9] which measures the balance between fibrin generation and lysis in a similar manner to that of CLT. Increased fibrin generation and reduced fibrinolysis were demonstrated compared with the control group. Our results are in keeping with this previous study as CLT results were prolonged in patients with APS at baseline. Lisman et al.[46] showed a clear association between plasma hypofibrinolysis, assessed by CLT, and the risk of developing a first venous thrombosis. They showed that despite an association between TAFI antigen levels and CLTs, the contribution of TAFI levels to CLTs is small. In other words, the CLT in a given plasma sample is determined by the total fibrinolytic capacity of the plasma, which is determined by the balance of the levels of all fibrinolytic proteins. Accordingly, we found a parallel increase in CLT and TAFI throughout the different pregnancy periods.

No differences were found between groups on evaluating TAFI levels and CLT during the second and third trimester of pregnancy when obstetric complications develop. Interestingly, Cellai et al.[47] and Schoeder et al.[48] reported similar data in other thrombotic pathologies such as acute coronary artery disease and acute pulmonary embolism, which may be explained by TAFI plasma half-life in humans despite the lack of studies to this respect. The very short-life of activated TAFI *in vitro* is well known.[5] It may therefore be hypothesized that in thrombotic

conditions such as pregnancy complications in the second and third trimester, increased thrombin generation may lead to TAFI activation and consumption thereby hindering the detection of previously increased plasma levels.

In our study, baseline was considered as 4–6 months after delivery rather than before pregnancy which may be a possible limitation. However, several previous studies have shown a significant drop in TAFI levels in the immediate post-partum period with a rapid return to basal levels a few days after delivery,[27–29,32] with a significant drop in the immediate post-partum period. Therefore, samples obtained 4–6 months after delivery, as in our study, may be considered as baseline samples.

Genetic factors may explain more than 50% of the variation in plasma levels of TAFI.[15–17] Both, TAFI levels and TAFI polymorphisms have been related to arterial and venous thrombosis, although with no conclusive results.[15] We determined two of these polymorphisms: one in the encoding region that results in an amino acid substitution, Ala 147Thr (505 A/G), and another located in the 3′-UTR region, +1542 C/G. We found no differences in the frequency distribution of TAFI polymorphisms in either group or according to pregnancy outcome or the risk of thrombosis. This finding indicates that the relationship between TAFI genotypes, TAFI levels and the risk of thrombotic complications is complex.[15]

Besides intensive research about the mechanisms of APS and its obstetric complications, treatment has not significantly changed over the last several years and many treated pregnant patients develop complications such as PE, uteroplacental insufficiency, IUFGR, fetal loss and pre-term birth, despite treatment.[2–3] In order to elucidate the exact role of TAFI in obstetric complications and thrombosis in APS patients, future studies investigating functional TAFI activity and other TAFI gene polymorphisms are clearly needed.

Conclusions

In conclusion, patients with APS have impairment in fibrinolysis demonstrated by prolonged baseline CLT compared with controls. These fibrinolytic changes cannot be attributed to differences in TAFI levels. Therefore, CLT, TAFI antigen levels and TAFI polymorphisms may not be useful as markers of thrombotic complications in APS pregnancies.

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ESTUDIO 4

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“Clot lysis time and thrombin activatable fibrinolysis inhibitor in severe preclampsia with or without associated antiphospholipid antibodies”.

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Clot lysis time and thrombin activatable fibrinolysis inhibitor in severe preeclampsia with or without associated antiphospholipid antibodies

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ABSTRACT

We investigated clot lysis time, thrombin activatable fibrinolysis inhibitor antigen (TAFI) levels and TAFI gene polymorphisms in pregnant patients with severe preeclampsia, with or without associated antiphospholipid syndrome (APS). The study groups included 82 pregnant patients without antiphospholipid antibodies with severe preeclampsia (PE group) and 10 pregnant APS patients who developed severe preeclampsia (APS-PE group). Controls included 76 primary pregnant APS patients (APS group) and 89 healthy pregnant patients (NOR group) with uneventful term pregnancy and delivery. Patients in the APS-PE, APS and NOR groups were sampled during each trimester of pregnancy and at 4–6 months and 12 months after delivery. Patients in the PE group were sampled during the third trimester and after delivery. Significantly prolonged clot lysis time after delivery was found in the PE, APS-PE and APS groups compared to the NOR group. The PE and APS-PE groups had longer clot lysis time than the APS group. Levels of TAFI were found to be higher after delivery in patients of the PE and APS-PE groups compared to the APS and NOR groups. Allele distribution of the TAFI gene polymorphisms was similar among the four study groups. We conclude that increased TAFI antigen levels and impaired fibrinolysis are pathogenetic factors in preeclampsia, regardless of whether or not preeclampsia is associated with the presence of antiphospholipid antibodies.

1. Introduction

Preeclampsia is an important pathological syndrome of pregnancy, being a major cause of maternal and perinatal morbidity and mortality that affects 3–5% of all pregnancies. Although the pathogenesis of preeclampsia is not clearly understood, impaired utero-placental circulation due to increased microthrombogenesis is accepted (Roberts and Cooper, 2001). Previous studies have described coagulation and fibrinolysis alterations in women with preeclampsia (Alfirevic et al., 2002). Recently there has been a growing interest in the association of impaired fibrinolysis with PE. Several studies have identified specific derangements in the fibrinolytic capacity associated with this pregnancy complication (Sucak et al., 2006; Sartori et al., 2007). Moreover, several polymorphisms of genes encoding inhibitors of fibrinolysis

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have been associated with preeclampsia (Wiwanitkit, 2006).

The antiphospholipid syndrome (APS), an acquired autoimmune condition, is characterized by the association of thrombosis (arterial or venous) and/or pregnancy morbidity with the presence of a variety of heterogeneous circulating autoantibodies called antiphospholipid antibodies. As previously reported by our group (Carmona et al., 2001) and others (Branch et al., 1992), preeclampsia is one of the principal obstetrical complications responsible for the increased perinatal morbidity and mortality associated with primary APS. Although the association of antiphospholipid antibodies and pregnancy morbidity is well established, the characteristics and pathogenic mechanisms of these antibodies remain unclear. Pregnancy complications have been partly attributed to thrombosis of the utero-placental vessels and placental infarction. A number of mechanisms by which antiphospholipid antibodies may promote thrombotic events have been proposed (Espinosa et al., 2003). Importantly, abnormalities in fibrinolysis have been described in APS patients and may be responsible for thrombotic events (Espinosa et al., 2003; Forastiero and Martinuzzo, 2008; Carmona et al., 2006).

Thrombin activatable fibrinolysis inhibitor (TAFI) is a procarboxypeptidase B-like proenzyme, which is a main inhibitor of fibrinolysis and a known contributing factor in the development of thrombotic events (Van Tilburg et al., 2000). Activated TAFI down-regulates fibrinolysis by removing C-terminal lysine residues from fibrin, thereby reducing adsorption of tissue plasminogen activator (tPA) and plasminogen to fibrin, and decreasing plasmin formation (Van Tilburg et al., 2000). Several polymorphisms have been identified in the TAFI gene. Among TAFI polymorphisms, +505A/G, and +1542C/G (Franco et al., 2001; Henry et al., 2001) have been the most thoroughly studied, and strong associations have been reported between these polymorphisms and plasma concentrations of TAFI (Browers et al., 2001). Both, TAFI levels and TAFI polymorphisms have been related to arterial and venous thrombosis (Browers et al., 2001). Routine laboratory assays of fibrinolytic factors provide static data and therefore have been of limited diagnostic use. Global coagulation assays may provide an alternative source of information that assesses changes over time in the balance of the fibrinolytic system (Curnow et al., 2006). Clot lysis time is a test to evaluate fibrinolysis. The suppression of fibrinolysis by TAFI slows clot lysis time (Wang et al., 1998).

Some studies have investigated TAFI levels and clot lysis time in normal pregnancies (Chetaille et al., 2000; Chabloz et al., 2001; Mousa et al., 2004; Watanabe et al., 2004; Goldenberg et al., 2005) and complicated pregnancies with preeclampsia (Antovic et al., 2002; Alacacioglu et al., 2004; Sucak et al., 2006) with contradictory results. Moreover, one previous study that investigated TAFI polymorphisms as a genetic risk factor of preeclampsia showed no association (De Maat et al., 2004). To the best of our knowledge there is no previous report on TAFI levels or clot lysis time in patients diagnosed with severe preeclampsia with or without antiphospholipid antibodies.

Therefore, the current study was undertaken to investigate clot lysis time, TAFI levels and TAFI gene polymorphisms in pregnant patients with severe preeclampsia associated with APS or not.

2. Materials and methods

2.1. Patients

This study was a retrospective analytic investigation of frozen blood samples prospectively collected and stored between May 2003 and February 2008 at the Hospital Clínic of Barcelona. A total of 285 patients with frozen plasma samples still stored at the time of this investigation, and fulfilling inclusion and blood sampling criteria reported below, were initially included. All the women involved gave informed consent to participate in the present study, which was approved by the Ethics Committee of our hospital. Four groups of patients were considered. There were two study groups: pregnant patients diagnosed as having severe preeclampsia during the third trimester of pregnancy according to the criteria reported below (PE group) and primary APS pregnant patients that developed severe preeclampsia (APS-PE group). There were two control groups: a positive control group including pregnant patients who had previously been diagnosed with primary APS but with uneventful term pregnancy and delivery (APS group), and a normal control group (NOR group) comprising healthy pregnant patients having a normal term pregnancy and delivery. All pregnant APS patients involved were identified in the Obstetrics Department and were followed until the final outcome of their pregnancy. During the follow-up period patients who were diagnosed as having severe preeclampsia in the third trimester of pregnancy were included in the APS-PE group. Patients who did not develop severe PE and had uneventful term pregnancies and delivery comprised the APS Group. Healthy pregnant controls were selected over the same study period from our low-risk pregnancy outpatient clinic and matched by age (±2 years) to women diagnosed with APS. Pregnant patients diagnosed with severe preeclampsia were recruited when they were admitted for suspected complications (such as edema or hypertension).

Severe preeclampsia was defined as preeclampsia with at least 1 of the following features: systolic blood pressure ≥160 mm or diastolic ≥110 mm Hg; acute-onset renal failure; proteinuria >5 g in a 24-h urine collection; oliguria (500 ml or less in 24 h); grand mal seizures (eclampsia); pulmonary edema; HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome; thrombocytopenia (less than 100,000 cells/μL); symptoms suggesting significant end-organ involvement: headache, visual disturbances, or epigastric or right upper quadrant pain; fetal intra-uterine growth restriction or oligohydramnios (ACOG, 2002a).

The diagnosis of APS was based on the International Consensus Statement of the classification criteria for definite antiphospholipid syndrome (Miyakis et al., 2006). The patients that had been diagnosed before the consensus was published fulfilled all the updated international consensus criteria. Patients had fulfilled the consensus criteria before the index pregnancy. These patients were managed as having high-risk pregnancies and were treated with low-dose

aspirin alone or together with low-molecular-weight heparin, according to a previous protocol reported by our group (Carmona et al., 2001; Balasch et al., 2002). Briefly, our standard treatment for APS consists of low-dose aspirin (100 mg/day) started whenever possible from 1 month before attempting conception and maintained throughout the pregnancy. Low-molecular-weight heparin is used in combination with low-dose aspirin in cases of previous vascular thrombotic history or after treatment failure of aspirin alone in a previous pregnancy. Thus, all patients in the APS and APS-PE group were treated with low-dose aspirin throughout the pregnancy and the study period.

To be included in this study, patients had to carry pregnancies beyond 26 weeks of gestation, thus reaching fetal viability (ACOG, 2002b). Exclusion criteria included hypertension or other significant medical diseases. A history of thrombosis or thrombophilia in patients in the PE and NOR groups was considered as an exclusion criteria. To this end, at recruitment, all patients and controls were checked for procoagulant factor V Leiden or prothrombin G20210A genetic variations, lupus anticoagulant and anticardiolipin antibodies, and protein C and protein S levels. Receiving drugs, except for iron supplementation during gestation, was also considered as exclusion criteria.

Frozen plasma samples were initially available from 285 patients fulfilling clinical inclusion criteria. However, 28 were excluded due to reasons indicated in Fig. 1 (associated thrombophilias other than antiphospholipid antibodies in the APS group, or pregnancy ending in miscarriage). Thus, there were 257 patients included in the final analysis: 82 patients in the PE group, 10 patients in the APS-PE group, 76 patients in the APS group and 89 patients in the NOR group. No patient received hormonal treatment after delivery or was breastfeeding at the sampling time after delivery. A number of patients in the APS-PE, APS and NOR groups had participated in a previously published study (Martínez-Zamora et al., 2009).

In the APS group ($n = 76$), 46 patients were treated with low-dose aspirin alone and 30 patients were treated with low-dose aspirin and low-molecular-weight heparin. In the APS-PE group all patients received low-dose aspirin and three patients were also treated with heparin, thus the percentage of patients receiving both drugs was similar in APS and APS-PE groups (39 and 30%, respectively).

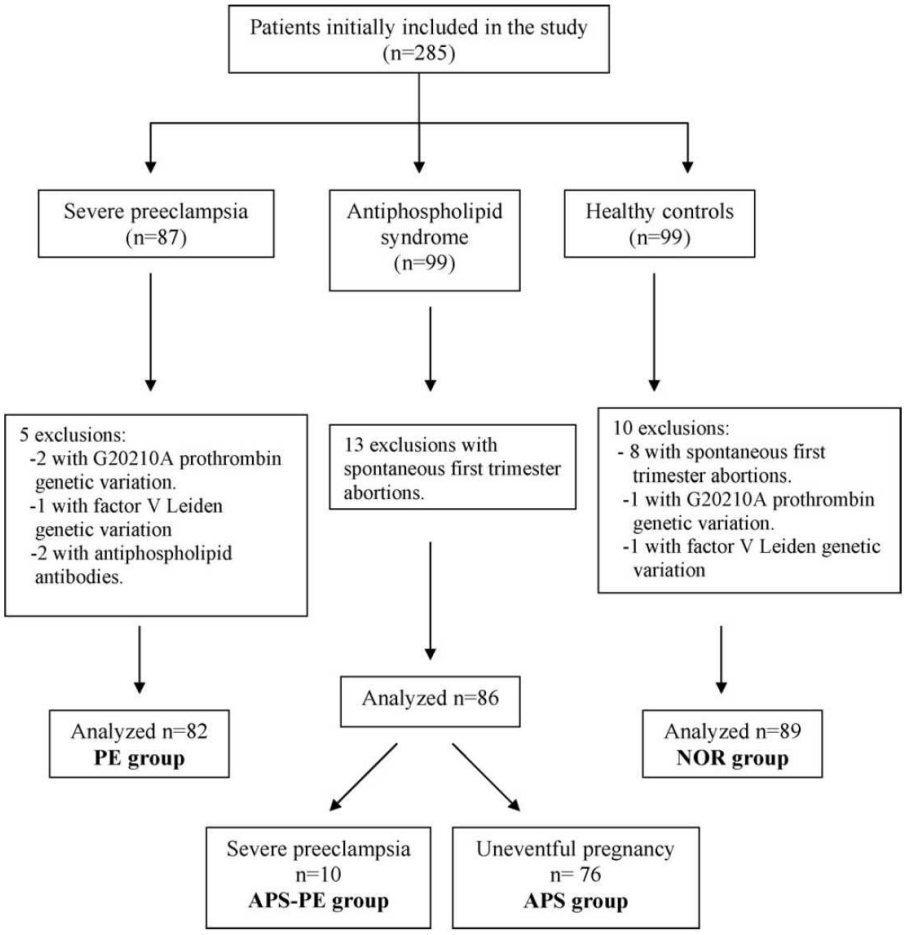

Fig. 1. Flow chart of inclusion and drop-out of patients included in this study.

2.2. Blood collection

Five blood samples were obtained in the PE-APS, APS and NOR groups: at each trimester of gestation (between weeks 6–10, 18–20, and 28–32), at 4–6 months after delivery (considered "postpartum" samples) and 12 months after delivery (considered "non-pregnant" samples). Three blood samples were obtained in the PE group: at the time of diagnosis during the third trimester, 4–6 months after delivery and 12 months after delivery.

Venous blood samples for coagulation and fibrinolysis studies were obtained as previously reported (Martínez-Zamora et al., 2009), between 0800 and 0900 h, after overnight fasting from food, liquids, and smoking (only water was allowed).

2.3. Antiphospholipid antibody testing

Lupus anticoagulant (LA) was detected using activated partial thromboplastin time, diluted Russell's viper venom time, and 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 (Brandt et al., 1995). The anticardiolipin antibodies were quantified using an enzyme linked immunosorbent assay (ELISA) (Cheshire Diagnostics, Cheshire, UK).

2.4. Fibrinolysis parameters

TAFI antigen levels were determined by an ELISA known to detect all the isoforms of TAFI (Gils et al., 2003) (Asserachrom TAFI, Diagnostica Stago, Asnieres, France).

The clot lysis time, which is the lysis of a thrombin-induced fibrin clot by exogenous t-PA, was studied by monitoring changes in turbidity during clot formation and subsequent lysis. Plasma clot lysis time was measured as previously described (Lisman et al., 2002) with modifications (Martínez-Zamora et al., 2009).

2.5. Genetic analyses

For detection of polymorphisms, genomic DNA was extracted from 100 μL of whole blood by a silica gel column method (QIAamp DNA blood mini kit, Qiagen GmbH, Hilden, Germany). TAFI Ala147Thr (+505A/G) and +1542C/G polymorphisms were determined by allele-specific PCR as previosly reported (Henry et al., 2001) with minor modifications (Martínez-Zamora et al., 2009).

2.6. Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences software, Release 15.0 for Windows (SPSS, Chicago, IL, USA). Comparison of quantitative variables was performed using ANOVA with Bonferroni's posthoc analysis. Comparison of qualitative variables was carried out using the χ^2 test. Statistical significance was defined as $p < 0.05$. Results are presented as mean ± SD.

3. Results

The clinical characteristics of the four groups studied are shown in Table 1. The mean age was similar in the four groups. As expected, patients in the APS-PE and APS groups had more previous abortions and fetal deaths compared to the PE and NOR groups. There were more preterm deliveries, a lower gestational age, and consequently a lower birth weight, in the PE and APS-PE groups. There were more cesarean sections among patients in the PE, APS-PE and APS groups compared to the NOR group. Timing of blood sampling over the study period was similar in the four groups studied (Table 1).

Fig. 2 shows TAFI levels and clot lysis time values throughout the study period in the four groups studied. There was a progressive increase in TAFI levels and clot lysis time from the first to the third trimester, dropping after delivery. There were no statistical differences in TAFI levels and clot lysis time between the "postpartum" value and the "non-pregnant" value. Five patients in the PE group had HELLP syndrome according to well-established diagnostic criteria (ACOG, 2002a).

Clot lysis time showed no statistical differences between groups during pregnancy. Significantly prolonged clot lysis time after delivery was found in the PE, APS-PE and APS groups compared to the NOR group. Patients in the PE and APS-PE group had longer clot lysis time compared to the APS group (Fig. 2).

TAFI levels showed no statistical differences between groups during pregnancy. Levels of TAFI were significantly higher in patients in group PE compared with groups APS and NOR in the "postpartum" and "non-pregnant" samples (Fig. 2). TAFI levels were significantly higher in patients in the APS-PE group compared with the NOR group in the "postpartum" and "non-pregnant" samples, and were higher than in the APS group, although the differences did not reach statistical significance (Fig. 2).

The comparison of TAFI levels and clot lysis time in the PE and APS-PE groups did not show differences during pregnancy, postpartum or non-pregnant determinations (Fig. 2).

The four groups studied were similar regarding allele frequency of the two TAFI polymorphisms studied (Table 2). As expected, TAFI antigen levels were higher in patients with the homozygote alleles *+1542 GG* and *+505 AA* (data not shown).

4. Discussion

The present study shows that patients with severe preeclampsia and APS patients have impaired fibrinolysis as evidenced by a prolonged clot lysis time after delivery. This derangement is stronger in patients with severe preeclampsia, regardless of whether preeclampsia is associated with APS or not, compared with APS patients without severe preeclampsia. This impairment in fibrinolysis can be

Table 1
Clinical characteristics and pregnancy outcome of the study groups.

Parameter	PE group ($n=82$)	APS-PE group ($n=10$)	APS group ($n=76$)	NOR group ($n=89$)
Age (years)	31.2 ± 4.3	34.1 ± 3.5	33.6 ± 3.7	32.5 ± 3.9
Previous pregnancies	69 (0.8;0–3)[a,c]	39 (2.9;0–7)[a,b]	157 (2.6;0–8)[c,d]	64 (0.74;0–5)[b,d]
Previous abortions <10 weeks	28 (0.3;0–3)[a,c]	29 (1.5;0–6)[a,b]	98 (1.6;0–6)[c,d]	29 (0.33;0–3)[b,d]
Previous fetal deaths >10 weeks	4 (0.1;0–2)[a,c]	4 (0.3;0–1)[a,b]	20 (0.4;0–2)[c,d]	1 (0.02;0–1)[b,d]
Previous neonatal death	0 (0;0–0)	1 (0.1;0–1)	10 (0.2;0–1)	0 (0;0–0)
Gestational age at delivery (weeks)	33.4 ± 3.6[a,b]	33.6 ± 3.9[c,d]	37.4 ± 2.7[a,c]	39.6 ± 2.7[b,d]
Neonatal birthweight (g)	1593 ± 661[a,b]	1650 ± 430[c,d]	2970 ± 815[a,c]	3275 ± 432[b,d]
Cesarean section	57 (69.5)[a]	8 (80)[b]	39 (51)[c]	12 (13.5)[a,b,c]
Preterm delivery	63 (76.8)[a,b]	7 (70)[c,d]	9 (12)[a,c]	9 (10.1)[b,d]
Neonatal mortality	7 (8.5)	1 (10)	2 (2.6)	0
Gestational age at first trimester sampling (weeks)	–	10.1 ± 1.3	9.6 ± 1.6	9.2 ± 1.2
Gestational age at second trimester sampling (weeks)	–	21.4 ± 2.3	20.4 ± 2.1	19.3 ± 1.7
Gestational age at third trimester sampling (weeks)	30.4 ± 0.9	29.4 ± 2.7	29.6 ± 1.9	29.5 ± 1.5
Time at "postpartum" sampling (weeks)	25.1 ± 2.9	22.4 ± 4.1	23.1 ± 3.6	25.7 ± 2.7
Time at "non-pregnant" sampling (weeks)	48.3 ± 1.6	47.5 ± 5.1	49.1 ± 2.3	51.1 ± 3.1

PE group, patients with severe preeclampsia without antiphospholipid antibodies; APS-PE group, antiphospholipid syndrome pregnant patients with severe preeclampsia; APS group, antiphospholipid syndrome patients without pregnancy complications, and NOR group, healthy pregnant controls. Values are mean ± SD or n (mean;range) or n (%). Results with common superscripts were statistically different. [a,b,c,d] $p<0.05$.

partially attributed to higher TAFI levels in patients with preeclampsia with or without APS, but not in APS patients without severe PE.

Studies investigating TAFI levels and clot lysis time during pregnancy in patients with thrombotic complications such as preeclampsia and/or fetal intra-uterine growth restriction (Antovic et al., 2002; Alacacioglu et al., 2004), have shown contradictory results. Thus, while it has been reported that TAFI levels were similar in healthy pregnant patients and pregnant women with preeclampsia (Alacacioglu et al., 2004), other authors have found that TAFI levels during the third trimester of pregnancy were reduced and clot lysis time increased in pregnancies complicated with preeclampsia (Antovic et al., 2002). Unfortunately, no determinations of TAFI levels or clot lysis time were performed after delivery or before pregnancy in these studies. Although there is no definite explanation for the contradictory results between these studies, differences in sample size, inclusion criteria and study design could explain these different results. The sample size is higher in the current investigation than in previous studies (Antovic et al., 2002; Alacacioglu et al., 2004); only patients with severe preeclampsia were included in the current investigation while mild cases were studied in previous reports (Antovic et al., 2002; Alacacioglu et al., 2004) and we carried out a sequential evaluation of patients throughout pregnancy, while other authors used different patients in each gestational period (Chabloz et al., 2001; Mousa et al., 2004; Watanabe et al., 2004).

We included two study points after delivery in order to minimize the limitation of considering results after delivery as baseline results, because real baseline samples before pregnancy were not determined and are difficult to obtain. Several previous studies demonstrated a rapid decrease in TAFI at basal levels within a few hours/days after delivery (Chabloz et al., 2001; Mousa et al., 2004; Watanabe et al., 2004; Alacacioglu et al., 2004), with a significant drop in the immediate postpartum period. Therefore, in our study, samples obtained 4–6 months and 12 months after delivery could be considered as baseline samples. Moreover, TAFI and clot lysis time levels are similar in both periods.

Several studies have shown that increased baseline TAFI levels are associated with an increased risk of thrombotic events such as ischemic stroke (Santamaría et al., 2003), acute coronary artery disease (Morange et al., 2003), recurrent venous thromboembolism (Eichinger et al., 2004) and deep vein thrombosis (Van Tilburg et al., 2000). This is in agreement with the current study in which TAFI levels were increased over baseline after delivery in patients in the PE group, demonstrating a possible association between TAFI and severe PE. Moreover, the evaluation of TAFI levels and clot lysis time during the third trimester of pregnancy when the pathologic condition is evident, showed no differences between groups. Interestingly, Cellai et al. (2006) and Schroeder et al. (2003) reported similar data in the

Table 2
Distribution of thrombin activatable fibrinolysis inhibitor (TAFI) polymorphisms in the four groups of patients studied.

Group	TAFI A+505G			TAFI C+1542G		
	AA	GA	GG	GG	CG	CC
PE	4 (5)	33 (40)	45 (55)	9 (11)	32 (39)	41 (50)
APS-PE	1 (10)	4 (40)	5 (50)	1 (10)	3 (30)	6 (60)
APS	5 (6)	33 (44)	38 (50)	4 (5)	29 (38)	43 (57)
NOR	7 (8)	43 (48)	39 (44)	8 (9)	32 (36)	49 (55)

PE group, patients with severe preeclampsia without antiphospholipid antibodies; APS-PE group, antiphospholipid syndrome pregnant patients with severe preeclampsia; APS group, antiphospholipid syndrome patients without pregnancy complications, and NOR group, healthy pregnant controls. Values are n (%).

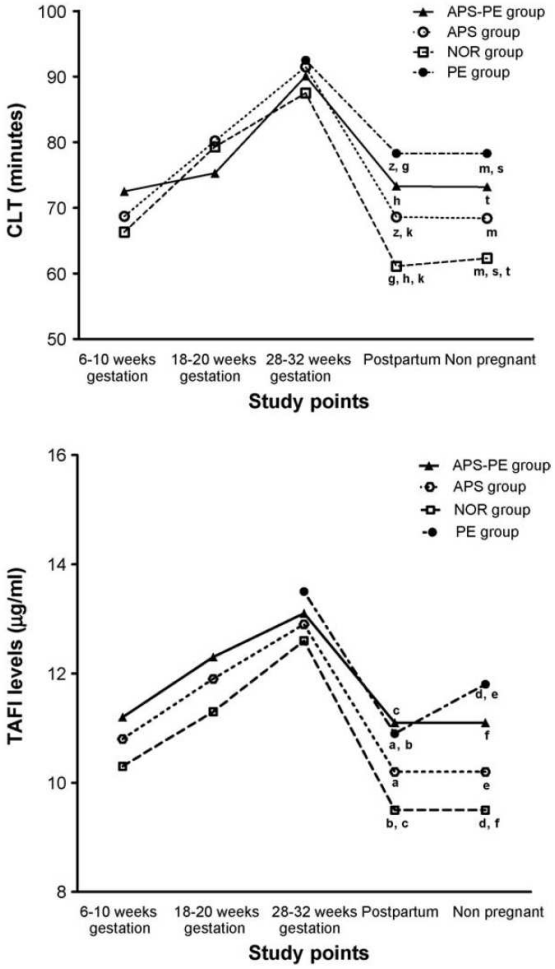

Fig. 2. Clot lysis time and thrombin activatable fibrinolysis inhibitor (TAFI) levels during the three trimesters of pregnancy, 'postpartum' and 'non-pregnant' values in antiphospholipid syndrome patients with severe preeclampsia (APS-PE group), antiphospholipid syndrome patients without pregnancy complications (APS group) and controls (NOR group), and clot lysis time and TAFI during the third trimester and 'postpartum' and 'non-pregnant' values in patients with severe preeclampsia without antiphospholipid antibodies (PE group). Statistical differences between groups are indicated with common superscripts ($^{a,b,d,e,z,g,s}p < 0.0001$; $^{c,f}p < 0.02$; $^{h,p}p < 0.005$; $^{k}p < 0.0003$; $^{m}p < 0.006$; $^{t}p < 0.009$). Results are given as mean ± SD.

acute phase of other thrombotic pathologies such as acute coronary artery disease and acute pulmonary embolism, showing no differences. This may be explained by the short TAFI plasma half-life in humans (Bouma and Meijers, 2003). Although appropriate studies in this respect are not available, the very short-life of activated TAFI *in vitro* is well known (Schatteman et al., 2000). Furthermore, TAFI levels are increased in the acute phase of the thrombotic event and thus, this phase is not considered to be useful to discriminate the risk (Bouma and Meijers, 2003). It could be hypothesized that in thrombotic conditions such as pregnancy complications in the third trimester, increased thrombin generation may lead to TAFI activation and consumption, thereby blunting the detection of previously increased plasma levels.

Genetic factors are estimated to explain between 20 and 62% of the variation in plasma levels of TAFI (Franco et al., 2001; Henry et al., 2001; Brouwers et al., 2001). Both TAFI levels and TAFI polymorphisms have been related to arterial and venous thrombosis, albeit with no conclusive results (Franco et al., 2001; Akatsu et al., 2004). We investigated two polymorphisms: one in the encoding region that results in an amino acid substitution, Ala 147Thr (505A/G), and another located in the 3′-UTR region, +1542C/G. We found no differences in the frequency distribution of TAFI polymorphisms in any of the groups. Our results are in agreement with those of De Maat et al. (2004) who compared TAFI polymorphisms and other common variants of thrombophilia genes among healthy pregnant controls and patients with preeclampsia and found no association between polymorphisms and the risk of PE. These findings indicate that the relationship between TAFI genotypes, TAFI levels and risk of thrombotic complications is complex (Franco et al., 2001), and other factors apart from TAFI polymorphisms may influence TAFI levels.

Our study has two possible limitations. The first is that the sample size was decided arbitrarily but in keeping with previous studies investigating TAFI polymorphisms (Franco et al., 2001; Henry et al., 2001; Browers et al., 2001). Nevertheless, we found differences in TAFI antigen levels and clot lysis time in the groups studied, although no differences were found regarding TAFI polymorphisms. We analyzed the TAFI A+505G and TAFI C+1542G polymorphisms because previous studies demonstrated that both polymorphisms are significantly and independently associated with plasma TAFI antigen levels and together, both explain 62% of the variance (Henry et al., 2001). In the current investigation, the four groups studied were similar regarding allele frequency of the two TAFI polymorphisms studied and as expected, TAFI levels were as expected considering each allele, being higher in homozygote alleles *+1542 GG* and *+505 AA* as previously reported by other authors (Franco et al., 2001; Henry et al., 2001; Browers et al., 2001). Other polymorphisms identified are in strong linkage disequilibrium with each other, making it difficult to clearly differentiate the effect of these other polymorphisms on TAFI levels (Henry et al., 2001).

On the other hand, it could be argued that patients were treated with low-dose aspirin and/or heparin and this may represent a bias in our study. However, all patients in the APS and APS-PE groups were taking low-dose aspirin, and the percentage of patients receiving low-dose aspirin and heparin was similar in both groups. Moreover, previous studies (Bjornsson et al., 1989; Ajjan et al., 2009) have demonstrated that aspirin intake enhances fibrinolysis due to acetylation in the fibrinogen molecule, correlating inversely with clot lysis time *in vitro*. Considering these studies, if low-dose aspirin has any effect, it would be by shortening the clot lysis time. Thus, the differences observed in our study between patients and controls would be even greater, further supporting our contention. Furthermore, a previous study (Colucci et al., 2002) evaluated the effect of heparin on fibrinolysis and demonstrated that heparin is unable to stimulate fibrinolysis through a TAFI-

dependent mechanism. Therefore, a confounding effect of low-dose aspirin and/or heparin treatment is unlikely.

The present investigation expands our previous work supporting an impairment in fibrinolysis in patients with APS as shown by impaired factor XIIa-dependent activation of fibrinolysis and prolonged baseline clot lysis time (Carmona et al., 2006; Martínez-Zamora et al., 2009). The results in the current investigation show an impairment in fibrinolysis as evidenced by increased TAFI levels and longer clot lysis time in severe preeclampsia patients, with or without APS. Nevertheless, APS patients without severe preeclampsia had normal TAFI levels in association with prolonged clot lysis time. These findings can be explained on the basis that the contribution of TAFI levels to clot lysis time is limited, despite an association between these two parameters (Lisman et al., 2005). On the other hand, it should be emphasized that the pathogenesis of pregnancy complications in APS is multifactorial (Espinosa et al., 2003; Forastiero and Martinuzzo, 2008), with disruption of fibrinolysis being just one of multiple pathophysiological mechanisms for thrombotic events proposed in pregnant patients. In contrast, increased TAFI levels may play a role in the pathogenesis of preeclampsia in pregnant patients lacking antiphospholipid antibodies, as previously suggested by other authors (Alacacioglu et al., 2004).

In conclusion, we find that pregnant women with severe preeclampsia and APS patients have impaired fibrinolysis evidenced by a prolonged clot lysis time after delivery. This impairment in fibrinolysis is more intense in patients with severe preeclampsia with or without APS, compared with APS patients without severe preeclampsia, and may be partially attributed to higher TAFI levels in the former but not in the latter patients.

Disclosure statement

The authors have nothing to disclose.

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ESTUDIO 5

“Riesgo de eventos tromboembólicos tras aborto de repetición en pacientes con síndrome antifosfolipídico: estudio caso-control”.

“Risk for thromboembolic events after recurrent miscarriage in antiphospholipid syndrome: a case-control study”.

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Extended report

Risk of thromboembolic events after recurrent spontaneous abortion in antiphospholipid syndrome: a case–control study

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ABSTRACT

Objective To investigate whether patients having antiphospholipid syndrome (APS) as the only aetiological factor for recurrent spontaneous abortion (RSA) are at increased risk of thrombosis later in life.

Methods A case–control study at a tertiary university referral centre. The study group consisted of 57 primary APS and RSA women (APS–RSA group). Control groups included: 86 patients with RSA of unknown aetiology (uRSA group), 42 patients with RSA and thrombophilic genetic defects as the only aetiologic factor for RSA (tRSA group) and 30 antiphospholipid antibody (aPL) positive but otherwise healthy women (aPL group). The main measurement was the thrombosis rate after long-term follow-up.

Results APS–RSA patients had a significantly higher 12-year cumulative thrombotic incidence rate compared with the three comparator groups (19.3% vs 4.8%, 0.0% and 0.0%, respectively (log rank), $p<0.001$). Patients in the APS–RSA group had 25.6 thrombotic events per 1000 patient-years (95% CI 12.8 to 45.9). The OR of thrombosis in relation to the presence (APS–RSA group) or absence (uRSA and tRSA groups) of aPL in patients with RSA was 15.06 (95% CI 3.2 to 70.5).

Conclusions Our data indicate that a history of RSA associated with aPL is a risk factor for subsequent thrombosis in the long term.

The antiphospholipid syndrome (APS) is an autoimmune, multisystemic disorder associated with vascular thrombosis and/or pregnancy morbidity in the presence of antiphospholipid antibodies (aPL), namely the lupus anticoagulant (LA) and anticardiolipin antibodies (aCL).[1] Women with aPL are considered at increased risk of both recurrent spontaneous abortion (RSA) and thrombotic events.[2] Remarkably, while pregnancy loss may be the sole manifestation of disease in such women and autoimmune disease is subclinical mainly in association with fetal wastage,[1] it has been reported that outside of pregnancy, women with a history of RSA are considered to be in a prothrombotic state.[3]

However, aPL frequently persists for many years, without apparent harm. In addition, aPL can be detected in the sera of asymptomatic individuals years before developing a full-blown disease. Therefore, an unresolved critical question is what additional factors lead to the sudden development of thrombosis, occurring in only a minority of patients with these antibodies.[4–7]

A 'two-hit hypothesis' has been proposed to explain why thrombotic events occur only occasionally, despite the persistent presence of aPL. The first hit (aPL) would increase the thrombophilic risk and a second hit is required to trigger the clotting.[6 7] Interestingly, two short communications suggested, for the first time, a high incidence of non-pregnancy-related vascular thrombosis in APS patients who present with pregnancy loss (ie, the second hit) as their only manifestation of APS.[8 9] Both reports, however, were retrospective including 65 and 52 women, respectively, treated with aspirin and, are in contrast with a retrospective case–control study using questionnaires and concluding that both idiopathic and APS-associated RSA were associated with a similar long-term risk of thrombosis.[10]

This study was undertaken to investigate whether patients with APS as the only aetiological factor for RSA are at increased risk of thrombosis later in life. This was done using appropriate case patients and three comparator groups.

MATERIALS AND METHODS

Study population and design

This case–control study involved a total of 215 women attending the Hospital Clínic of Barcelona identified from a list of patients who underwent thrombophilia testing in our laboratory from 1996 to 2005. Thirty-eight eligible individuals could not be contacted and appropriate information obtained and they were therefore not included. Therefore, 185 patients with a history of RSA who underwent all the diagnostic tests reported below and 30 healthy women fulfilling the inclusion criteria reported below were included. The survey was performed 4–12 years after the patients had attended our hospital for RSA work-up or after diagnosis with circulating positive aPL, being otherwise healthy women. All the women gave informed consent to participate in the present study, which was approved by the ethics committee of our hospital.

Four groups of patients were included. The study group consisted of 57 women previously diagnosed with primary APS who had had three or more consecutive spontaneous abortions before 10 weeks' gestation (APS–RSA group). The diagnosis of APS was based on the international consensus statement of the updated classification criteria for definite APS.[11] Patients who had been diagnosed

before the consensus had been published fulfilled all the later updated international consensus criteria. As the current investigation is a long-term follow-up study, all patients tested positive for aPL on three or more occasions at least 12 weeks apart. Patients with LA, medium to high levels of immunoglobulin (Ig) G and/or IgM aCL, or both, were included. Those with only low IgG and/or IgM aCL were excluded. No other aetiological factor was identified in the fertility studies including routine screening for systemic diseases, diabetes mellitus, thyroid dysfunction, polycystic ovary disease, a chromosome assessment of the woman and her partner, uterine abnormalities, endometrial and hormonal luteal phase defects, endometrial and cervical infection and thrombophilia other than aPL (plasma levels of protein S and C, antithrombin III, factor V Leiden and prothrombin G20210A mutations, acquired protein C resistance).

The first control group included 86 patients with three or more consecutive spontaneous abortions of unknown aetiology before 10 weeks' gestation (uRSA group). All tested negative for the above-mentioned investigations. The second control group included 42 patients with three or more consecutive spontaneous abortions before 10 weeks' gestation with thrombophilic genetic defects as the only aetiological factor for their pregnancy losses (tRSA group). Thrombophilia in this group was defined as factor V Leiden (heterozygote) mutation (n=17), prothrombin G20210A gene (heterozygote) mutation (n=12), protein C deficiency (n=9), or protein S deficiency (n=4). No woman in this group had combined thrombophilia (two or more findings). The last comparator group included 30 aPL-positive but otherwise healthy women with no history of thrombotic or obstetric morbidity (aPL group).

In the aPL group, 19 (63%) patients had had successful pregnancies. These patients were found to be aPL positive as a result of thrombophilia screening because of familial thrombophilia (n=12), prolonged activated partial thromboplastin time on routine screening (n=11), rheumatological disease screening in patients with arthralgia(n=5) and false-positive plasma reagin tests during the first trimester of pregnancy (n=2). Eleven patients (19%) in the APS–RSA group, 19 (22%) in the uRSA group and nine (21%) in the tRSA group had had successful pregnancies before RSA. Eleven patients in the APS–RSA group and 18, six and three in uRSA, tRSA and aPL groups, respectively, comprised the 38 individuals who could not be contacted.

All patients had regular menstrual cycles and no history of infertility. Laboratory determinations were performed 6 months or more after the last pregnancy and always when patients attended our hospital but not at the time of the survey. Exclusion criteria included any other form of autoimmune disorder or significant comorbidity.

Laboratory evaluation

aPL were determined by measuring LA and aCL. LA was determined according to the Subcommittee for the Standardization of Lupus Anticoagulants of the International Society of Thrombosis and Hemostasis guidelines.[12] The aCL were measured using standardised ELISA (Cheshire-Diagnostics, Cheshire, UK). Results were expressed in GPL and MPL units, and medium or high titres (>40 and >60 GPL or MPL, respectively) were considered positive.

Activated protein C resistance was measured in plasma using a coagulative assay as previously reported (Chromogenix, Mölndal, Sweden).[13 14] Patients showing a positive activated protein C resistance test were tested for the presence of factor V Leiden mutation.

Factor V Leiden mutation was determined with a nucleic acid sequence-based amplification assay (Organon Teknika, Boxtel, The Netherlands) until March 1999, by PCR and allele-specific oligonucleotide hybridisation (mDx Factor V Leiden; Bio-Rad Laboratories Diagnostics Group, Hercules, California, USA) until December 2003, and by restriction fragment length polymorphism PCR (Ampli-set-FV Leiden; BIRD Srl, Sienna, Italy) thereafter.

For prothrombin G20210A mutation detection, PCR was performed as previously reported.[15]

Antithrombin III and protein C activities were measured by colorimetric assays (Dade-Behring, GmbH, Marburg, Germany and Chromogenix, respectively).[16 17] Free protein S was measured by ELISA (Stago, Asnières, France).[18]

Patient follow-up and outcome assessment

Detailed medical histories for the periods before and after the initial laboratory testing, laboratory data, results of imaging studies and medications during the study period were obtained for all patients. In addition, patients or their surrogates were contacted by a doctor involved in the current study in person or by telephone and information was collected using a standardised data sheet. The structured questionnaire designed to elicit any thrombotic event and cardiovascular risk factors used in this study is also used in our institution, and was obtained after achieving consensus between gynaecologists, rheumatologists, internists and haematologists. The study period was defined as the interval from initial laboratory testing to the time of patient interview (minimum follow-up 4 years).

All patients and controls answered a structured questionnaire designed to elicit any thrombotic episode. The following factors were examined to determine their influence on thrombosis risk: ethnicity, hypertension, diabetes mellitus, cancer, family history of thrombosis, smoking, weight, height, high cholesterol, high triglycerides, oral contraceptive use. No woman had received hormone replacement therapy. Current oral contraceptive use was defined as use within the 3 months before the date of the survey.[19 20] Current smoking was defined as smoking five or more cigarettes a day in the year before the date of the survey.[21] Patients who had not been previously diagnosed with hypertension, hypercholesterolaemia and diabetes were given an appointment at our hospital to check all these parameters by means of non-fasting blood tests and blood pressure measurement. Women were classified as having hypertension, diabetes or hypercholesterolaemia according to criteria previously reported.[22 23] Some patients in the APS–RSA and aPL groups had taken low-dose aspirin since the time of testing positive for aPL and were still on treatment at the time of being contacted for the specific purpose of this study.

The primary outcome was incident vascular thrombotic events verified by checking hospital records and confirmed by accepted imaging, Doppler and laboratory studies, as previously reported.[23] Deep vein thrombosis was confirmed by Doppler studies and/or phlebography. Pulmonary embolism was confirmed by ventilation/perfusion pulmonary scintigraphy or spiral CT. Peripheral arterial thrombosis was confirmed by arteriography. Cerebrovascular accident and cerebral venous thrombosis were defined by CT and/or brain MRI. Myocardial infarction was confirmed by raised cardiac enzymes and appropriate electrocardiographic changes.

Statistical analysis

Statistical analysis was performed with the statistical package for the social sciences software, release 15.0 for

Windows and the open source epidemiological statistics for public health, version 2.3.1. www.OpenEpi.com, updated 19 September 2010. OR were calculated with the Woolf–Haldane modification, which adds 0.5 to all cells to accommodate possible zero counts when appropriate.[24] Comparison of quantitative variables was performed using analysis of variance with Bonferroni's post-hoc analysis. Comparison of qualitative variables was undertaken using the $\chi 2$ test. Cumulative event curves were generated with the Kaplan–Meier method and compared with the log-rank test of significance. All statistical tests were two-sided, and differences were considered statistically significant at $p<0.05$. Results are presented as mean±SD (and range when appropriate) or n (%).

RESULTS

Table 1 summarises the demographic variables, risk factors, comorbidities and clinical characteristics in the four groups studied. No differences were observed for any of the parameters considered except aspirin treatment. Twelve and nine patients in the APS–RSA (21%) and aPL (30%) groups, respectively, were given prophylactic daily low-dose aspirin but no woman in the uRSA or tRSA groups received this treatment ($p<0.0001$). No patient was taking hydroxychoroquine as all patients included had primary APS, as mentioned above.

The mean observation time was similar in the four groups of patients(table 2). Eleven patients in the APS–RSA group(19.3%) and two patients in the tRSA group(4.8%) had a thrombotic event during the study period. No subsequent thrombotic episodes were recorded during the study interval, and there were two thrombotic deaths (due to cerebral arterial infarction and massive pulmonary embolism) in the APS–RSA group. The rate of thrombosis in the APS–RSA group was significantly higher than in the control groups (table 2, figure 1). Patients in the APS–RSA group had 25.6 thrombotic events per 1000 patient-years (95% CI 12.8 to 45.9) while the corresponding figure in the tRSA group was 7.1 thrombotic events per 1000 patient-years (95% CI 0.8 to 25.5). Patients with APS–RSA had a significantly higher 12-year cumulative thrombotic incidence rate compared with the three comparator groups (19.3% vs 4.8%, 0.0% and 0.0%, respectively (log rank), $p<0.001$) (figure 1).

The OR of thrombosis in relation to the presence (APS–RSA group) or absence (uRSA and tRSA groups) of aPL in patients with RSA was 15.06 (95% CI 3.2 to 70.5, $p<0.0001$). This was still true when only patients with thrombophilic disorders other than aPL (tRSA group) were considered (OR 4.8, 95% CI 1 to 22.8, $p<0.05$). Accordingly, the OR was even higher when the APS–RSA group was compared with the uRSA group alone (OR 42.8, 95% CI 2.5 to 742, $p<0.0001$).

The occurrence of thrombotic events among women with RSA (APS–RSA, uRSA and tRSA groups) treated with aspirin (16% or 2/12 patients) did not differ from patients who did not receive this treatment (6% or 11/173 women, $p=0.2$) (OR 2.9, 95% CI 0.5 to 15.1). One patient in the APS–RSA group presented with cerebral arterial infarction during the puerperium despite taking low-dose aspirin.

Remarkably, five out of seven (71.4%) patients in the APS–RSA group diagnosed with arterial thrombosis had concomitant thrombosis risk factors (hypertension, one subject; hypertension and hypercholesterolemia, one subject; heavy smoker (≥20 cigarettes/day) and hypercholesterolaemia, one subject; heavy smoker and hypertension, one subject; heavy smoker, one subject) and one out of four (25%) who presented with venous thrombosis had hypertension.

Several patients (6/13, 46%) were diagnosed as having thrombotic events in our hospital. In three cases(23%) it was possible to check medical records in two hospitals located in the metropolitan area of Barcelona, while in the remaining four patients the diagnosis was confirmed by colleagues who provided a copy of diagnostic evidence with pertinent imaging and laboratory studies. Therefore, there were no equivocal cases among the 13 patients diagnosed as having this condition.

Table 1 Demographic features, clinical characteristics and risk factors of the four groups studied

Parameter	APS–RSA group (n=57)	uRSA group (n=86)	tRSA group (n=42)	aPL group (n=30)	p Value
Caucasian*	57 (100)	86 (100)	42 (100)	30 (100)	NS
Age at RSA diagnosis, years†	32.8±3.5 (26–43)	34.5±4.5 (24–45)	34.3±5.1 (23–42)	31.9±5.6 (24–42)‡	NS
Aspirin treatment*	12 (21)	0 (0)	0 (0)	9 (30)	<0.0001
First trimester spontaneous abortions†	3.7±1.2 (3–8)	4.3±2.0 (3–15)	3.8±1.1 (3–6)	0±0 (0)	NS
Second and/or third trimester pregnancy losses†	0.1±0.4 (0–2)	0.03±0.2 (0–1)	0.1±0.4 (0–1)	0±0 (0)	NS
Uneventful deliveries†	1.0±0.7 (0–2)	0.7±0.6 (0–2)	0.9±0.7 (0–2)	1.3±0.9 (0–3)	NS
BMI†	24.3±3.9 (17–32)	25.2±4.2 (17–34)	24.7±5.3 (17–34)	25.7±5.8 (18–33)	NS
Smoker*					NS
Never	48 (84.2)	66 (76.7)	35 (83.3)	20 (67)	
Past	3 (5.3)	7 (8.2)	2 (4.8)	6 (20)	
Current	6 (10.5)	13 (15.1)	5 (11.9)	4 (13)	
Oral contraceptives*					NS
Never-users	57 (100)	57 (100)	42 (100)	22 (73)	
Past-users	0 (0)	0 (0)	0 (0)	8 (27)	
Current-users	0 (0)	0 (0)	0 (0)	0 (0)	
Hypercholesterolaemia*	2 (3.5)	4 (4.6)	2 (4.7)	4 (13)	NS
Hypertension*	4 (7)	3 (3.5)	2 (4.8)	1 (3)	NS
Diabetes*	0 (0)	1 (1.2)	0 (0)	1 (3)	NS
Malignancy*	0 (0)	0 (0)	0 (0)	0 (0)	NS
Thrombosis family history*	3 (5)	2 (2)	3 (7)	2 (7)	NS

Values are *n (%) or †mean±SD (range).
‡ Age at study inclusion.
aPL group, patients with positive tests for antiphospholipid antibodies (aPL) without pregnancy or thrombotic morbidity (laboratory testing for aPL was performed at the time of patients attending our hospital. See Materials and methods section for more details); APS–RSA group, antiphospholipid syndrome patients with recurrent spontaneous abortion; BMI, body mass index; NS, not significant; RSA, recurrent spontaneous abortion; tRSA group, patients with recurrent spontaneous abortion without aPL but with other known thrombophilias; uRSA group, patients with recurrent spontaneous abortion without aPL or other known thrombophilias.

Table 2 Thrombotic events in the four groups studied

Parameter	APS–RSA group (n=57)	uRSA group (n=86)	tRSA group (n=42)	aPL group (n=30)	p Value
Follow-up, years	7.3±3.54–12	6.0±2.0 (4–10)	6.2±1.3 (4–9)	8.2±4.3(6–12)	NS
Patients with thrombotic event	11 (19.3)*†‡	0 (0)*	2 (4.8)†	0(0)‡	<0.0001*‡
Cerebral arterial infarction	4	0	0	0	<0.05†
Deep vein thrombosis	2	0	2	0	
Pulmonary embolism	2	0	0	0	
Ischaemic infarction	2	0	0	0	
Cerebellar arterial infarction	1	0	0	0	

Values are mean±SD or n (%). Statistical comparisons are indicated by common superscripts.
aPL group, patients with positive tests for antiphospholipid antibodies (aPL) without pregnancy or thrombotic morbidity; APS–RSA group, antiphospholipid syndrome patients with recurrent spontaneous abortion; NS, not significant; tRSA group, patients with recurrent spontaneous abortion without aPL but with other known thrombophilias; uRSA group, patients with recurrent spontaneous abortion without aPL or other known thrombophilias.

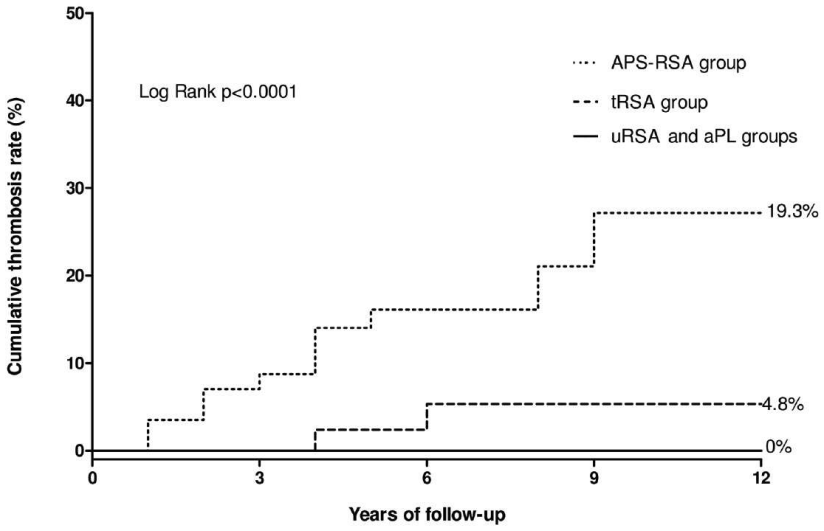

Figure 1 Kaplan–Meier curves of 12-year cumulative incidence of thrombotic events in the four groups studied. aPL group, patients with positive tests for antiphospholipid antibodies (aPL) without pregnancy or thrombotic morbidity (laboratory testing for aPL was performed at the time of patients attending our hospital; APS–RSA group, antiphospholipid syndrome patients with recurrent spontaneous abortion; tRSA group, patients with recurrent spontaneous abortion without aPL but with other known thrombophilias; uRSA group, patients with recurrent spontaneous abortion without aPL or other known thrombophilias.

Apart from those 38 individuals who could not be contacted among all eligible patients initially selected, accurate information was obtained from each subject through a personal or phone interview carried out by a doctor involved in the current study and the structured questionnaire designed to elicit any thrombotic episode. The patients included were thus only those for whom appropriate information was available, thereby avoiding possible missed cases among those not reporting thromboses.

In the whole study population, 43% and 57% of aCL-positive patients had medium and high antibody titres, with no differences in this respect between the three groups presented in table 3 (data not shown). aCL titres were also similar on comparing patients developing thrombosis or not (data not shown). No difference was found regarding aPL and isotype distribution in aPL-positive subjects developing thrombosis or not (table 3).

DISCUSSION

This study shows that patients with RSA associated with the presence of aPL have an increased long-term risk of thrombosis, with an approximately 15-fold greater risk of presenting with a thrombotic event than RSA patients without aPL. Our data also suggest that thrombosis in APS–RSA patients is frequently associated with concomitant cardiovascular risk factors. The most striking feature of the current investigation was the high incidence of non-pregnancy-related vascular thrombosis in APS patients who presented with RSA as their only manifestation of APS. Our study has several strengths. First, retrospective studies cannot exclude the possibility that a thromboembolic event may, in fact, promote the development of aCL.[25] At the time the plasma samples were obtained in our study, participants had no history of thrombotic events. Therefore, the potential confounders of systemic disease or pre-existing vascular injury seem to have been avoided in the current investigation. Second, we included only well-selected case patients according to both international criteria and laboratory guidelines for definite APS.[11 12] In addition, we used three appropriate control groups including recurrent aborters of unknown aetiology according to a complete diagnostic work-up, patients with RSA associated with inherited thrombophilia, and healthy women testing positive for aPL. Remarkably, the annual incidence of the first episode of venous thromboembolism reported in the tRSA group in

Table 3 LA and aCL isotype distribution in aPL-positive patients developing thrombosis or not*

Parameter	APS–RSA group without thrombosis (n=46)	APS–RSA group and thrombosis (n=11)	aPL group (n=30)
aCL	26 (56%)	5 (46%)	20 (67%)
aCL IgM	5 (19%)	0 (0%)	4 (20%)
aCL IgG	15 (58%)	3 (60%)	12 (60%)
aCL IgM + IgG	6 (23%)	2 (40%)	4 (20%)
LA	11 (24%)	3 (27%)	7 (23%)
aCL + LA	9 (20%)	3 (27%)	3 (10%)
aCL IgM + LAC	3 (33%)	1 (33%)	0 (0%)
aCL IgG + LAC	5 (56%)	2 (67%)	3 (100%)
aCL IgG + IgM + LAC	1 (11%)	0 (0%)	0 (0%)

Values are n (%).
* p Value: NS (by χ^2 test).
aCL, anticardiolipin antibodies; aPL group, patients with positive tests for antiphospholipid antibodies (aPL) without pregnancy or thrombotic morbidity; APS–RSA group, antiphospholipid syndrome patients with recurrent spontaneous abortion; IgG, immunoglobulin G; IgM, immunoglobulin M; LA, lupus anticoagulant.

our study is clearly within the range of estimated risk reported in the literature for patients with inherited thrombophilia.[26] Finally, this was a long-term study with individual follow-up ranging from 4 to 12 years.

This study has several limitations. This is a retrospective analysis of prospectively collected material and only a relatively low number of subjects with unequal distribution in the four groups studied could be included. However, the importance of this study lies in the fact that the presence of aPL was demonstrated before the occurrence of the thrombotic event. In fact, very little is known about the risk of thrombosis in aPL-positive individuals who are still free of thrombosis.[4 7] Therefore, our study represents a relevant contribution in this respect. It should be noted that patients were not checked for the presence of β2-glycoprotein-1 antibodies because all were diagnosed and recruited before the 2006 updated classification criteria for APS.[11] A review including 28 studies and analysing 60 associations between β2-glycoprotein-1 antibodies and thrombosis concluded that the results were partly controversial, but measurement of these antibodies may still be practical and useful in some situations.[27] A recent multicentre follow-up study[28] including only aPL carriers, concluded that hypertension and LA (but not β2-glycoprotein-1 antibodies) are independent risk factors for thrombosis. This notwithstanding, by majority, the last consensus paper on the subject agreed that IgG and IgM anti-β2-glycoprotein-1 should be included as part of the modified Sapporo criteria for thrombosis and pregnancy complications.[11] The consensus also stressed that testing for anti-β2-glycoprotein-1 can be helpful for APS diagnosis, particularly when aCL and LA are negative and APS is strongly suspected.[11]

Overall, our results are in line with previous reports suggesting that non-pregnant women with a history of RSA are in a prothrombotic state.[3 10 29] In addition, a large retrospective study of 130 000 women reported that a history of first trimester spontaneous abortion was associated with a significantly increased risk of maternal ischaemic heart disease.[30] The authors hypothesised that this may reflect common determinants, such as thrombophilic genetic defects and aCL.[30] Our study shows that the presence of circulating aPL rather than thrombophilic genetic defects in patients with RSA is the main determinant of thrombotic events in later life. Therefore, we found that patients with RSA and aPL have an approximately fivefold greater risk of presenting with a thrombotic event than RSA patients with thrombophilic genetic defects. Interestingly, only 19.3% of patients in the APS–RSA group and no woman in the aPL group developed a thrombotic event in the long term, despite aPL and aCL and their isotype distribution being similar in aPL-positive patients developing thrombosis or not and irrespective of being recurrent aborters or healthy controls (table 3).

There is currently no way to predict when or which aPL patients will develop thrombosis. The above notwithstanding, in this respect, our study is in agreement with previous reports[28 31–33] suggesting that aPL-positive individuals should be risk-stratified according to traditional cardiovascular risk factors, which can be responsible for triggering acute thrombosis.

The above data have potentially interesting implications. First, our results provide further support to the 'two-hit hypothesis'[5 6] in which aPL (first hit) increases the thrombophilic risk and the clotting takes place in the presence of another thrombophilic condition (ie, RSA associated with traditional cardiovascular risk factors or not, acting as the second hit). This would explain previous epidemiological studies suggesting that a woman's reproductive history may indicate future cardiovascular risk.[30 34] On the other hand, the aetiopathogenesis of APS is apparently multifactorial involving responses of both adaptive and innate immunity, being supported by a genetic background and triggered by environmental factors. In other words, genetically determined and environmental factors (second hits) may cooperate with aPL (first hit) in favouring thrombotic events.[5] Therefore, whether an individual will develop a thrombotic event depends on the concomitant presence of additional factors that may increase the whole thrombotic risk. This would explain why up to 80% of patients with RSA associated with aPL did not develop a thrombotic event.

In conclusion, our data indicate that a history of RSA associated with aPL is a risk factor for subsequent thrombosis in the long term. This may have clinical implications, mainly in those patients with traditional cardiovascular risk factors. If our results are confirmed by others, further studies would be warranted to assess the efficacy and risks of long-term thromboprophylaxis with aspirin and/or heparin in patients with RSA associated with aPL.

Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the ethics committee of the Hospital Clinic of Barcelona.

Provenance and peer review Not commissioned; externally peer reviewed.

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5. DISCUSIÓN

A pesar de los avances en medicina reproductiva, el fallo reproductivo humano sigue siendo un evento muy frecuente. La mortalidad embrionaria precoz es muy elevada en los humanos y se ha postulado que la causa principal es un fallo de la implantación (Bulleti et al., 1996; Bischof et al., 2006; Christiansen et al., 2006). La tasa de abortos espontáneos puede llegar a ser del 60-80% si se tienen en cuenta las pérdidas gestacionales que acontecen en las primeras semanas tras la concepción y que pasa desapercibida en muchas pacientes (Bulleti et al., 1996; Choudhury and Knapp, 2000).

Los abortos clínicos afectan al 15% de las mujeres, predominantemente en el primer trimestre, y mientras que la mayoría son esporádicos y no recurrentes, hay un subgrupo que engloba el 2-5% de las parejas que presenta abortos de repetición (AR) (Clark et al., 2001). Estas pérdidas gestacionales repetidas sugieren la presencia de una causa específica, y ello ha motivado gran cantidad de investigación científica intentado identificar y esclarecer su etiopatogenia. A pesar de estos esfuerzos, la literatura científica actual describe que sólo en un 50% de los casos de AR se identifica una causa responsable (Li et al, 2002; ACOG, 2002) lo que supone un problema de salud difícil de afrontar y resolver tanto para médicos como pacientes.

De la misma manera, el fallo de implantación tras transferencia embrionaria es un problema constante en las fecundaciones in vitro (FIV). Así pues, es desalentador que sólo alrededor del 20% de los embriones transferidos tras una FIV implanten en el útero a pesar de seleccionar embrio-

nes aparentemente normales (International Committee for Monitoring Assisted Reproductive Technology [ICMART], 2009; Andersen et al., 2009). Además, hay algunas mujeres que no pueden concebir con tratamientos de FIV a pesar de repetidas transferencias de embriones de buena calidad, y en estos casos son catalogadas como pacientes con fallos de implantación repetidos (FIR).

Es de destacar que recientemente se considera que tanto los AR como los FIR representan, en algunos casos, diferentes manifestaciones de un mismo espectro patogénico. Las vías de la hemostasia se involucran íntimamente con la ovulación, la implantación y la placentación, y por ello se han desarrollado hipótesis que postulan que en muchos casos los AR y los FIR son causados por el mismo defecto materno de hemostasia que conlleva una trombosis de la vascularización uteroplacentaria (Christiansen et al., 2006; Stern and Chamley, 2006). Por todo ello, se ha postulado que mujeres con alteraciones de la coagulación y/o fibrinolisis pueden tener un riesgo de aborto y de pérdida gestacional preclínica incrementados. Consecuentemente, la comunidad científica actual propone desarrollar estudios que permitan aclarar las causas asociadas a trombofilias que puedan conllevar AR y FIR (Christiansen et al., 2006; Stern and Chamley, 2006). Es bien conocido que la hipercoagulabilidad debida a la presencia de trombofilias, mayoritariamente mutaciones del factor V y de la protrombina se han asociado a complicaciones gestacionales tardías como preeclampsia, retraso de crecimiento intrauterino, desprendimiento de placenta y muerte intrauterina tardía debido a la trombosis de vasos maternos y una reducción de la perfusión del espacio intervelloso

(Preston et al., 1996; Kupferminc et al., 1999; Martinelli et al., 2000). En cambio, en la actualidad el mecanismo causal asociado a trombofilias que cause FIR es desconocido. De hecho, aunque se ha descrito un aumento de trombofilias diferentes del SAF en pacientes con FIR (Grandone et al., 2001; Azem et al., 2004; Qublan et al., 2006; Bellver et al., 2008), otros estudios prospectivos que valoraron el impacto del factor V Leiden, considerada la trombofilia hereditaria más frecuente, observaron que su presencia no afectaba al riesgo de FIR (Martinelli et al., 2003; van Dunne et al., 2005) o que incluso aumentaba las posibilidades de gestación (Göpel et al., 2001). Es decir, que la relación entre trombofilias genéticas y complicaciones gestacionales tardías parece clara pero respecto al FIR las publicaciones son escasas y contradictorias.

El papel de los defectos de la fibrinolisis en el fallo reproductivo recurrente es poco conocido pero parece que podría afectar a la migración trofoblástica (Sotiriadis et al., 2007). El sistema fibrinolítico incluye un amplio espectro de enzimas proteolíticas con funciones fisiológicas y patofisiológicas en diferentes procesos como el balance hemostático, el remodelado tisular, la invasión tumoral, la angiogénesis y la reproducción (Zorio et al., 2008). Ya en la implantación humana, el sistema fibrinolítico participa en la regulación de la migración e invasión trofoblástica (Coulam et al., 2006; Sotiriadis et al., 2007). La invasión trofoblástica durante la implantación supone una degradación de la matriz extracelular que requiere metaloproteasas. La expresión de las metaloproteasas en el lecho de implantación es estimulada por la proteasa

sérica plasmina (Coulam et al., 2006; Sotiriadis et al., 2007). La plasmina promueve la invasión trofoblástica también degradando ciertos componentes de la matriz extracelular de la decidua (Sotiriatidis et al., 2007). Por todo ello, las alteraciones de la fibrinolisis conllevarían un descenso de la invasión trofoblástica y un fallo de implantación. La plasmina es la enzima principal del sistema fibrinolítico y es la responsable de la degradación de la fibrina en productos de degradación solubles. La conversión del plasminógeno a plasmina está mediado por dos tipos de activadores diferentes: el activador del plasminógeno tipo urokinasa y el activador tisular del plasminógeno. La actividad de ambos está regulada por inhibidores de la activación del plasminógeno específicos. La plasmina puede ser inhibida por inhibidores específicos, principalmente la α2-antiplasmina y la α2-macroglobulina. La fibrinolisis puede también decelerarse mediante el PAI-1, que actúa como un inhibidor del activador tisular del plasminógeno. Es decir, que hay una plausibilidad biológica en que las alteraciones de la fibrinolisis puedan contribuir a los FIR.

El **estudio 1** es el primer trabajo de investigación que aporta información relevante acerca del papel del inhibidor de la fibrinolisis TAFI en pacientes con FIR, demostrando niveles elevados de TAFI en estas pacientes. Esto se asoció, además, con una disminución de la fibrinolisis demostrada por un incremento del CLT. Por tanto, nuestro estudio está de acuerdo con estudios previos que sugirieron que la hipofibrinolisis puede ser una situación

predisponente de fallo reproductivo precoz, ya sea en forma de FIR o de AR (Gris et al., 1997; Sotiriatidis et al., 2007). Hay que destacar que el **estudio 1** de la presente Tesis Doctoral incluyó sólo pacientes con tres o más FIR a las que se habían transferido embriones de buena calidad de acuerdo con los criterios habituales (morfología, simetría, crecimiento embrionario, etc.) de valoración del laboratorio de fecundación asistida (El-Toukhy and Taranissi, 2006). Este hecho da más valor a nuestros resultados ya que cada vez más expertos consideran que la incapacidad de producir embriones de buena calidad supone *per se*, un importante factor predisponente al FIR (Ola and Li, 2006). Además, el diseño del estudio caso-control en el que cada caso se comparó con dos controles secuenciales en el tiempo a partir de la fecha en la que la paciente con FIR fue incluida en el programa de FIV contribuye a evitar sesgos asociados a posibles cambios en las técnicas de laboratorio de FIV. También hay que destacar que las pacientes sólo fueron reclutadas para el estudio tras excluirse otras causas conocidas de FIR (Quenby et al., 2009).

La implicación potencial de los defectos fibrinolíticos en los AR fue sugerido por primera vez hace dos décadas (Gris et al., 1990; Folkeringa et al., 2009; Knol et al., 2009; Masini et al., 2009; Rai et al., 2003), pero su estudio sistemático sólo se ha realizado recientemente (Soriatidis et al., 2007). Esta revisión sistemática y metanálisis de la literatura demostró una asociación entre el déficit de factor XII y el riesgo de AR. El estudio, no obstante, mostró las limitaciones de los estudios analizados (son estudios retrospectivos en los que no se descartó la presencia de otras causas de AR y pudo haber un sesgo de

selección de las pacientes estudiadas) y la escasez de estudios que analicen el TAFI. Por otro lado, dos estudios publicados por el mismo grupo de investigación (Folkeringa et al., 2009; Knol et al., 2009) que analizaron los niveles de TAFI en pacientes con AR obtuvieron resultados poco concordantes: en el primero los niveles de TAFI no se relacionaron con el riesgo de AR mientras que en el segundo los niveles elevados de TAFI parecían constituir un elemento protector frente a AR.

Tal como se ha descrito ampliamente en la Introducción de esta Tesis Doctoral, aunque la asociación de los AAF con las pérdidas gestacionales y la morbilidad gestacional, tanto precoz como tardía, está ampliamente aceptada, no se conocen los mecanismos exactos que la producen (Levine et al., 2002). Una de las causas que se han propuesto son las alteraciones de la fibrinolisis. La formación de fibrina y la fibrinolisis están en constante equilibrio, ya que de lo contrario se llega a un estado protrombótico o prohemorrágico. Parece que los AAF podrían alterar la fibrinolisis y así contribuir a complicaciones vasculares trombóticas (Carmona et al., 2006). Se ha descrito una reducción de la actividad fibrinolítica en pacientes con SAF que podría ser responsable de eventos trombóticos (Espinosa et al., 2003; Pierangeli et al., 2008; Forastiero et al., 2008).

Algunos estudios publicados han analizado los niveles de TAFI (Folkeringa et al., 2009; Knol et al., 2009), los polimorfismos de TAFI (Masini et al., 2009) y/o la capacidad fibrinolítica global (Gris et al., 1990; Rai et al., 2003)

en pacientes con AR, pero no se conocían hasta el momento datos acerca de los niveles de TAFI y el CLT en pacientes con SAF como causa de AR. Esta hipótesis constituyó la base para el desarrollo del **estudio 2**, que es un estudio caso control que fue diseñado para analizar la asociación entre los niveles de TAFI, los polimorfismos de TAFI y el CLT en pacientes con SAF y tres o más AR. Los resultados de dicho estudio demostraron que tanto las pacientes con AR asociado a SAF como aquellas con AR idiopático presentaban una alteración de la fibrinolisis demostrada por un CLT prolongado comparado con controles fértiles sanas. La disminución de la fibrinolisis se relacionó parcialmente a cambios en los niveles de TAFI en las pacientes AR idiopáticas, pero no en las pacientes AR con SAF. Estos resultados añaden evidencia y apoyan los resultados encontrados en el **estudio 1** en los que TAFI parece tener un papel en el fallo reproductivo humano precoz en las mujeres sin AAF. La correlación entre el alargamiento del CLT y los niveles aumentados de TAFI observados en AR sin SAF pero no en AR con SAF se puede explicar por la contribución parcial de los niveles de TAFI al CLT a pesar de la asociación entre ambos parámetros (Lisman et al., 2005). Asimismo, hay que destacar que la patogénesis de las complicaciones obstétricas en el SAF es multifactorial (Pierangeli et al., 2008; Forastiero et al., 2008; Bouma et al., 2003) y que las alteraciones de la fibrinolisis no són más que uno de los múltiples mecanismos patofisiológicos para el desarrollo de los eventos trombóticos en pacientes gestantes.

Los factores genéticos pueden explicar entre el 20 y el 62% de la variación de los niveles de TAFI, aunque la relación entre los polimorfismos de TAFI, los niveles de TAFI y el riesgo de complicaciones trombóticas no es bien conocido en la actualidad (Franco et al, 2001; Henry et al., 2001). Ni en el **estudio 1** ni en el **estudio 2** se hallaron diferencias en la distribución de alelos de los polimorfismos de TAFI entre los diferentes grupos estudiados. Esto contrasta con los resultados publicados previamente por otros autores (Masini et al., 2009) que concluyeron que algunos polimorfismos pueden asociarse a un menor riesgo de AR. Estos autores, no obstante, no analizaron los niveles de TAFI ni el CLT e incluyeron pacientes con dos o más abortos entre los que hasta el 45% presentaba una causa conocida de AR.

Tal como se ha descrito en la Introducción de la presente Tesis Doctoral, la gestación normal se ha asociado con cambios en múltiples componentes de la hemostasia, con un incremento en la concentración de la mayoría de factores de coagulación, un descenso de la concentración de algunos anticoagulantes naturales, y una reducción de la actividad fibrinolítica global (Bremme et al., 2003; Brenner, 2004). Asimismo, durante el estado fisiológico de hipercoagulabilidad característico de la gestación normal, la existencia de trombofilias congénitas o adquiridas constituyen un factor de riesgo sobreañadido que puede incrementar el riesgo tromboembólico durante el embarazo y llevar al desarrollo de complicaciones obstétricas graves secundarias a trombosis placentarias como abortos de repetición,

preeclampsia, retraso de crecimiento intrauterino o desprendimiento de placenta.

Algunos estudios han investigado los niveles de TAFI y el CLT en gestantes sanas (Chetaille et al., 2000; Chabloz et al., 2001; Mousa et al., 2004; Watanabe et al., 2004; Goldenberg et al., 2005) y en gestaciones complicadas con preeclampsia (Antovic et al., 2002; Alacacioglu et al., 2004; Sucak et al., 2006) mostrando resultados contradictorios. Curiosamente, sólo un estudio previo investigó los polimorfismos de TAFI como factor de riesgo de preeclampsia y demostró una ausencia de asociación (De Maat et al., 2004). No se ha comunicado previamente en la bibliografía estudios sobre los niveles de TAFI o el CLT en pacientes con SAF aunque sí se han descrito en otras enfermedades autoinmunes con aumento de riesgo trombótico como la enfermedad de Behçet (Donmez et al., 2005; Ricart et al., 2008) o en el lupus eritematoso sistémico (Ringwald et al., 2007) y sólo en escasos estudios se ha descrito el TAFI o el CLT en pacientes con AAF pero sin SAF (Curnow et al., 2006; Ringwald et al., 2007; Patterson et al., 2006).

Por ello nuestra contribución con el **estudio 3** es de gran interés clínico-práctico ya que es el primero que describe los niveles de TAFI y sus polimorfismos y el CLT en pacientes gestantes con SAF y los relaciona con el resultado gestacional y los antecedentes de trombosis previas. Nuestros resultados indican que las pacientes con SAF tienen una alteración de la fibrinolisis demostrada por una prolongación del CLT basal comparado con

controles sanas. Esos cambios de la fibrinolisis no se relacionaron con diferencias en los niveles de TAFI. Asimismo, los niveles de TAFI o el CLT no se asociaron a diferentes resultados obstétricos o a antecedentes de trombosis en las pacientes con SAF. Estos resultados pueden atribuirse a diferentes factores. En primer lugar la patogénesis de las complicaciones obstétricas asociadas al SAF es multifactorial (Espinosa et al., 2003; Pierangeli et al., 2008; Forastiero et al., 2008; D´Ippolito et al., 2007) y las alteraciones de la fibrinolisis sólo constituyen uno de los mecanismos fisiopatogénicos propuestos implicados en las complicaciones obstétricas trombóticas asociadas al SAF. En segundo lugar, las complicaciones incluidas en el presente estudio fueron heterogéneas, englobando desde gestaciones complicadas con preeclampsias severas y/o retrasos de crecimiento intrauterino severos hasta trombosis maternas, con un número de complicaciones bajo en el grupo de pacientes con SAF.

Curiosamente, en el **estudio 3** no se hallaron diferencias en los niveles de TAFI o CLT durante el segundo o tercer trimestre de la gestación, que es el momento en que se desarrollan las complicaciones obstétricas. Estos resultados están de acuerdo con los de otros autores (Cellai et al., 2006; Schroeder et al., 2003) en los que tampoco se observaron diferencias de niveles de TAFI durante la fase aguda de otras patologías trombóticas fuera de la gestación como son la cardiopatía isquémica y el tromboembolismo pulmonar agudo, probablemente por una activación y aumento de consumo de TAFI en fase aguda trombótica.

Una de las potenciales limitaciones del **estudio 3**, es que se consideraron niveles basales aquéllos obtenidos a los 4-6 meses tras el parto en lugar de los obtenidos antes de la gestación. No obstante, algunos estudios previos habían demostrado un descenso significativo de los niveles de TAFI en el periodo postparto inmediato con una rápida disminución a los niveles basales sólo unos días tras el parto (Chabloz et al., 2001; Mousa et al., 2004; Watanabe et al., 2004; Antovic et al., 2002). Por ello, los niveles a los 4-6 meses tras el parto pueden ser considerados con mucha seguridad como determinaciones basales.

La preeclampsia es una de las complicaciones obstétricas principales responsables del incremento de la morbilidad y mortalidad materno-fetal asociada al SAF (Carmona et al., 2001). Aunque la patogénesis de la preeclampsia no se conoce con exactitud, una de las causas aceptadas es la alteración de la circulación útero-placentaria debido a un aumento de microtrombogénesis (Roberts and Cooper, 2001). Además, estudios previos han descrito alteraciones de la coagulación y de la fibrinolisis en mujeres con preeclampsia (Alfirevic et al., 2002) y, diferentes estudios han identificado alteraciones específicas en la capacidad fibrinolítica asociadas a esta complicación obstétrica (Sucak et al., 2006; Sartori et al., 2007).

En este contexto, y en base a lo expuesto previamente se llevó a cabo el **estudio 4** con el objetivo de investigar el CLT, los niveles de TAFI y sus polimorfismos genéticos en pacientes gestantes complicadas con una pre-

eclampsia severa asociada o no a SAF. De esta manera, incluyendo sólo pacientes con preeclampsia severa, evitábamos la limitación del **estudio 3** en cuanto a heterogeneidad de las complicaciones asociadas a SAF. En la misma línea que el **estudio 3**, el **estudio 4** demostró que tanto las pacientes con preeclampsia severa sin SAF como las pacientes con preeclampsia severa con SAF tenían una alteración de la fibrinolisis evidenciada por un CLT alargado tras el parto comparado con controles. Esta alteración del CLT también se evidenció en el **estudio 3** en las pacientes con SAF sin complicaciones obstétricas. Estos resultados pueden atribuirse parcialmente a niveles de TAFI más elevados en pacientes con preeclampsia severa con o sin SAF, pero no en pacientes con SAF sin preeclampsia severa. De nuevo, hay que enfatizar que los hallazgos del **estudio 4** se explican por una contribución parcial y limitada del TAFI sobre el CLT, a pesar de la clara y demostrada asociación entre ambos parámetros (Lisman et al., 2005). Por otro lado, hay que tener presente que la patogénesis de las complicaciones gestacionales en el SAF es multifactorial (Espinosa et al., 2003; Forastiero and Martinuzzo, 2008), siendo las alteraciones de la fibrinolisis simplemente uno de los múltiples mecanismos fisiopatológicos propuestos durante la gestación.

Hay que destacar que el **estudio 4** muestra que los niveles elevados de TAFI pueden jugar un papel en la fisiopatología de la preeclampsia severa en pacientes sin AAF, como se había sugerido con anterioridad (Alacacioglu et al., 2004). Dos estudios previos que analizaron los niveles de TAFI o el CLT durante la gestación complicada con preeclampsia o r etraso de crecimiento

intrauterino sin AAF (Antovic et al., 2002; Alacacioglu et al., 2004) presentaron resultados contradictorios, hallando niveles más bajos de TAFI y un alargamiento del CLT en el estudio de Antovic *et al.* y sin hallar diferencias en los niveles de TAFI en el estudio de Alacacioglu *et al*. Además, estos estudios no determinaron los valores de TAFI y CLT basales. Hay que destacar que en dichos estudios se analizaron grupos de pacientes difíciles de comparar con los de nuestro estudio y entre ellos ya que difieren en cuanto al número de casos, los criterios de inclusión y la gravedad de la preeclampsia.

Se ha comentado anteriormente que una de las posibles limitaciones del **estudio 3** fue la de considerar como niveles basales las determinaciones realizadas 4-6 meses tras el parto, ya que las determinaciones antes de la gestación son muy difíciles de obtener. En el **estudio 4**, con la intención de minimizar esta limitación se realizaron dos determinaciones a los 4-6 meses y a los 12 meses tras el parto. De esta manera, y habiendo descrito otros estudios (Chabloz et al., 2001; Mousa et al., 2004; Watanabe et al., 2004; Antovic et al., 2002) que los niveles de TAFI descienden rápidamente horas/días tras el parto a niveles basales, encontramos niveles similares en ambos momentos del estudio, que, por tanto, podrían considerarse muestras basales y avalan los datos de nuestro **estudio 3**.

Nuevamente, en el **estudio 4**, en concordancia con el **estudio 3**, y con otras publicaciones sobre TAFI en patologías trombóticas agudas (Cellai et al., 2006; Schroeder et al., 2003) no se hallaron diferencias en los niveles de TAFI

en fase aguda en el tercer trimestre, probablemente como reflejo de una vida media corta del TAFI activado (Schatteman et al., 2000), y posiblemente reflejando que la fase aguda de un evento trombótico no debe ser considerada la mejor para discriminar el riesgo (Bouma and Meijers, 2003) en parte por el consumo de factores de coagulación y fibrinolisis. Por otro lado estudios de la literatura reciente demuestran que los niveles basales elevados de TAFI aumentan el riesgo de eventos trombóticos como el riesgo de accidente vascular cerebral isquémico (Santamaría et al., 2003), de cardiopatía isquémica (Morange et al., 2003), de tromboembolismo venoso recurrente (Eichinger et al., 2004) y de trombosis venosa profunda (Van Tilburg et al., 2000).

A pesar de las diferencias halladas en los **estudios 3 y 4** en cuanto a los niveles de TAFI basales en los grupos de estudio, no se hallaron diferencias en los polimorfismos de TAFI. Estos resultados están en línea con los publicados previamente en pacientes complicadas con preeclampsia sin SAF (De Maat et al. 2004). Estos resultados indican que la relación entre los genotipos de TAFI, los niveles de TAFI y el riesgo de complicaciones trombóticas es complejo (Franco et al., 2001), y que otros factores aparte de los polimorfismos de TAFI pueden influenciar los niveles de TAFI.

Aunque las pacientes con SAF de los **estudios 3 y 4** recibieron tratamiento con ácido acetil salicílico a dosis bajas y/o heparina de bajo peso molecular, a diferencia de los grupos controles sin SAF, esto no puede considerarse como una limitación o sesgo de los resultados. En primer lugar

hay que tener en cuenta que las pacientes del grupo SAF que presentaron una preeclampsia grave recibieron el mismo tratamiento que las gestantes que no se complicaron. Por otro lado, estudios previos (Bjornsson et al., 1989; Ajjan et al., 2009) demostraron que la toma de ácido acetil salicílico a dosis muy altas (650 miligramos cada 12 horas) potenciaba la fibrinolisis debido a una acetilación de la molécula de fibrinógeno, correlacionando inversamente con el CLT. Considerando estos estudios, por tanto, si la ingesta de dosis bajas de ácido acetil salicílico hubiera producido algún efecto en los resultados de los **estudios 3 y 4**, hubiera sido en el sentido de acortar el CLT en las pacientes con SAF, y por tanto las diferencias observadas en los estudios presentados aún hubiese sido mayor. Además, en un estudio previo (Colucci et al., 2002) en el que se evaluó el efecto de la heparina sobre la fibrinolisis, se demostró que la heparina es incapaz de estimular la fibrinolisis a través de un mecanismo mediado por el TAFI. Por todo ello, el sesgo que podría suponer en este estudio el tratamiento con ácido acetil salicílico a dosis bajas y/o heparina en las pacientes con SAF es improbable.

Una de las grandes limitaciones del conocimiento del SAF en la actualidad sigue siendo la imposibilidad de discriminar aquéllos pacientes con SAF o portadores de AAF que van a desarrollar trombosis y/o complicaciones obstétricas. Ya se ha expuesto con anterioridad que las pacientes con antecedentes de AR tienen un estado protrombótico, aún fuera de la gestación, y se ha sugerido que los cambios de coagulación pueden preceder a la pérdida gestacional (Regan and Rai, 2002; Rai, 2003). En este sentido, en un estudio

descriptivo de gran tamaño se valoraron las placentas procedentes de pacientes con AR y se reportó un aumento significativo en la prevalencia de infartos placentarios entre aquéllas pacientes con AR (10%) respecto a las que no habían presentado AR ni complicaciones gestacionales (1%) (Sebire et al., 2003). Es de destacar que los estudios que incluyen pacientes con SAF exclusivamente obstétrico como pacientes de riesgo de presentar eventos trombóticos fuera de la gestación son escasos, retrospectivos o incluyen un bajo número de pacientes (Erkan et al., 2001; Tincani et al., 2002). Por otro lado, otras publicaciones no han demostrado un riesgo más elevado de trombosis en las AR con o sin SAF (Quenby et al., 2005). Ante esta controversia y en ausencia de datos concluyentes se realizó el **estudio 5**, que tuvo como objetivo investigar si las pacientes con AR como única manifestación clínica del SAF tenían un riesgo aumentado de trombosis a largo plazo. Para ello se escogieron tres grupos comparativos de pacientes: un grupo de AR idiopáticas, un grupo de AR con trombofilia genética y un grupo de portadoras sanas de AAF. Los resultados de este estudio demostraron, por primera vez, que las pacientes con AR y SAF tenían un riesgo 15 veces mayor de presentar un evento trombótico a largo plazo que aquéllas pacientes sin AAF con o sin otras trombofilias. Además las pacientes que presentaron eventos trombóticos arteriales tenían factores de riesgo cardiovascular asociados con mayor frecuencia respecto las que no los presentaron.

Los resultados del **estudio 5** están en concordancia con estudios previos que sugieren que las mujeres con antecedente de AR tienen un riesgo

trombótico elevado (Rai, 2003; Smith et al., 2003; Quenby et al., 2005). Por otro lado añade información interesante al diferenciar pacientes AR con SAF o con trombofilias sin AAF. Así permite conocer que las pacientes AR con AAF presentan un riesgo 5 veces mayor de presentar un evento trombótico que las pacientes con trombofilias hereditarias. Además permite apoyar la hipótesis en dos tiempos ("two-hit hypothesis") que defiende que los AAF (primer "hit") aumentan el riesgo o sustrato trombótico, pero que se requiere de un segundo "hit" (como puede ser el haber presentado AR asociado o no a factores de riesgo cardiovascular) para que se desencadene el evento trombótico (Meroni, 2008; Meroni and Shoenfeld, 2008). Este estudio puede tener importantes implicaciones clinico-prácticas en el sentido de plantear la necesidad de un tratamiento profiláctico anticoagulante a largo plazo en estas mujeres.

Nuestros estudios nos permiten llegar a las conclusiones que se exponen a continuación y que se corresponden con los 6 objetivos propuestos en el apartado correspondiente de esta Tesis Doctoral (ver páginas 31 y 32).

6. CONCLUSIONES

1. Las pacientes con fracaso implantatorio repetido tras fecundación in vitro (FIR) y con abortos de repetición (AR) de causa idiopática presentan una reducción del potencial fibrinolítico del plasma determinado mediante el tiempo de lisis del coágulo (CLT). Esta alteración de la fibrinolisis puede explicarse en parte por niveles de TAFI significativamente mayores en estos dos grupos de pacientes. La determinación de polimorfismos de TAFI no muestra diferencias en estos grupos.

2. Las pacientes con AR con o sin síndrome antifosfolipídico asociado (SAF) presentan una reducción del potencial fibrinolítico objetivada mediante una prolongación del CLT. Sólo las pacientes con AR sin SAF asociado presentan una elevación de los niveles de TAFI, por lo que, en este grupo de pacientes, la alteración global de la fibrinolisis puede atribuirse en parte a este hallazgo. No se han hallado diferencias en los polimorfismos de TAFI entre estos dos grupos.

3. Durante la gestación se produce un incremento de los niveles de TAFI y un alargamiento del CLT que regresan a niveles basales tras la gestación. Las pacientes con SAF presentan un alargamiento del CLT basal respecto a las gestantes sanas. No existen diferencias en la distribución de polimorfismos de TAFI ni en sus niveles entre gestantes sanas o con SAF.

4. Las gestantes con SAF no presentan diferencias en los niveles de TAFI, polimorfismos de TAFI o CLT en función del resultado perinatal o el antecedente de trombosis.

5. Las pacientes con SAF con o sin preeclampsia y las pacientes con preeclampsia sin SAF presentan una reducción del potencial fibrinolítico del plasma basalmente. Los niveles basales de TAFI se han encontrado significativamente más elevados en las pacientes con preeclampsia severa con o sin SAF que en las pacientes sin preeclampsia o en controles sanas.

6. Las pacientes con AR y SAF presentan un riesgo de trombosis a largo plazo 15 veces superior que las pacientes con AR sin SAF. El riesgo de eventos trombóticos arteriales es superior en aquéllas pacientes que además presentan factores de riesgo cardiovascular tradicionales.

Como consecuencia de todo ello y a modo de conclusión general de interés clínico-práctico, podemos afirmar que existe una alteración basal de la fibrinolisis en las pacientes con SAF, en las pacientes que han presentado una preeclampsia con o sin AAF, en las pacientes con AR y en las pacientes con FIR. Dicha alteración global de la fibrinolisis puede atribuirse parcialmente a un incremento de los niveles de TAFI en las pacientes con FIR, en las pacientes con AR sin AAF y en las pacientes complicadas con preeclampsia severa. Además de la alteración global de

la fibrinolisis, las pacientes con AR con SAF presentan un incremento significativo del riesgo trombótico a largo plazo en comparación con las pacientes con AR sin SAF.

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