UNIVERSIDAD DE SALAMANCA

FACULTAD DE BIOLOGÍA

DEPARTAMENTO DE BIOLOGÍA ANIMAL, PARASITOLOGÍA, ECOLOGÍA, EDAFOLOGÍA Y QUÍMICA AGRÍCOLA

VNiVERSiDAD
D SALAMANCA
CAMPUS DE EXCELENCIA INTERNACIONAL

TESIS DOCTORAL

El papel de la plasmina en la supervivencia de *Dirofilaria immitis* y en la patología vascular del hospedador durante la dirofilariosis cardiopulmonar

Tesis Doctoral presentada por el Licenciado **D. Javier González Miguel** para optar a la Mención de Doctor Europeo por la Universidad de Salamanca.

Salamanca, 2015.

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CERTIFICAN:

Que la Tesis Doctoral titulada **"El papel de la plasmina en la supervivencia de *Dirofilaria immitis* y en la patología vascular del hospedador durante la dirofilariosis cardiopulmonar"** ha sido realizado bajo su dirección, por **D. Javier González Miguel**, con D.N.I. 72098244-Y, licenciado en Biología por la Universidad de Salamanca, en el Área de Parasitología del Departamento de Biología Animal, Parasitología, Ecología, Edafología y Química Agrícola de la Universidad de Salamanca. Dicha Tesis Doctoral reúne las condiciones necesarias para ser defendida y optar a la Mención de Doctor Europeo por la Universidad de Salamanca.

Y para que así conste, a los efectos legales, expiden y firman el presente certificado en Salamanca, a 25 de Mayo de 2015.

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ÁREA DE PARASITOLOGÍA

La presente Tesis Doctoral está elaborada en el formato de compendio de artículos/publicaciones según la normativa aprobada por la Comisión de Doctorado y Posgrado de la Universidad de Salamanca el 15 de febrero de 2013 y consta de las siguientes publicaciones:

1. **Excretory/secretory antigens from *Dirofilaria immitis* adult worms interact with the host fibrinolytic system involving the vascular endothelium**

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   Molecular & Biochemical Parasitology 181 (2012) 134-140.

   Factor de impacto (2012): 2,734. doi:10.1016/j.molbiopara.2011.10.010

2. **Surface associated antigens of *Dirofilaria immitis* adult worms activate the host fibrinolytic system**

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   Veterinary Parasitology 196 (2013) 235-240

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3. **Surface-displayed glyceraldehyde 3-phosphate dehydrogenase and galectin from *Dirofilaria immitis* enhance the activation of the fibrinolytic system of the host**

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4. **Can the activation of plasminogen/plasmin system of the host by metabolic products of *Dirofilaria immitis* participate in heartworm disease endarteritis?**

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   Parasites & Vectors (2015) 8: 194.

   Factor de impacto (2014): 3,25. doi:10.1186/s13071-015-0799-0

5. **Fibrinolysis and Proliferative Endarteritis: Two Related Processes in Chronic Infections? The Model of the Blood-Borne Pathogen *Dirofilaria immitis***

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AUTORIZAN:

Que la Tesis Doctoral titulada **"El papel de la plasmina en la supervivencia de *Dirofilaria immitis* y en la patología vascular del hospedador durante la dirofilariosis cardiopulmonar"** sea presentada en la modalidad de compendio de artículos/publicaciones (Comisión de Doctorado y Posgrado, 15 de febrero de 2013).

Y para que así conste, a los efectos legales, expiden y firman el presente certificado en Salamanca, a 25 de Mayo de 2015.

Fdo.: **Fernando Simón Martín** Fdo.: **Rodrigo Morchón García**

Javier González Miguel ha sido beneficiario de una **Beca adscrita a Contrato Art. 83 LOU** suscrito con la empresa Chemical Ibérica Productos Veterinarios S. L. durante 8 meses (enero-septiembre 2008), de una **Beca de postgrado del Programa Nacional de Formación de Profesorado Universitario (FPU)** del Ministerio de Ciencia e Innovación (AP2009-3479) durante 4 años (julio 2009-julio 2013) y actualmente se encuentra contratado como **Personal Docente Investigador a través de un Proyecto Art. 83 LOU** por la Universidad de Salamanca.

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“La ciencia es respecto del alma lo que es la luz respecto de los ojos,

y si las raíces son amargas, los frutos son muy dulces”

Aristóteles

“Heavenly Fruits” – Vladimir Kush

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INTRODVCCIÓN

La dirofilariosis cardiopulmonar causada por *Dirofilaria immitis* es una parasitosis de transmisión vectorial que afecta a las poblaciones de cánidos y félidos domésticos y silvestres de todo el mundo. Además, *D. immitis* puede transmitirse al hombre; de hecho, casos de dirofilariosis humana se detectan con frecuencia creciente, por lo que a la indudable importancia de la dirofilariosis desde el punto de vista veterinario, hay que añadir el interés médico de las infecciones zoonósicas.

D. immitis se ha adaptado en diversos grados a sus distintos hospedadores, estableciendo con ellos un amplio espectro de relaciones. Si bien existen precisas descripciones de las consecuencias patológicas de la infección tanto en perros y gatos como en humanos, disponemos de mucha menos información sobre las moléculas y células, tanto del parásito como de sus hospedadores, implicadas en esas relaciones, así como de los mecanismos en los que intervienen.

Al igual que las filarias tropicales, los vermes adultos de *D. immitis* presentan una gran longevidad, pese a vivir en un entorno tan hostil como la sangre. Este hecho, necesariamente, debe estar asociado a mecanismos de supervivencia desarrollados por el parásito, que le permitan modificar en beneficio propio su entorno inmediato, constituido por la propia sangre y la pared arterial. Además de los mecanismos antiinflamatorios previamente estudiados por nuestro equipo, en la actualidad, la activación del sistema fibrinolítico de los hospedadores por parte de los organismos invasores es considerada un mecanismo de supervivencia clave en las relaciones que se establecen entre los patógenos sanguíneos y sus hospedadores, ya que no solo contribuye a mantener la hemostasia, hecho fundamental para los patógenos hemáticos, sino que también se ha relacionado con su capacidad invasiva.

En la dirofilariosis crónica, el aparente equilibrio que se prolonga durante largos períodos de tiempo, beneficioso tanto para *Dirofilaria* como para el hospedador, puede alterarse por la influencia de diversos factores derivados tanto del parásito (presencia física de los vermes, acción de sus productos metabólicos y la muerte de algunos de ellos), como del hospedador (respuesta inmune y conformación del sistema vascular), dando comienzo a la patología vascular, cuyos eventos principales son la aparición de endarteritis proliferativa y la formación de coágulos. Aunque se han propuesto diversos factores desencadenantes de la formación de vellosidades en la pared arterial durante la

dirofilariosis animal, no existen hasta el momento estudios experimentales orientados a la identificación de los mecanismos moleculares implicados. Recientemente, investigaciones realizadas en patología vascular humana han relacionado la sobreproducción de plasmina, molécula clave del sistema fibrinolítico, con la proliferación y migración de las células de la pared arterial, así como con la destrucción de la matriz extracelular. Dichos procesos presentan una evidente similitud con los que conducen a la aparición de endarteritis proliferativa en la dirofilariosis cardiopulmonar.

Considerando todos estos hechos, en la presente Tesis Doctoral se han investigado las moléculas de *D. immitis* implicadas en la activación del sistema fibrinolítico con la consecuente producción de plasmina y el posible papel de esta enzima en el desarrollo de la endarteritis proliferativa durante la dirofilariosis cardiopulmonar.

REVISIÓN BIBLIOGRÁFICA

1. SISTEMÁTICA Y FILOGENIA

Dirofilaria immitis (Leidy, 1856) es un nematodo filaroideo incluido dentro de la familia Onchocercidae. Esta familia comprende a su vez dos subfamilias, la subfamilia Onchocercinae, con los géneros *Onchocerca*, *Brugia*, *Wuchereria* y *Mansonella*; y la subfamilia Dirofilariinae, con los géneros *Dirofilaria* y *Loa* (Marquardt *et al*., 2000; Bain, 2002). No obstante, esta clasificación basada en caracteres morfológicos ha sido cuestionada desde el punto de vista molecular. Xie *et al*. (1994) propusieron una relación más cercana entre los géneros *Loa* y *Mansonella* por una parte, y entre *Onchocerca* y *Dirofilaria* por otra, comparando la región espaciadora del ARN ribosomal 5S. Estos resultados fueron indirectamente corroborados gracias al estudio filogenético de las bacterias endosimbiontes del género *Wolbachia* presentes en las filarias (Sironi *et al*., 1995; Bandi *et al*., 1998), y más recientemente mediante análisis filogenéticos basados en las secuencias mitocondriales correspondientes a la citocromo oxidasa I y al ARN ribosomal 12S de las propias filarias (Casiraghi *et al*., 2001; Huang *et al*., 2009).

2. BIOLOGÍA Y CICLO DE *D. IMMITIS*

Los vermes adultos de *D. immitis* son largos, delgados, de aspecto filiforme y con un marcado dimorfismo sexual. Las hembras miden 250-300 mm de longitud por 1-1,3 mm de diámetro, mientras que los machos, más pequeños y con su extremo posterior enrollado en espiral, miden 120-200 mm de longitud por 0,7-0,9 mm de diámetro (Manfredi *et al*., 2007). Poseen un ciclo biológico indirecto que implica a un hospedador definitivo vertebrado y a un vector (Figura 1). Su escasa especificidad de hospedador determina que *D. immitis* pueda parasitar numerosas especies de mamíferos (Barriga, 1982). De todas ellas, los perros y los cánidos silvestres sirven de reservorios de la enfermedad, al ser los hospedadores a los que mejor se ha adaptado el parásito. Pese a que se trata de un hospedador menos adecuado para su desarrollo, el gato puede albergar la fase adulta del parásito, mientras que el hombre también puede verse afectado, aunque de manera accidental y sin ninguna implicación para la transmisión (McCall *et al*., 2008; Simón y Genchi, 2000). En el hospedador humano, los vermes inmaduros de *D. immitis* pueden alcanzar una rama de la arteria pulmonar, donde estimulan una reacción inflamatoria y tromboembólica que destruye los vermes y causa nódulos pulmonares

(Simón *et al*., 2005). La poca especificidad de *D. immitis* también se ve reflejada a nivel de su hospedador intermediario. Al menos 70 especies de mosquitos culícidos son consideradas como vectores potenciales del parásito, aunque solo se ha demostrado la capacidad vectorial real en algunas de ellas, pertenecientes a los géneros *Culex*, *Aedes*, *Culiseta*, *Mansonia* y *Coquilletidia* dentro de la subfamilia Culicinae y al género *Anopheles* dentro de la subfamilia Anophelinae (Cancrini y Kramer, 2001; Cancrini *et al*., 2006).

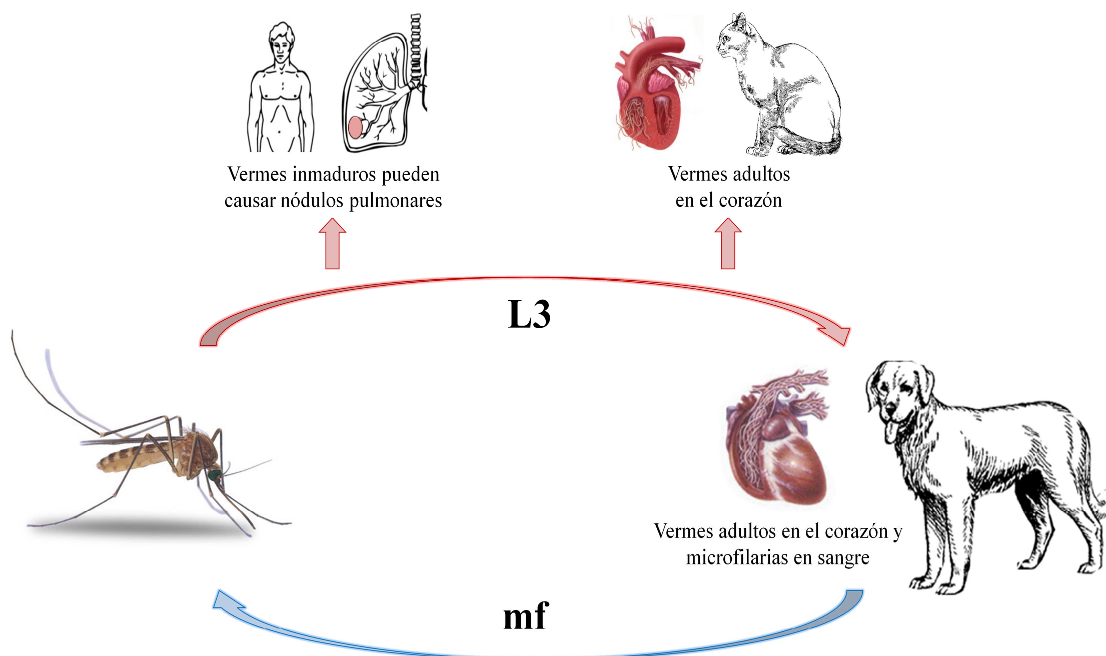

Figura 1. Ciclo biológico de *D. immitis*. L3, larvas de 3er estadio; mf, microfilarias.

2.1. Desarrollo de *D. immitis* en los vectores

Cuando las hembras de mosquitos susceptibles se alimentan sobre reservorios infectados, ingieren microfilarias (larvas de primer estadio) presentes en la sangre periférica de estos. Aproximadamente 24 horas después, las microfilarias, que miden 290-330 µm de longitud por 5-7 µm de diámetro (Venco *et al*., 2011), pasan del tubo digestivo del vector a los tubos de Malpighi, donde comienzan el desarrollo a larvas infectantes. Dicho proceso dura entre 10 y 15 días, dependiendo de diferentes condiciones ambientales, principalmente de la temperatura y comprende dos mudas. La primera da lugar a las larvas de segundo estadio (L2) y se produce aproximadamente entre los días

8º y 10º después de la infección. La siguiente, que tiene como resultado la larva de tercer estadio (L3) y que implica un importante aumento de tamaño, tiene lugar unos 3 días después. Las L3 completan su desarrollo emigrando hacia la región cefálica del vector. Una vez allí, se acumulan en las piezas bucales y son inoculadas en el tejido subcutáneo de un nuevo hospedador en la siguiente toma de sangre (Manfredi *et al.*, 2007).

2.2. Desarrollo de *D. immitis* en los hospedadores definitivos

Los mosquitos parasitados depositan una gota de hemolinfa con las L3 durante la toma de sangre. Estas penetran en la piel por sus propios medios mudando a larvas de cuarto estadio (L4) entre 3 y 12 días post-infección, con un considerable aumento de tamaño asociado. La siguiente muda dará lugar a los vermes adultos inmaduros (50-70 días post-infección), los cuales alcanzan su localización definitiva en las arterias pulmonares y el ventrículo derecho del corazón entre los días 70º y 85º después de la infección (Figura 2). La fecundación comienza a partir de los 120 días post-infección, cuando se completa el desarrollo de los vermes adultos, alcanzando estos la madurez sexual (Manfredi *et al.*, 2007). Una vez realizada la fecundación, las hembras liberan microfilarias al torrente circulatorio. Estas aparecen en la circulación periférica entre los 6 meses y medio y 7 meses post-infección. La microfilaremia aumenta durante los 10 meses siguientes y se mantiene constante durante varios años, desapareciendo después progresivamente. La longevidad de las microfilarias puede alcanzar los dos años, mientras que los vermes adultos pueden vivir 7 años o más en su localización definitiva (McCall *et al.*, 2008). Por otra parte, algunos perros infectados no presentan microfilarias en sangre (infecciones amicrofilarémicas), situación que puede deberse a distintos factores como el envejecimiento de las hembras, infecciones por vermes de un solo sexo y/o la respuesta inmune del hospedador (Simón y Genchi, 2000).

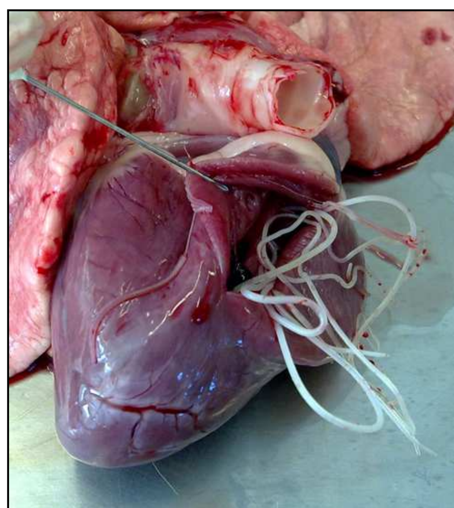

Figura 2. Vermes adultos (macho y hembra) de *D. immitis* en el corazón de un perro con dirofilariosis (Simón et al., 2012).

2.3. Composición proteica de *D. immitis*

El conocimiento de la composición proteica de un patógeno es esencial para poder definir las relaciones que establece con su hospedador a nivel molecular. No obstante, esta información es aún escasa en el caso de *D. immitis*. La base de datos de secuencias proteicas del *National Center for Biotechnology Information* (NCBI) contiene 277 resultados para *D. immitis*, un número escaso en comparación con el de otros nematodos en los que se han desarrollado muchos más estudios moleculares (*Caenorhabditis elegans*: 85050 resultados; *Brugia malayi*: 34705 resultados).

Durante la década de los 80 del pasado siglo se llevaron a cabo los primeros trabajos de identificación proteica en *D. immitis*, con el objetivo de buscar moléculas que pudieran ser empleadas en el diagnóstico inmunológico de la dirofilariosis cardiopulmonar. Se caracterizaron tanto bioquímica como inmunológicamente numerosos antígenos circulantes en suero de perros infectados naturalmente (Ehrenberg *et al*., 1987), así como otros antígenos presentes en la superficie de larvas L3 (Philipp y Davis, 1986; Ibrahim *et al*., 1989) y vermes adultos (Scott *et al*., 1988).

En la década siguiente, la mejora de las técnicas de biología molecular permitió un aumento considerable de los estudios de identificación y caracterización proteica en *D. immitis*. Muchos de ellos fueron realizados por el grupo de investigación liderado por los doctores Glenn R. Frank y Robert B. Grieve (*Heska Corporation*, EEUU). Entre los más relevantes se encuentran la identificación y clonación de un grupo de antígenos que han servido para el diagnóstico de la dirofilariosis cardiopulmonar, como P20, P22L y P22U (Frank y Grieve, 1991; Frank *et al*., 1996; Frank *et al*., 1999) o un inhibidor de la aspartil proteasa (Dit33) (Frank *et al*., 1998). Además, llevaron a cabo la clonación de una proteína similar a la glutatión peroxidasa (Di29) excretada por vermes adultos (Tripp *et al*., 1998), así como la identificación de un gran número de antígenos de L3 y L4 con características proteolíticas (Richer *et al*., 1992) o posibles aplicaciones inmunoprofilácticas (Grieve *et al*., 1992). Otros trabajos realizados en esta década permitieron comparar los repertorios antigénicos de las L2, L3 y L4 (Scott *et al*., 1990) o la identificación y clonación molecular de la proteína de choque térmico p27, una peroxirredoxina, una paramiosina, un factor quimiotáctico de neutrófilos o la ciclofilina Dicyp-3, entre otras (Limberger y McReynolds, 1990; Owhashi *et al*., 1993; Lillibridge *et al*., 1996; Hong *et al*., 1998; Zipfel *et al*., 1998).

A partir del año 2000, frente a la producción de proteínas recombinantes de forma individual, algunas con importantes funciones antioxidantes (Chandrashekar *et al*., 2000), relacionadas con la muda (Crossgrove *et al*., 2002), la estructura (Harris y Fuhrman, 2002), la proliferación y diferenciación (Shea *et al*., 2004) o como posibles dianas terapéuticas (Yates y Wolstenholme, 2004), las nuevas técnicas de identificación masiva han permitido aumentar notablemente el conocimiento sobre las proteínas de *D. immitis*. Mediante la combinación de electroforesis bidimensional y espectrometría de masas, nuestro grupo ha llevado a cabo los primeros estudios de proteómica en los extractos antigénicos de *D. immitis* (Oleaga *et al*., 2009; González-Miguel *et al*., 2010a y b) (Figura 3). Partiendo de un extracto proteico soluble de vermes adultos y con el objetivo de conocer qué antígenos son reconocidos por el sistema inmune de perros, gatos o humanos infectados con *D. immitis*, se identificaron 39 proteínas inmunógenas del parásito (Tabla 1). Estas proteínas pertenecen principalmente a cuatro grupos funcionales que incluyen enzimas metabólicas, enzimas detoxificantes o con potencial redox, moléculas relacionadas con respuesta al estrés y proteínas estructurales. Esto sugiere la importancia que tiene para el parásito la generación de energía mediante procesos metabólicos tales como la glicolisis anaerobia, o los mecanismos defensivos mediante el empleo de un repertorio de enzimas relacionadas con la detoxificación o la respuesta al estrés. Destaca también el gran número de proteínas que han sido previamente estudiadas en otros parásitos por su papel como receptores del plasminógeno, como la actina, la enolasa, la fructosa-bifosfato aldolasa (FBAL) o la gliceraldehído-3-fosfato deshidrogenasa (GAPDH) (González-Miguel *et al*., 2010b). Bastantes de las proteínas identificadas están representadas por diversas isoformas, algunas de las cuales no son reconocidas por el sistema inmune de los hospedadores, lo que parece indicar que *D. immitis* posee sistemas bioquímicos redundantes que hacen más difícil su interferencia por el sistema inmune del hospedador (Simón *et al*. 2012).

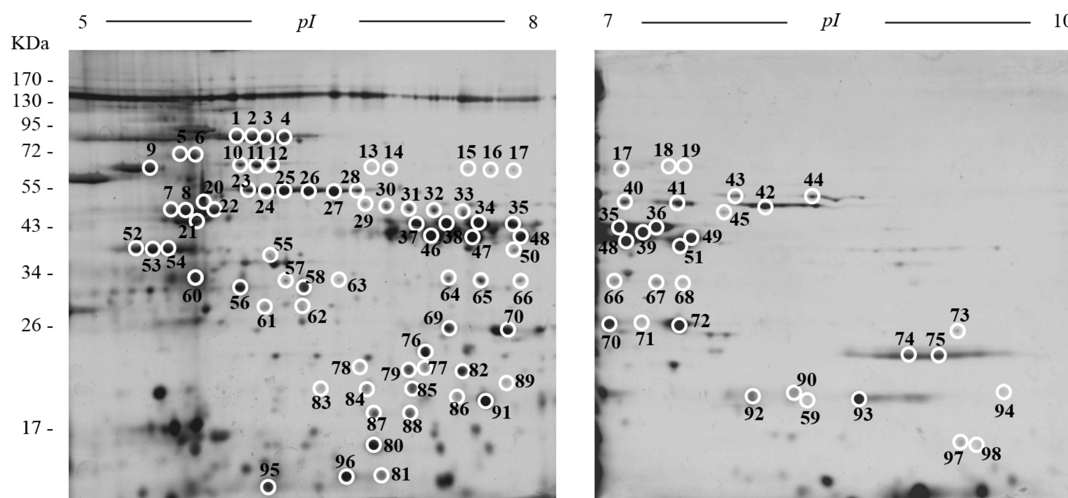

Figura 3. Electroforesis bidimensional representativa de 40 µg de un extracto de proteínas somáticas de vermes adultos de *D. immitis*. Geles de pH 5-8 y 7-10 y 12% de acrilamida teñidos con nitrato de plata. Los *spots* identificados por espectrometría de masas están numerados (Oleaga *et al*., 2009; González-Miguel *et al*., 2010a y b).

Más recientemente, la publicación de una base de datos sobre el transcriptoma de *D. immitis* (Fu *et al*., 2012), los nuevos datos disponibles sobre el genoma del parásito (Godel *et al*., 2012), así como la aparición de técnicas más efectivas de identificación están permitiendo completar el mapa proteico del parásito. Geary *et al*. (2012) identificaron 110 proteínas en el secretoma de *D. immitis* mediante la combinación de cromatografía en fase líquida con la espectrometría de masas (LC-MS). Aplicando una metodología similar nuestro grupo identificó un total de 108 y 16 proteínas, respectivamente, en los compartimentos somático y de superficie del parásito (Morchón *et al*., 2014). Atendiendo a las funciones de estas proteínas, de nuevo aquellas enzimas relacionadas con el metabolismo energético, los procesos redox, las proteínas con funciones estructurales o respuesta al estrés fueron las más representadas (Figura 4).

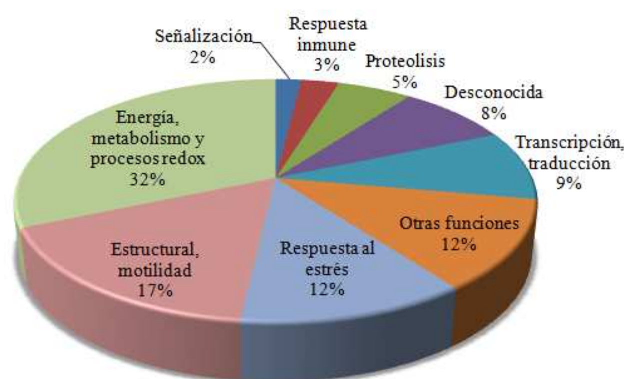

Figura 4. Comparación de la abundancia relativa de los grupos funcionales asignados a las proteínas de *D. immitis* identificadas por LC-MS en el compartimento antigénico somático del parásito (Morchón et al., 2014).

Número de *spot*	Código de acceso (NCBI)	Proteína	Especie	Proceso biológico
1-4	AAF32254	Proteína de choque térmico Hsp 70	*Wuchereria bancrofti*	Respuesta al estrés
5-7	CAA34719	Actina	*Caenorhabditis elegans*	Motilidad celular
8	P30162	Actina-1	*Onchocerca volvulus*	Motilidad celular
9	CAE11787	Disulfuro isomerasa	*Brugia malayi*	Homeostasis redox
10-12	AAC24752	Precursor de transglutaminasa	*Dirofilaria immitis*	Homeostasis redox
13,14	AAV33247	Fosfoglicerato mutasa	*Onchocerca volvulus*	Glicolisis
15-17	A8NLA3	Oxidorreductasa dependiente de FAD	*Brugia malayi*	Transporte electrónico
18,19	EDP29666	Glucosa fosfato isomerasa	*Brugia malayi*	Glicolisis
20	EDP37909	Inhibidor α de la disociación del Rab GDP	*Brugia malayi*	Transporte proteico
21	P30163	Actina-2	*Caenorhabditis elegans*	Motilidad celular
22	1D4X_A	Cadena A de la Mg-Atp actina	*Caenorhabditis elegans*	Motilidad celular
23-28	XP_001896281	Enolasa	*Brugia malayi*	Glicolisis
29-32	XP_001670614	Proteína hipotética CBG05397	*Caenorhabditis briggsae*	-
33-39	AAB52600	Fructosa-bifosfato aldolasa	*Onchocerca volvulus*	Glicolisis
40-42	XP_001891892	Fosfoglicerato quinasa	*Brugia malayi*	Glicolisis
43-45	XP_001900957	Fumarasa	*Brugia malayi*	Metabolismo aeróbico
46-49	XP_001899850	Gliceraldehído-3-fosfato deshidrogenasa	*Brugia malayi*	Glicolisis
50	XP_001900208	Lactato deshidrogenasa	*Brugia malayi*	Glicolisis anaerobia
51	XP_001897743	Aldo/ceto oxidorreductasa	*Brugia malayi*	Procesos redox
52-54	XP_001899521	Proteína de desorganización muscular 1	*Brugia malayi*	Adhesión celular
55	AAZ42332	Subunidad β de la proteína G	*Caenorhabditis remanei*	Transducción de señales
56-59	Q27384	Precursor del inhibidor de pepsina Dit33	*Dirofilaria immitis*	-
60	NP_508842	Actina-4	*Caenorhabditis elegans*	Motilidad celular
61	AAB08736	Proteína pequeña de choque térmico p27	*Dirofilaria immitis*	Respuesta al estrés
62	P52033	Precursor de la Glutatión peroxidasa Di29	*Dirofilaria immitis*	Respuesta al estrés oxidativo
63-68	AAF37720	Galectina	*Dirofilaria immitis*	Respuesta inmune
69-72	XP_001897269	Triosafosfato isomerasa	*Brugia malayi*	Glicolisis
73	CAA73325	Glutatión transferasa	*Brugia malayi*	Detoxificación
74-75	AAD11968	P22U	*Dirofilaria immitis*	-
76-77	AAC38831	Tiorredoxina peroxidasa	*Dirofilaria immitis*	Homeostasis redox
78-81	CAA61152	Proteína pequeña de choque térmico	*Brugia pahangi*	Respuesta al estrés
82	AA799423	Peptidil-prolil isomerasa	*Taenia solium*	Plegamiento proteico
83-88	CAA48632	Proteína OV25-1	*Onchocerca volvulus*	Respuesta al estrés
89,90	BAA96354	Proteína de unión a fosfatidiletanolamina	*Dirofilaria immitis*	Transducción de señales
91	XP_001899662	Precursor del antígeno OV-16	*Brugia malayi*	Transducción de señales
92,93	AAC47233	Ciclofilina Ovcyp-2	*Onchocerca volvulus*	Plegamiento proteico
94	XP_001902628	Bmcyp-2	*Brugia malayi*	Plegamiento proteico
95,96	BAA02004	Precursor del factor quimiotáctico de neutrófilos Di-NCF	*Dirofilaria immitis*	-
97,98	XP_001901495	Nucleósido-difosfato quinasa	*Brugia malayi*	Metabolismo de nucleótidos

Tabla 1. Proteínas inmunógenas de *D. immitis* identificadas por MALDI-TOF MS (Oleaga *et al*., 2009; González-Miguel *et al*., 2010a y b). La numeración de *spots* hace referencia a su localización en el mapa proteómico de *D. immitis* ilustrado en la figura 3. Se incluye el nombre de la proteína, especie en la que ha sido identificada, número de acceso a la información disponible en la base datos del NCBI, y proceso biológico asignado a cada proteína según las bases de datos *Gen Ontology* (http://www.geneontology.org) y *Swiss-Prot/Uniprot* (http://beta.uniprot.org).

2.4. La bacteria simbionte intracelular *Wolbachia*

D. immitis alberga bacterias endosimbiontes del género *Wolbachia* (Kozek *et al*., 2007). Estas bacterias fueron descubiertas en los años 70 mediante el empleo de la

microscopía electrónica (McLaren *et al*., 1975; Vincent *et al*., 1975; Kozek y Figueroa, 1977) y "redescubiertas", dos décadas después, en vermes adultos de *D. immitis* permitiendo demostrar su pertenencia al género *Wolbachia*, orden Rickettsiales (alfa 2 proteobacterias), mediante el empleo de técnicas de biología molecular (Sironi *et al*., 1995). *Wolbachia* se ha encontrado en hexápodos, crustáceos y quelicerados, en los que causa alteraciones reproductivas. No obstante, con los nematodos de la familia Onchocercidae ha establecido una relación simbiótica obligatoria al ser su presencia crucial para la embriogénesis y la muda de las filarias, mientras que estas proporcionan aminoácidos para el crecimiento de las bacterias (Lamb *et al*., 2004; Foster *et al*., 2005; Fenn y Blaxter, 2006).

Wolbachia se transmite por vía materna representando un componente estable de la estructura de *D. immitis*, al encontrarse en todas sus fases evolutivas. Es especialmente abundante en las larvas que se desarrollan en los hospedadores vertebrados (L3 y L4), en los cordones hipodérmicos de los adultos de ambos sexos y en los órganos genitales de las hembras (McGarry *et al*., 2004). Recientemente, ha sido detectada también en las gónadas somáticas o en la pared intestinal de nuevas especies de filarias de la familia Onchocercidae, lo que sugiere que la relación entre las bacterias y las filarias es más compleja y diversa de lo que se suponía hasta el momento (Ferri et al., 2011).

3. IMPORTANCIA EPIDEMIOLÓGICA DE LA DIROFILARIOSIS

La dirofilariosis es una enfermedad cosmopolita que afecta a poblaciones caninas y felinas, tanto domésticas como silvestres de áreas templadas y tropicales de todo el mundo. Además, en los últimos años la dirofilariosis está experimentando una expansión hacia áreas con climas más fríos, introduciéndose en países donde no se había denunciado la transmisión, a la vez que aumentan las prevalencias en las zonas históricamente endémicas. Diversos factores, tanto dependientes del comportamiento humano en relación con las mascotas, como climáticos, pueden influir en la expansión de la enfermedad. Entre ellos, el movimiento sin control de animales infectados, la introducción de nuevas especies de mosquitos capaces de actuar como vectores o el cambio climático, causado por el calentamiento global y el desarrollo de la actividad

humana en nuevas áreas. Todo ello provoca que la dirofilariosis cardiopulmonar sea considerada actualmente un problema veterinario de primera magnitud y una enfermedad emergente en algunas áreas (Genchi *et al*., 2009; Morchón *et al*., 2012a).

El conocimiento de la distribución de la infección canina es fundamental desde el punto de vista epidemiológico, ya que la transmisión de *Dirofilaria* en un área dada depende de la existencia de perros parasitados que actúen como reservorios de la enfermedad (Figura 5) (Simón *et al*., 2012). En comparación con los numerosos estudios llevados a cabo en perros, la información disponible sobre la distribución de la dirofilariosis felina es bastante escasa. No obstante, las infecciones felinas por *D. immitis* tienden a detectarse en las mismas áreas que la dirofilariosis canina, si bien con prevalencias entre el 5 y el 20% de las que presentan los perros en las mismas áreas (Newcombe y Ryan, 2002). Con respecto a la dirofilariosis humana, puesto que algunos vectores de la enfermedad se alimentan indistintamente de reservorios animales o de humanos, en áreas donde la dirofilariosis canina es endémica, la dirofilariosis pulmonar humana existe y puede suponer un problema desde el punto de vista médico. Además, debido tanto a su carácter asintomático, como a su localización en órganos internos, la revisión retrospectiva de los casos publicados no proporciona una distribución real de la enfermedad. En los últimos años, la mejora en las herramientas diagnósticas, junto con el creciente interés de la comunidad científica ha permitido aumentar notablemente el número de casos denunciados.

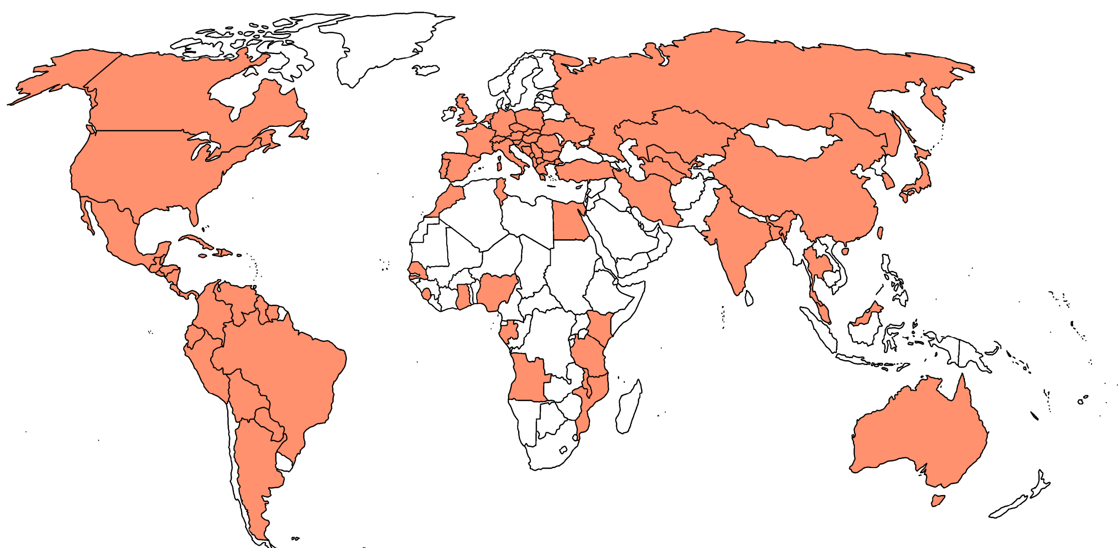

Figura 5. Distribución geográfica de los casos de dirofilariosis cardiopulmonar canina denunciados en el mundo.

3.1. Distribución de la dirofilariosis cardiopulmonar canina

3.1.1. América

La dirofilariosis canina ha sido descrita en perros de prácticamente todo el continente americano a excepción de Chile donde no fue encontrada, y en la Guayana Francesa y Uruguay donde no se han realizado estudios (Labarthe y Guerrero, 2005; Guerrero *et al*., 2006; Lee *et al*., 2010). Las prevalencias varían dependiendo de la climatología: Canadá (0,24%) (Slocombe y Villeneuve, 1993), Costa del Golfo de Estados Unidos (48,8%) (Levy *et al*., 2011), Estado de Amazonas en Brasil (57,6%) (Soares *et al*., 2014), Cuba (63,2%) (Duménigo *et al*., 1988) o zonas rurales del norte de Argentina (74%) (Vezzani *et al*., 2006).

En Estados Unidos, los mapas de incidencia realizados por la *American Heartworm Society* desde el año 2001 han permitido conocer, además, la dinámica de la distribución. Se ha observado que la dirofilariosis se ha extendido desde los estados de las costas Atlántica y del Golfo hasta los más occidentales, encontrándose actualmente en todos e incrementando su prevalencia en la mayor parte de ellos, aunque la transmisión en Alaska no ha sido bien documentada (Nelson *et al*., 2005; Guerrero *et al*., 2006; Lee *et al*., 2010).

3.1.2. Europa

La presencia del parásito en Europa ha sido históricamente denunciada en países de la cuenca mediterránea y del sur, como Grecia, Italia, Francia, España y Portugal en los que la enfermedad es endémica. En Grecia se han denunciado prevalencias en torno al 10-15% por todo el territorio (Haralampidis, 2003). En Italia *D. immitis* presenta prevalencias superiores al 50% en el área hiperendémica del valle del río Po, al norte del país (Genchi *et al*., 2001), mientras que en Francia se localiza principalmente en el sur, a lo largo de la costa Mediterránea (Doby *et al*., 1986; Guerrero *et al*., 1992). En España la distribución de la dirofilariosis canina se reparte zonalmente por todo el territorio, aunque es especialmente prevalente en zonas asociadas a regadíos como la ribera del Tormes en Salamanca (33,3%), delta del río Ebro (35,8) o Huelva (36,7%). También se han observado altas prevalencias en las islas, donde la influencia marina proporciona la humedad adecuada para las poblaciones de mosquitos, como la isla de Gran Canaria (23,87%) o Ibiza (39%) (Guerrero *et al*., 1989; Montoya *et al*., 2007; Simón *et al*., 2014).

En Portugal se han denunciado prevalencias medias en torno al 15-20% a lo largo de todo el país, encontrándose la más alta en la isla de Madeira (30%) (Araujo, 1996; Genchi *et al*., 2001; Vieira *et al*., 2014a y b).

Por otra parte, un gran número de estudios llevados a cabo en la última década demuestra que la dirofilariosis canina se ha expandido hacia países del centro y norte del continente, donde anteriormente no se conocía o donde solo se habían encontrado casos esporádicos (Morchón *et al*., 2012a; Simón *et al*., 2012). Esto ha permitido denunciar recientemente la presencia de *D. immitis* en países como Alemania, Ucrania o Rusia (Kartashev *et al*., 2011; Hamel *et al*., 2013; Genchi *et al*., 2014).

3.1.3. África

La escasez de estudios epidemiológicos realizados en el continente africano junto con la variedad de los métodos de diagnóstico empleados y el número de especies de filarias existentes, no permiten conocer la distribución real de la dirofilariosis canina en África (Simón *et al*., 2012). Datos revisados por Genchi *et al*. (2001) han permitido reportar la presencia del parásito en Marruecos, Túnez, Egipto, Tanzania, Kenia, Mozambique, Malawi, Senegal, Angola, Gabón, Nigeria y Sierra Leona. Más recientemente se ha descrito la enfermedad en perros de zonas urbanas en Ghana (Clarke *et al*., 2014).

3.1.4. Asia y Australia

Un número mayor de estudios, aunque realizados de manera esporádica, ha permitido localizar la presencia de *D. immitis* en muchas regiones de Asia y Australia, con prevalencias variables. En Irán la dirofilariosis canina se reparte por todo el territorio, alcanzando prevalencias del 51,4% en las zonas más húmedas del norte (Khedri *et al*., 2014) y así mismo, en la India se localiza principalmente en el noreste del país (Chakravarty y Chaudhuri, 1983; Patnaik, 1989). En la parte más oriental del continente se han denunciado prevalencias elevadas del parásito en China (21,6%) (Sun *et al*., 2012), Taiwan (57%) (Wu y Fan, 2003), Japón (59%) (Tanaka *et al*., 1985), Corea del Sur (69,5%) (Song *et al*., 2003) y Malasia (70%) (Lok, 1988). En Australia, *D. immitis* es endémica a lo largo de las áreas costeras del norte y oeste, así como en la parte oriental de los estados de Queensland, Nueva Gales del Sur y Victoria (Kendall *et al*., 1991; Bidgood y Collins, 1996).

4. LAS RELACIONES PARÁSITO/HOSPEDADOR EN LA DIROFILARIOSIS

El conocimiento de los mecanismos que rigen las interacciones entre los parásitos y sus hospedadores, así como la implicación de los tejidos del hospedador y sus alteraciones patológicas es fundamental para establecer las pautas adecuadas de manejo y control de las parasitosis. Estas relaciones resultan especialmente complejas en la dirofilariosis como consecuencia de dos factores: la capacidad de *D. immitis* para infectar distintos hospedadores en los que el parásito muestra diversos grados de adaptación y desarrollo; y la presencia de la bacteria simbionte *Wolbachia* que afecta a la inmunidad desarrollada por el hospedador, a la patogenia y consecuentemente al cuadro clínico de la enfermedad (Simón et *al*., 2009).

Pese a que la patología en general y la patología vascular en particular de la dirofilariosis cardiopulmonar han sido ampliamente descritas, los estudios sobre las relaciones que establece *D. immitis* con sus hospedadores a nivel molecular (mecanismos patogénicos, respuesta inmune y estrategias de supervivencia) son relativamente escasos.

4.1. Tejidos del hospedador afectados por la patología vascular. La pared arterial

Las arterias pulmonares lobares constituyen la localización definitiva primaria de los vermes adultos de *D. immitis*. Allí los parásitos pueden sobrevivir durante largos períodos de tiempo (más de 7 años), en números que pueden variar entre uno y más de 250 individuos. La pared arterial es, por tanto, el tejido del hospedador con el que el parásito interacciona de manera inmediata, estableciendo un amplio intercambio molecular y causando las primeras y algunas de las más graves alteraciones patológicas de la dirofilariosis cardiopulmonar.

La pared de las arterias pulmonares, cuya estructura ha sido recientemente revisada en profundidad por Townsley (2012), presenta, al igual que la de los demás vasos sanguíneos, tres capas concéntricas (Figura 6). La túnica íntima está formada por un revestimiento de células endoteliales dispuestas longitudinalmente en contacto directo

con la sangre, la membrana basal y una región de tejido conectivo subendotelial donde se pueden encontrar células musculares lisas de manera esporádica. A continuación y separada de la íntima por una lámina elástica interna se encuentra la túnica media. Es la capa de mayor grosor y está compuesta principalmente por células musculares lisas que se disponen concéntricamente y están embebidas en una matriz extracelular rica en colágenos, elastina, fibrilina y proteoglicanos. Finalmente la túnica adventicia es la capa más alejada del lumen vascular y consta principalmente de fibroblastos dispuestos longitudinalmente dentro de una matriz que contiene colágeno y elastina separada del medio externo por una lámina elástica (Figura 7).

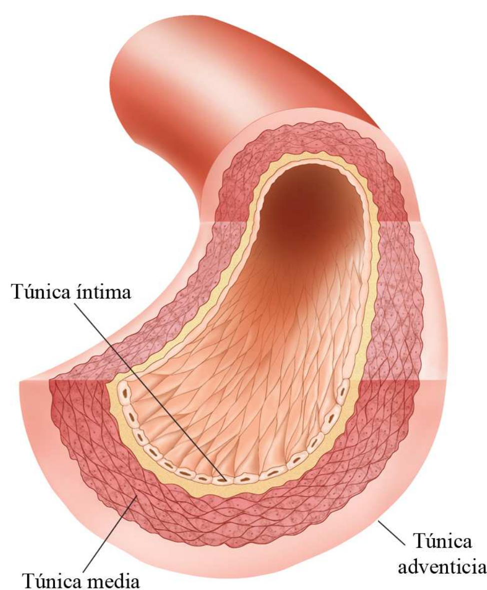

Figura 6. Imagen representativa de la disposición en capas de una arteria. Tomada de Encyclopædia Britannica Online. <http://global.britannica.com/EBchecked/media/95216>

Pese a esta descripción de la pared arterial constituida por tres capas estructuralmente distintas, la evidencia indica que los componentes celulares y extracelulares de estas capas están ampliamente interconectados, de tal manera que los límites que se han marcado tradicionalmente son inciertos. Esto es particularmente evidente si se considera que dichas interconexiones juegan un papel clave en la capacidad funcional de la pared arterial. Así, a lo largo de gran parte de la red arterial pulmonar, la lámina elástica interna se compone de membranas fenestradas que permiten el desarrollo de proyecciones endoteliales hacia la túnica media facilitando la comunicación entre el endotelio y el músculo liso perivascular. Estas interacciones célula-célula se consideran esenciales para la integración local de la vasoconstricción y de la vasodilatación, así como para la coordinación de las respuestas vasculares en la red de vasos interconectados (Martinez-Lemus, 2012; Townsley, 2012).

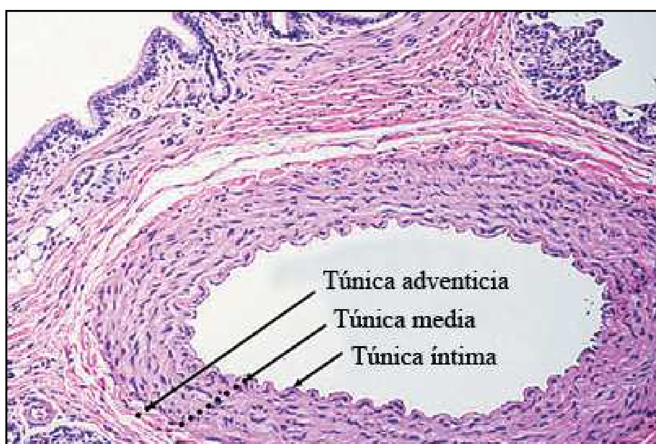

Figura 7. Corte histológico mostrando una arteria pulmonar normal canina. La túnica íntima, media y adventicia muestran un grosor y estructura celular normal. Tinción: hematoxilina-eosina. Aumento: 10X (Quinn y Williams, 2011).

4.1.1. El endotelio vascular

El endotelio es una monocapa continua formada por células unidas a la vez entre sí y a la membrana basal subyacente. Las células endoteliales son células polarizadas, con un dominio apical en contacto con la sangre y uno distal basal en contacto con el subendotelio (Dejana *et al*., 1995). El endotelio vascular está considerado el principal órgano de regulación de numerosas funciones vasculares teniendo, además, un papel clave en la regulación de la homeostasis al constituir la interfase entre la sangre y los tejidos. Cualquier alteración tanto en la composición de la sangre como en el flujo sanguíneo puede convertir un endotelio sano con características antitrombóticas, antiinflamatorias y vasodilatadoras en un tejido donde predomine la coagulación, la inflamación y la vasoconstricción (Michiels, 2003).

4.1.2. El músculo liso perivascular

El músculo liso perivascular constituye el componente mayoritario de la túnica media y se presenta generalmente en forma de haces de células musculares lisas de apariencia fusiforme y con un núcleo alargado. Estas se encuentran rodeadas de una lámina externa e interconectadas por uniones en hendidura. La principal función de las células musculares lisas dentro de la túnica media es la de controlar el diámetro vascular mediante los procesos de contracción y relajación celular. Para ello se disponen concéntrica y perpendicularmente al eje longitudinal del vaso (Martinez-Lemus, 2012; Townsley, 2012). Además, estas células juegan un papel fundamental en otros procesos fisiológicos como el mantenimiento del tono vascular, reparación, cicatrización y desarrollo arterial, así como en las principales enfermedades vasculares, incluyendo la

formación de la placa de ateroma (Schwartz, 1997). Las células musculares lisas vasculares tienen la capacidad de modular su fenotipo o estado de diferenciación. Por ello, en arterias adultas intactas, las células musculares son normalmente quiescentes y presentan un estado diferenciado conocido como fenotipo contráctil, el cual es esencial para la estabilidad hemodinámica. En contraste, la formación de lesiones derivadas de aterosclerosis o estenosis implica la diferenciación de las células musculares lisas a un estado conocido como fenotipo sintético. Este proceso incluye una reorganización estructural visible dentro de las células que permite aumentar su capacidad para proliferar, migrar hacia la túnica íntima y secretar componentes de la matriz extracelular (Dupont *et al*., 2005).

4.1.3. La matriz extracelular

La matriz extracelular constituye uno de los mayores componentes de los vasos sanguíneos, suponiendo más de la mitad de la masa de la pared de arterias y venas (Hungerford y Little, 1999). Pese a estar formada principalmente por colágenos y elastinas, otros componentes como la fibronectina, microfibrillas (principalmente fibrilinas) y abundante material soluble o amorfo como proteoglicanos y glicoproteínas pequeñas ricas en leucina están presentes entre los espacios extracelulares de la pared de los vasos, siendo cruciales para su integridad (Bou-Gharios *et al*., 2004).

La matriz extracelular define las propiedades mecánicas críticas para una correcta función del sistema vascular. Además, el adecuado balance entre su producción y degradación es crucial para el mantenimiento de la estructura del tejido, así como para su desarrollo y reparación (Wagenseil y Mecham, 2009). Los diferentes tipos celulares de los vasos sanguíneos son los encargados de la síntesis de los componentes de la matriz extracelular. Se asume que las células endoteliales son las principales responsables de la síntesis y deposición de los componentes de la matriz extracelular de la túnica íntima (Davis y Senger, 2005). En la túnica media el material extracelular, incluido el colágeno y las fibras elásticas es producido principalmente por las células del músculo liso durante el desarrollo, mientras que en la túnica adventicia, el colágeno es sintetizado y secretado por los fibroblastos, como en otros tejidos conectivos (Bou-Gharios *et al*., 2004; Wagenseil y Mecham, 2009).

La degradación de la matriz extracelular es realizada por un grupo de enzimas conocidas como metaloproteasas de matriz que incluye colagenasas, gelatinasas, estromelisinas, metaloelastasas, así como cualquier otra proteína capaz de degradar específicamente algún componente de la matriz extracelular (Galis y Khatri, 2002). Estas enzimas, cuya síntesis es realizada por las células endoteliales, musculares lisas y por los fibroblastos, son capaces de degradar completamente la matriz extracelular y, por tanto, sus mecanismos de acción han de ser estrictamente regulados (Bou-Gharios *et al*., 2004). Además, puesto que otras muchas moléculas que no forman parte de la matriz extracelular son sustratos potenciales de las metaloproteasas, su actividad puede afectar a procesos tan importantes como la migración celular, diferenciación, crecimiento, procesos inflamatorios, neovascularización o apoptosis (Nagase *et al*., 2006).

4.2. Características clínicas de la dirofilariosis cardiopulmonar

La dirofilariosis cardiopulmonar está descrita en el perro, principal hospedador de *D. immitis*, como una enfermedad grave, que potencialmente puede causar la muerte del hospedador. Presenta un desarrollo generalmente crónico muy complejo, afectando progresivamente al sistema vascular, al parénquima pulmonar y en sus últimas fases, a las cámaras derechas del corazón (Furlanello *et al*., 1998; Venco, 2007; McCall *et al*., 2008). Los daños provocados en el hospedador se atribuyen principalmente a los vermes adultos. Estos, localizados en las arterias pulmonares, inducen las primeras lesiones en las paredes de los vasos, hecho clave para la evolución de la enfermedad y para el desarrollo posterior de la patología pulmonar y cardíaca (Simón *et al*., 2012) (Figura 8).

4.2.1. Daños vasculares

Con la llegada de los vermes a las arterias pulmonares comienzan los primeros daños en el tejido que está en contacto con los parásitos, el endotelio. Como respuesta al trauma mecánico, se producen cambios anatómicos en la pared arterial, aumentando el tamaño tanto de las células endoteliales como el de los espacios intracelulares, a la vez que se desorientan los ejes longitudinales de las células (Venco y Vezzoni, 2001). La desorganización del endotelio facilita la infiltración de células inflamatorias, principalmente neutrófilos, hacia los espacios perivasculares. Por otra parte, la exposición del subendotelio favorece la activación plaquetaria. Todo ello desemboca en un proceso

de endarteritis proliferativa, fenómeno de hiperplasia característico de la dirofilariosis cardiopulmonar (Figura 9).

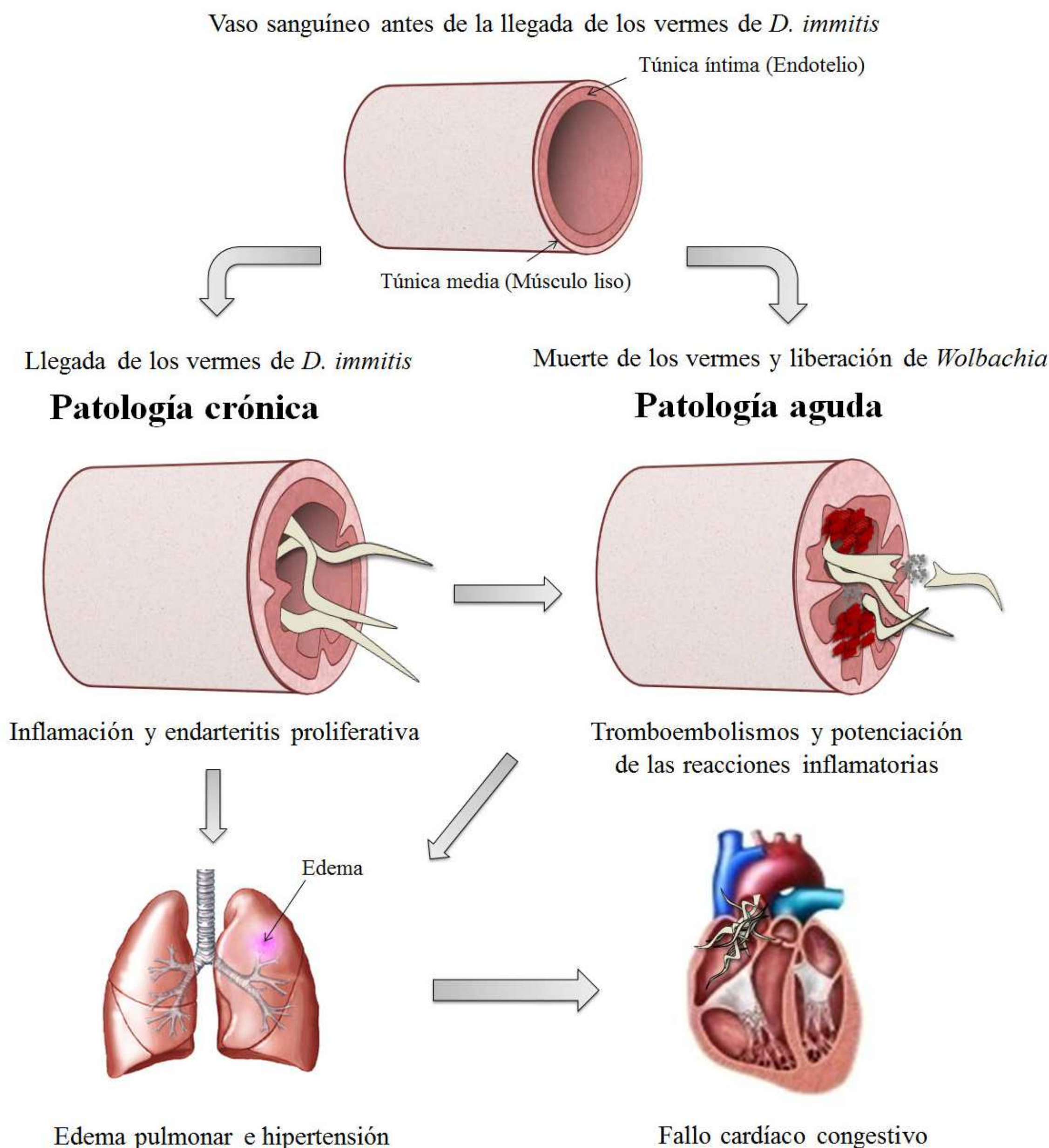

Figura 8. Progresión de la patología de la dirofilariosis cardiopulmonar. La enfermedad tiene habitualmente un curso crónico afectando inicialmente al sistema vascular, para luego extenderse al tejido pulmonar y a las cámaras derechas del corazón. La muerte simultánea de un gran número de vermes adultos puede desencadenar la forma aguda de la enfermedad (Simón *et al*., 2012).

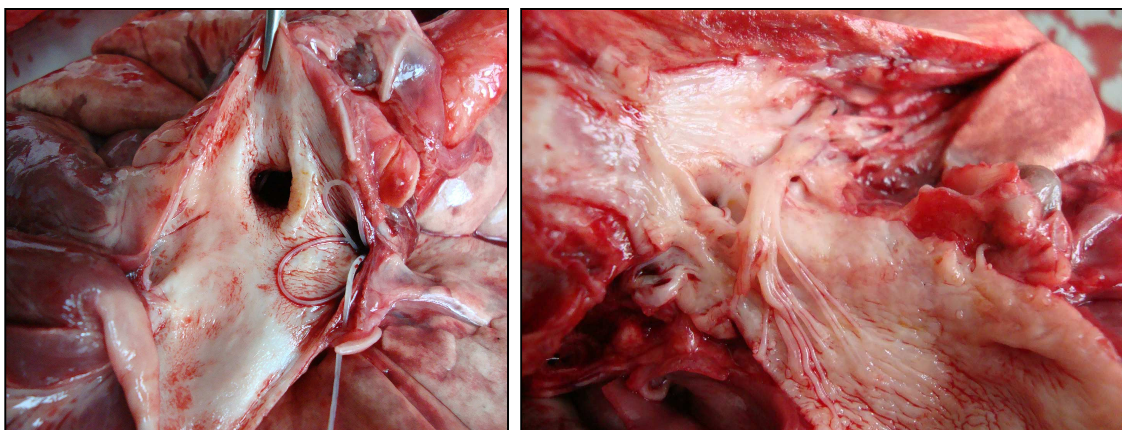

Figura 9. Imágenes representativas de la presencia de endarteritis proliferativa en la arteria pulmonar de un perro con dirofilariosis. Se puede observar el endotelio engrosado con presencia de vellosidades y coloración purpúrea del mismo (Carretón *et al*., 2012).

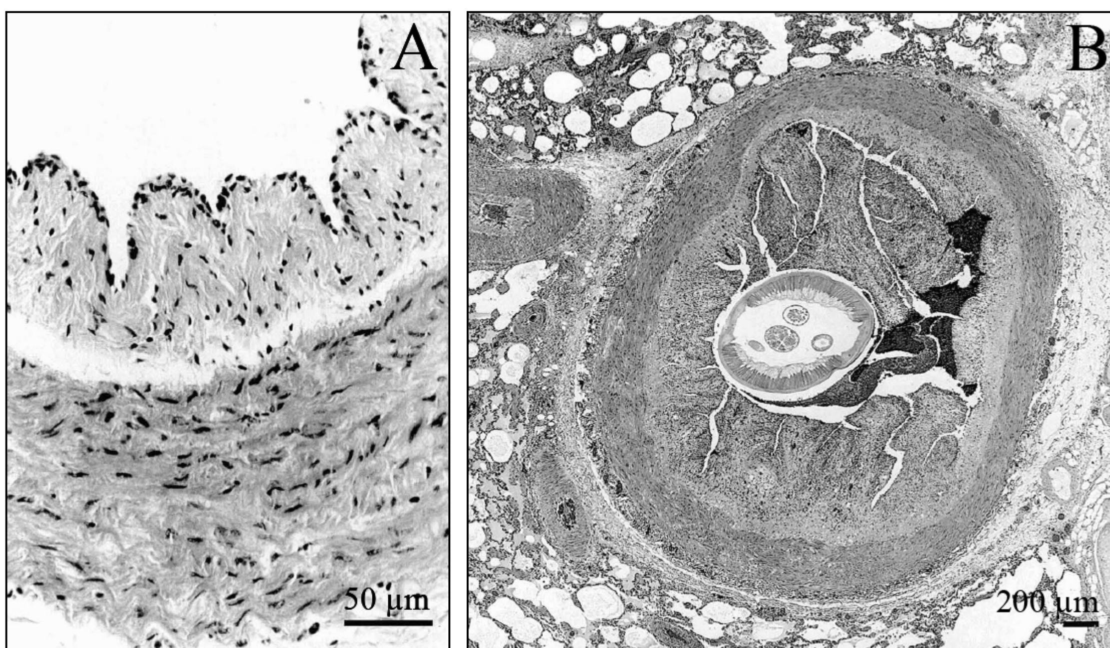

Figura 10. Endarteritis proliferativa. (A) Sección de la arteria pulmonar de un gato con dirofilariosis donde se pueden observar numerosas microvellosidades debido al engrosamiento de la túnica íntima y a la hiperplasia de las células endoteliales. (B) Arteria pulmonar de un gato con dirofilariosis donde se aprecia un verme de *D. immitis* rodeado en parte por un trombo. Se observa una gran proliferación vellosa con infiltración de células inflamatorias. Tinción: hematoxilina-eosina (McCracken y Patton, 1993).

La endarteritis proliferativa está causada por la proliferación y migración de células de la pared arterial hacia el lumen vascular, por lo que la pared de la arteria deja de ser lisa y blanca y desarrolla microvellosidades intravasculares, que dan a la superficie de la arteria pulmonar un aspecto rugoso y una tonalidad púrpura (Venco, 2007; Carretón *et al*., 2012). Se ha descrito que este proceso va acompañado de proliferación y migración, tanto de células endoteliales como del músculo liso perivascular (Adcock, 1961; Atwell *et al*., 1986; Hidaka *et al*., 2004; Kawabata *et al*., 2008), así como de degradación de la matriz extracelular (Wang *et al*., 2005) (Figura 10).

Además, se pierde la capacidad de contracción/relajación de la pared arterial, las arterias lesionadas pierden elasticidad, se vuelven tortuosas y aunque sufren dilatación, la luz disminuye debido al engrosamiento provocado por la formación de microvellosidades. Esto puede provocar oclusión de las arteriolas más estrechas por embolización (Kaiser *et al*., 1989; Carretón *et al*., 2012).

4.2.2. Desarrollo posterior de la enfermedad

La patología pulmonar aparece de manera secundaria, provocada por los cambios vasculares. El aumento de la permeabilidad vascular, la disminución del calibre de los vasos y las obstrucciones que alteran el flujo sanguíneo conducen a un aumento de la presión en la arteria pulmonar, produciéndose hipertensión pulmonar (Venco y Vezzoni, 2001; Venco *et al*., 2011). Esta, aunque suele ser moderada, puede triplicarse en la arteria pulmonar durante el ejercicio debido a la pérdida de elasticidad de la pared arterial (Kittleson, 1998). Por otra parte, la salida de líquido y antígenos parasitarios desde los vasos dañados hacia el parénquima pulmonar perivascular provoca edema e inflamación (Figura 11A). La presencia de eosinófilos y neutrófilos conforma infiltrados intersticiales y alveolares que provocan una fibrosis irreversible y esta a su vez, la disminución del área de intercambio gaseoso y el aumento de la resistencia vascular pulmonar (Rawlings, 1986). Además, la aparición de enfisema y de hipertrofia de la musculatura bronquial asociada también ha sido descrita (Dillon *et al*., 1995; Venco *et al*., 2011).

En animales sometidos a ejercicio físico y con una gran carga parasitaria, el estado de hipertensión pulmonar provoca una dilatación del ventrículo derecho acompañada de hipertrofia compensatoria para mantener la alta presión de perfusión y mover la sangre a los pulmones (Venco, 2007; Wang *et al*., 2005) (Figura 11B). La persistencia de la

hipertensión pulmonar, junto con el aumento del ritmo cardíaco producido por el ejercicio o por fenómenos tromboembólicos, puede generar una dilatación irreversible de la parte derecha del corazón, determinando una insuficiencia cardíaca congestiva. Diferentes complicaciones como edema miocárdico, fibrosis, isquemia o insuficiencia valvular como consecuencia del agrandamiento de las cámaras atrio-ventriculares o por la presencia de los vermes en el interior del corazón pueden agravar finalmente la situación (Venco y Vezzoni, 2001; Venco, 2007). Además de la patología cardiopulmonar, la dirofilariosis puede causar alteraciones en órganos y tejidos tan dispares como el riñón, el hígado, el cerebro, la cámara anterior del ojo o la cavidad peritoneal, debido entre otras cosas a localizaciones ectópicas o aberrantes del parásito (McCall *et al*., 2008).

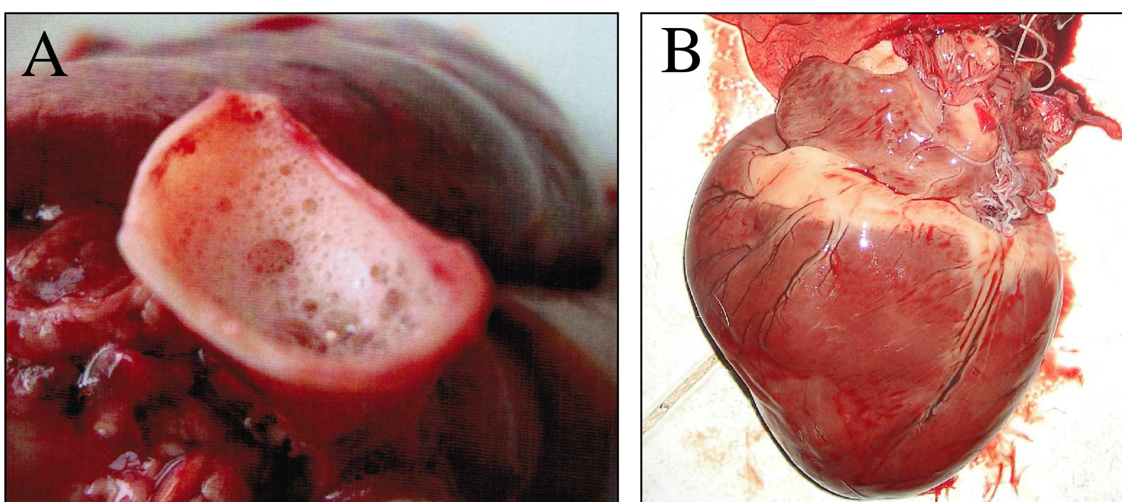

Figura 11. (A) Edema pulmonar en un perro con dirofilariosis (Carretón et al., 2012). (B) Cardiomegalia con dilatación de las cámaras cardíacas derechas del corazón en un perro con dirofilariosis. Se puede observar la presencia de vermes adultos de *D. immitis* saliendo a través del tronco de la arteria pulmonar (Carretón et al., 2012).

4.2.3. Patología aguda

De forma paralela al desarrollo crónico de la enfermedad descrito hasta el momento, pueden producirse procesos agudos que suponen un riesgo inmediato para la vida de los animales que los padecen (Venco, 2007). Aparecen cuando se produce la muerte súbita y simultánea de muchos vermes adultos, de manera natural o como consecuencia de un tratamiento filaricida. Los parásitos vivos tienen capacidad para controlar la formación de trombos, pero cuando mueren, la liberación masiva de productos antigénicos al torrente circulatorio produce una trombosis masiva y una

exacerbación de las reacciones inflamatorias en el endotelio vascular (Venco y Vezzoni, 2001) (Figura 12). Los vermes muertos son arrastrados distalmente hacia las arterias más finas, mientras que los fragmentos del parásito se calcifican y son parcialmente incorporados a la pared de la arteria, con la posterior cicatrización y formación de gran cantidad de tejido conectivo fibroso. Todo ello provoca un grave deterioro del flujo sanguíneo, hipoxia e inflamación granulomatosa de la pared arterial con un crecimiento exagerado de las vellosidades y un aumento de la permeabilidad, con la formación de edemas perivasculares (Carretón *et al*., 2012).

Por último, dentro de la forma aguda de la enfermedad, destaca el síndrome de la vena cava. Esta variante clínica suele presentarse en animales de pequeño tamaño y con una alta carga parasitaria, se presenta de improviso y su pronóstico es de reservado a grave, presentando una alta mortalidad. Consiste en la acumulación masiva de vermes adultos en el atrio ventricular derecho. Las turbulencias que aparecen en el torrente sanguíneo inducen daños mecánicos en la pared de los eritrocitos provocando hemólisis. Además, la masa de parásitos se opone al retorno venoso al corazón, lo que provoca un estado de shock cardiocirculatorio (Kitagawa *et al*., 1987; Furlanello *et al*., 1998; Carretón *et al*., 2012).

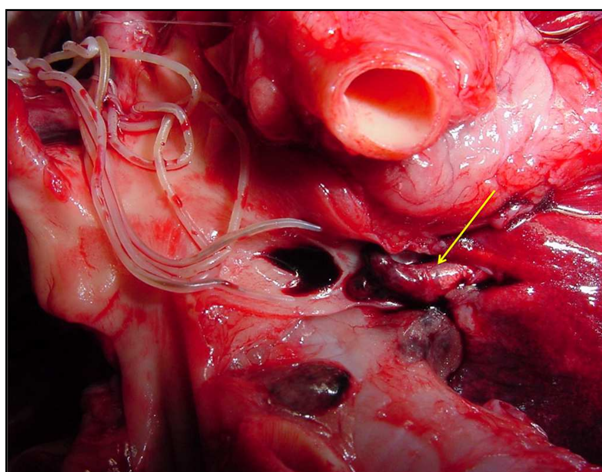

Figura 12. Tromboembolismo (flecha amarilla) localizado en la arteria pulmonar de un perro que murió de dirofilariosis cardiopulmonar (Cortesía de L. Venco, Ospedale Veterinario "Città di Pavia", Pavia, Italia).

4.2.4. Sintomatología

En la dirofilariosis cardiopulmonar canina, los síntomas, en general, van apareciendo progresivamente en consonancia con el desarrollo crónico habitual de la enfermedad. La mayor parte de los perros infectados no presenta síntomas durante meses o años, y estos dependen de la carga parasitaria, la reactividad individual y el ejercicio físico al que son sometidos (Dillon *et al*., 1995). Cuando aparecen los síntomas, estos incluyen tos, disnea y taquipnea, intolerancia al ejercicio, pérdida de peso, síncope, hemoptisis o epistaxis y ascitis (Figura 13). Además, aparecen sonidos pulmonares en los

lóbulos caudales, arritmias y ruidos cardíacos por insuficiencia de la tricúspide. Cuando se desarrolla la insuficiencia cardíaca congestiva derecha se presenta, además, pulso venoso yugular e ingurgitación de las yugulares, hepatomegalia, edema pulmonar y derrame pleural (Carretón *et al*., 2012).

En relación con la variante aguda de la enfermedad y ante la aparición de tromboembolismos tras la muerte masiva de vermes adultos de *D. immitis*, los perros pueden mostrar disnea y hemoptisis potencialmente fatales. La muerte súbita es rara pero puede sobrevenir como consecuencia de una insuficiencia respiratoria, caquecsia o fenómenos tromboembólicos graves (Venco y Vezzoni, 2001). El síndrome de vena cava se manifiesta con signos de colapso cardiovascular, disnea, soplo cardíaco y hemoglobinuria debida a hemólisis mecánica como consecuencia de las turbulencias causadas por la masa de vermes al dificultar el flujo sanguíneo (Venco *et al*., 2011).

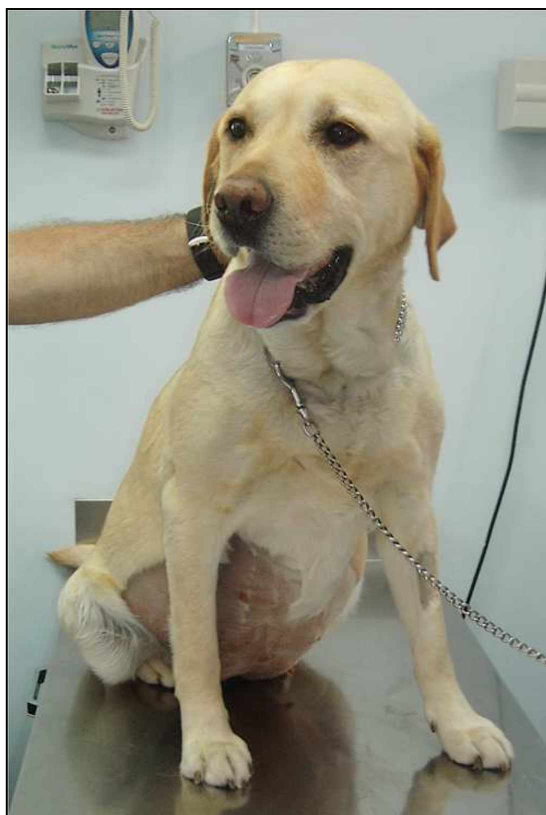

Figura 13. Ascitis en un perro con dirofilariosis (Cortesía de J. A. Montoya-Alonso, Facultad de Veterinaria, Universidad de Las Palmas de Gran Canaria, Las Palmas de Gran Canaria, España).

4.3. Mecanismos patogénicos

Históricamente, la patogénesis de la dirofilariosis ha sido atribuida a la presencia física de los vermes adultos en las arterias pulmonares de los hospedadores. Sin embargo, la complejidad del cuadro clínico de la dirofilariosis cardiopulmonar no puede explicarse sin otros factores relacionados con el parásito y sus moléculas excretadas. La idea de explicar los mecanismos patogénicos de la dirofilariosis a través de la alteración de la función fisiológica del endotelio causada por productos excretados por las filarias, fue postulada en los años 80 y 90 del pasado siglo por un equipo multidisciplinar de la Universidad de Michigan (EEUU). Estos investigadores propusieron que los vermes adultos de *D. immitis* contribuyen a la alteración de la capacidad de relajación de las arterias pulmonares. Dicho proceso estaría mediado por la liberación de productos parasitarios que cambian el comportamiento de las células endoteliales vasculares, pero no del músculo liso asociado. Identificaron al óxido nítrico (NO), los productos de la actividad de una ciclooxigenasa (que no fueron especificados), la prostaglandina D2 y la histamina como las moléculas responsables de dicha alteración (Kaiser *et al*., 1989 y 1992; Mupanomunda *et al*., 1997; Kaiser y Williams, 1998).

En el mismo sentido, no se conocen con exactitud los mecanismos patogénicos que provocan la formación de microvellosidades. Se ha postulado que el daño mecánico causado por la sola presencia de los vermes adultos en las arterias pulmonares podría participar en el proceso patológico (Atwell *et al*., 1985). También se ha señalado que la pared arterial podría responder a sustancias activas o factores de crecimiento secretados por las plaquetas que invaden el espacio perivascular, como consecuencia del daño en la superficie arterial. Otros dos mecanismos que podrían contribuir a la progresión de la endarteritis proliferativa son, en primer lugar, las reacciones antígeno-anticuerpo que activan el complemento, alterando la función endotelial y aumentando la permeabilidad vascular, y, en segundo lugar, el aumento de la lipoproteína de baja densidad (LDL) en el plasma, lo que contribuiría a estimular la proliferación de las células del músculo liso hacia la íntima (Furlanello *et al*., 1998; Venco, 2007; Venco *et al*., 2011). No obstante, puesto que la gravedad de la proliferación vellosa está directamente relacionada con la duración de la infección y la carga parasitaria (Venco, 2007), es posible que productos excretados por el parásito puedan influir en el proceso.

Otra gran parte de los hallazgos realizados sobre los mecanismos patogénicos de la dirofilariosis cardiopulmonar se han centrado en la inmunopatología de la enfermedad (Figura 14). Los datos obtenidos indican que los hospedadores infectados o inmunizados con *D. immitis* desarrollan una respuesta inmune dual Th_1/Th_2 (Marcos-Atxutegui *et al*., 2003; Kramer *et al*., 2005). La respuesta de tipo Th_1, proinflamatoria, es estimulada por la bacteria simbionte *Wolbachia* e induce la expresión del ARNm de la óxido nítrico sintasa inducible (iNOS), del interferón gamma (IFN-γ), así como la producción de NO y de anticuerpos IgG2a. La respuesta de tipo Th_2, antiinflamatoria, está dirigida preferentemente contra los antígenos de *D. immitis* (Marcos-Atxutegui *et al*., 2003; Morchón *et al*., 2007a y b). Esta polarización de la respuesta inmune se ha observado también en infecciones naturales. Una respuesta de tipo Th_2, caracterizada por una intensa expresión del ARNm de las citoquinas IL-4 e IL-10 y la producción de IgG, se ha descrito en infecciones caninas microfilarémicas. Una respuesta de tipo Th_1, caracterizada por la ausencia de expresión de IL-10 y una intensa expresión de iNOS y la producción de IgG2, predomina en las infecciones caninas amicrofilarémicas. Estos datos sugieren que las microfilarias circulantes pueden estimular una respuesta de tipo Th_2 ineficiente, permitiendo la supervivencia a largo plazo de los vermes adultos (Morchón *et al*., 2007b).

El descubrimiento de que *Wolbachia* es clave para el desarrollo de las reacciones inflamatorias en la dirofilariosis ha supuesto un gran avance en este aspecto. Se ha demostrado que la mayor parte de los pacientes con dirofilariosis pulmonar presenta una respuesta exclusiva de IgG1 (Th_1) contra la proteína mayoritaria de la cubierta de *Wolbachia* (WSP) (Simón *et al*., 2003; Simón *et al*., 2007). Otros trabajos indican que la WSP activa la quimiotaxis de neutrófilos (Bazzocchi *et al*., 2003) y que es capaz de inhibir su apoptosis *in vitro*, lo que podría contribuir a prolongar la reacción inflamatoria (Bazzocchi *et al*., 2007). Además, se ha demostrado en otras filariosis que *Wolbachia* interacciona con los macrófagos a través de la familia de receptores *Toll-like* (TLR2, TLR4 o TLR6) (Brattig *et al*., 2004; Turner *et al*., 2009). Por otra parte, cultivos de células endoteliales estimulados con WSP mostraron un aumento significativo en la expresión de los eicosanoides proinflamatorios, tromboxano B2 y leucotrieno B4, así como en la de las enzimas responsables de su síntesis (ciclooxigenasa-2 y 5-lipooxigenasa). Ambos eicosanoides alcanzaron niveles máximos en infecciones felinas experimentales a los 180 días post-infección, momento en el que comienzan las reacciones inflamatorias más graves (Morchón *et al*., 2007c). Además, el tromboxano B2 presenta elevados niveles

tanto en infecciones crónicas caninas y felinas, como en pacientes humanos con dirofilariosis pulmonar, lo que se correlaciona, en todos los casos, con elevados títulos de anticuerpos IgG anti-WSP, indicando una intensa liberación de *Wolbachia* por la destrucción de los vermes (Morchón *et al*., 2006, 2007c y 2009). Finalmente, la expresión de iNOS y NOS endotelial (eNOS), así como la de otras moléculas relacionadas tanto con la adhesión y transmigración de leucocitos (VCAM, ICAM y PECAM), como con la proliferación celular (E-cadherina y VEGF), también se vieron aumentadas en cultivos de células endoteliales estimulados con WSP (Morchón *et al*., 2008). No obstante, el hecho de que muchas de estas moléculas también se vean activadas en cultivos similares estimulados con antígenos somáticos parasitarios (Simón *et al*., 2008) o que filarias que carecen de *Wolbachia* también induzcan reacciones inflamatorias, parece sugerir que además de las bacterias, las propias filarias participan también en la generación de la patología inflamatoria de la dirofilariosis cardiopulmonar (Simón *et al*., 2012).

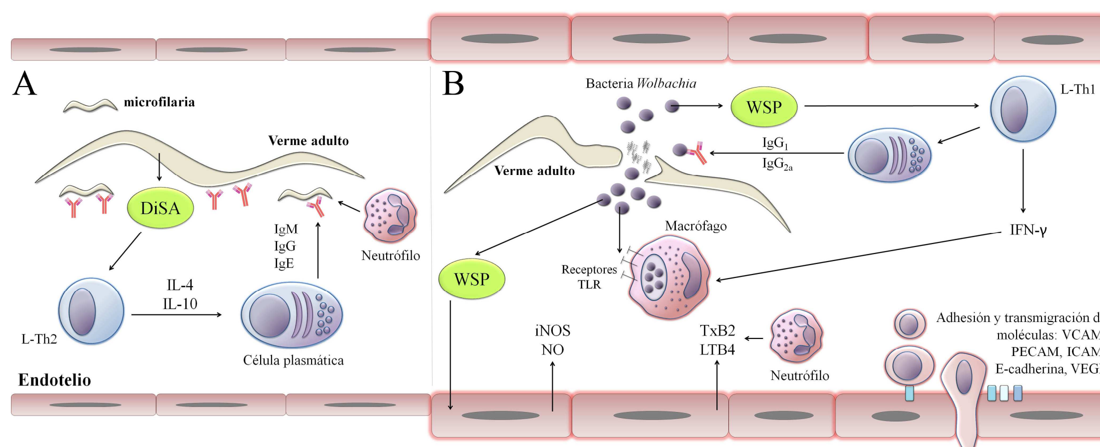

Figura 14. Mecanismos inmunopatogénicos en la dirofilariosis cardiopulmonar mediados por una respuesta inmune dual Th_1/Th_2. (A) La respuesta de tipo Th_2, antiinflamatoria, es estimulada por los antígenos de *D. immitis* y la presencia de microfilarias. Se incrementa la expresión de IL-4 e IL-10, así como los niveles de anticuerpos relacionados con la respuesta Th_2, IgG (en perros) o IgE (en humanos). (B) La respuesta de tipo Th_1, proinflamatoria, es estimulada por las bacterias *Wolbachia* liberadas a partir de vermes muertos. La proteína mayoritaria de la superficie de *Wolbachia* (WSP) estimula la producción de IFN-γ, interacciona con macrófagos (probablemente a través de receptores TLR) e inhibe su apoptosis. *Wolbachia* estimula además la producción de anticuerpos típicos de la respuesta Th_1 y la expresión de mediadores proinflamatorios en células endoteliales vasculares. En estas mismas células los niveles de expresión de moléculas de adhesión/transmigración (VCAM, PECAM, ICAM, E-cadherina y VEGF) también se ven aumentados. Algunos de estos estímulos también son producidos por un extracto de antígenos somáticos de *D. immitis* (DiSA) (Simón *et al*., 2012).

4.4. Respuesta inmune

El sistema inmune es una sofisticada y compleja herramienta que ha evolucionado para destruir a los patógenos invasores (Sorci *et al*., 2013). Sin embargo, frente a la fuerte respuesta inmune desarrollada por los hospedadores parasitados, los helmintos en particular son capaces de persistir en el hospedador, produciendo generalmente infecciones de tipo crónico (Moreau y Chauvin, 2010). En este sentido, el desarrollo de las larvas infectantes hasta vermes adultos en la dirofilariosis cardiopulmonar y el carácter crónico en la mayor parte de la infecciones, sugiere una escasa eficiencia de la respuesta inmune y/o una habilidad del parásito para evadir los mecanismos de control del hospedador. No obstante, está generalmente aceptado que el hospedador es capaz de controlar la carga parasitaria manteniéndola dentro de unos límites compatibles con su propia supervivencia, destruyendo una parte de las larvas adquiridas por las reinfecciones (Simón *et al*., 2001 y 2012).

En la dirofilariosis los datos experimentales parecen demostrar que la respuesta inmune está influenciada por la presencia de microfilarias y por el estatus clínico de los hospedadores infectados (Simón *et al*., 2012). En perros con dirofilariosis, la microfilaremia se ha relacionado con niveles significativamente superiores de anticuerpos IgG anti-*D. immitis* y anti-*Wolbachia* en comparación con lo que ocurre en infecciones amicrofilarémicas (Grieve *et al*., 1979; Morchón *et al*., 2007b; Marcos-Atxutegi *et al*., 2004). En el mismo sentido, una mayor respuesta de anticuerpos IgG anti-*Wolbachia* fue encontrada en la orina de perros microfilarémicos con glomerulonefritis asociada (Morchón *et al*., 2012b). Por otra parte, comparando ambos tipos de anticuerpos en perros amicrofilarémicos, la respuesta es mayor en aquellos con tromboembolismos pulmonares masivos que en los que no presentan sintomatología (Kramer *et al*., 2005; Simón *et al*., 2007).

En estudios llevados a cabo con gatos infectados experimentalmente se ha observado una respuesta IgG moderada y de corta duración solo en los 2 primeros meses post-infección contra los antígenos de L3 y una respuesta IgG intensa entre los 2 y los 6 meses post-infección contra los antígenos de los vermes adultos (Bazzocchi *et al*., 2000; Prieto *et al*., 2001 y 2002). Esta respuesta desciende en gatos tratados con ivermectina, mientras que las IgG anti-*Wolbachia* se incrementan, probablemente como consecuencia

de la muerte de las larvas y la liberación de *Wolbachia* al organismo del hospedador (Prieto *et al*., 2002).

En las infecciones humanas, pacientes diagnosticados de dirofilariosis pulmonar presentan una respuesta de IgG o IgM frente a los complejos antigénicos somático y excretor, además de niveles elevados de IgG anti-*Wolbachia* (Simón *et al*., 1991, Simón *et al*., 2003), mientras que en seropositivos asintomáticos (sin nódulos pulmonares) predominan las IgE y los anticuerpos anti-*Wolbachia* se mantienen en niveles más bajos (Sato *et al*., 1985; Espinoza *et al*., 1993; Simón *et al*., 2003).

4.5. Mecanismos de evasión y supervivencia

Pese a la grave patología que produce, *D. immitis* es capaz de sobrevivir durante años en el sistema vascular de su hospedador definitivo asegurando su reproducción, interaccionando con su entorno inmediato a través de los antígenos de la interfase parásito/hospedador. Por ello, la larga esperanza de vida de *D. immitis* puede ser considerada como un reflejo de la evolución de estrategias altamente efectivas relacionadas con sus mecanismos de evasión y supervivencia.

Los pocos datos disponibles muestran que cada fase evolutiva de *D. immitis* ha desarrollado diferentes estrategias para evadir la respuesta inmune del hospedador (Simón *et al*., 2001) (Figura 15). Se ha demostrado que las L3 eliminan entre el 10 y el 20% de su contenido antigénico de superficie (constituido principalmente por dos moléculas de 6 y 35 kDa), no siendo repuesto posteriormente. Esto permite ofrecer un bajo perfil antigénico difícil de detectar, en un estadio de corta duración pero de vital importancia para el establecimiento de la infección en el hospedador definitivo (Ibrahim *et al*., 1989). Por su parte, los vermes adultos expresan en su superficie glicolípidos no inmunógenos, y son capaces de retener plaquetas y adsorber albúmina, IgG y la fracción C3 del complemento como mecanismo de enmascaramiento y así evitar el reconocimiento de los antígenos cuticulares (Scott *et al*., 1988; Bilge *et al*., 1989; Kadispasaoglu y Bilge, 1989). Además, se han detectado proteasas con capacidad para hidrolizar anticuerpos IgG en la epicutícula de las microfilarias (Tamashiro *et al*., 1987). En cuanto a los productos secretados por *D. immitis*, se ha demostrado que la estimulación de cultivos de células endoteliales vasculares con los antígenos excretores/secretores del parásito produce un aumento significativo en la producción de prostaglandina E2 (PGE2), así como un

significativo descenso en la transmigración de monocitos (Morchón *et al*., 2010). La PGE2 es un eicosanoide derivado del ácido araquidónico que produce efectos inmunosupresores y antiinflamatorios (Liu y Weller, 1990), por lo que altos niveles de esta molécula pueden relacionarse con la supervivencia del parásito, lo que ha sido postulado también en otras especies de filarias (Liu, *et al*., 1990; Brattig *et al*., 2006).

Finalmente, como se ha señalado con anterioridad, los estudios proteómicos están generando abundante información sobre la composición proteica de *D. immitis*. El análisis mediante esta tecnología de los diferentes compartimentos antigénicos del parásito parece indicar que *D. immitis* posee un gran número de enzimas cuyas funciones están directamente relacionadas con su supervivencia. Diferentes isoformas de proteínas de choque térmico, peroxirredoxinas, moléculas antioxidantes y detoxificantes y enzimas implicadas en la generación de energía están ampliamente representadas en el repertorio antigénico del parásito (Oleaga *et al*., 2009; González-Miguel *et al*., 2010a y b; Geary *et al*., 2012; Morchón *et al*., 2014).

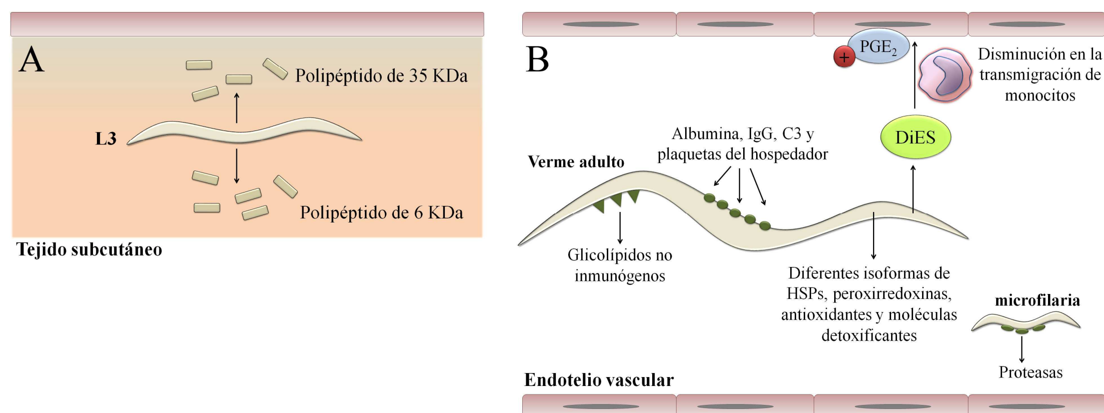

Figura 15. Mecanismos de evasión y supervivencia descritos en *D. immitis*. (A) Las larvas infectantes L3 evaden la respuesta inmune del hospedador liberando grandes cantidades de dos polipéptidos de superficie de 6 y 35 KDa. (B) En localización vascular, los vermes adultos pueden enmascarar su superficie mediante la adsorción de moléculas del hospedador. Además, poseen glicolípidos no inmunógenos y un gran número de isoformas de proteínas de choque térmico, peroxirredoxinas, antioxidantes y moléculas detoxificantes. En el endotelio sus productos de excreción/secreción estimulan la expresión del eicosanoide antiinflamatorio PGE2 y la disminución en la transmigración de monocitos. Las microfilarias poseen además proteasas de superficie con capacidad para digerir anticuerpos del hospedador (Simón *et al*., 2012).

5. EL SISTEMA FIBRINOLÍTICO

La coagulación sanguínea es un complejo evento enzimático que culmina con la formación de una proteína insoluble de aspecto filamentoso llamada fibrina (Figura 16). Junto con las plaquetas, la fibrina conforma un tapón que evita el sangrado cuando se produce un daño vascular. El conjunto de mecanismos encargados de lisar el coágulo de fibrina una vez formado y, por tanto, de restaurar el estado fisiológico normal que permita una correcta permeabilidad vascular, constituye el sistema fibrinolítico (Mosher, 1990; Gaffney y Longstaff, 1994).

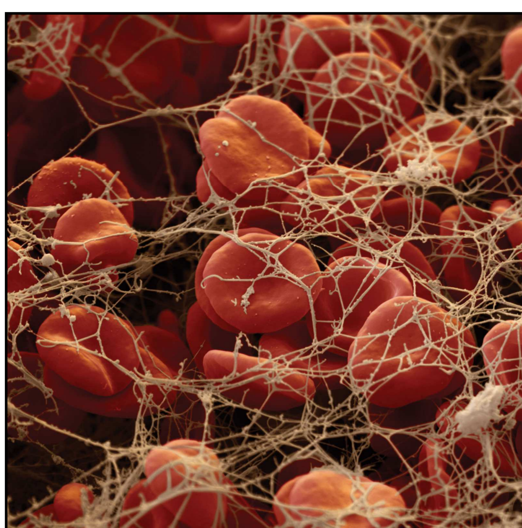

Figura 16. Glóbulos rojos atrapados en una malla de filamentos de fibrina. Tomado de Encyclopædia Britannica Online. http://global.britannica.com/EBchecked/topic/69202/bleeding-and-blood-clotting#ref64585

Como se ha descrito anteriormente, *D. immitis* provoca un daño vascular tanto por procesos mecánicos como enzimáticos, lo que sin duda estaría relacionado con una intensa actividad de la cascada de la coagulación. No obstante, *D. immitis* sobrevive durante largos períodos en el sistema vascular de hospedadores inmunocompetentes sin verse afectado, aparentemente, por este proceso. Por otra parte, cuando mueren los vermes adultos pueden producirse los graves tromboembolismos que caracterizan la patología aguda y que comprometen de inmediato la vida del hospedador. Por tanto, es razonable asumir que los vermes adultos interaccionan en vida con su entorno intravascular mediante mecanismos de supervivencia, ya que dichos fenómenos tromboembólicos, además de producir graves consecuencias para la vida del animal parasitado, pueden ser nocivos para el propio parásito. Por ello, es probable que, como ocurre en otros patógenos sanguíneos, *D. immitis* interaccione de algún modo con el sistema fibrinolítico de su hospedador.

Se revisan a continuación las principales características bioquímicas y funcionales de los componentes más importantes del sistema fibrinolítico, la naturaleza de su interacción con diferentes grupos de patógenos, así como sus implicaciones fisiopatológicas.

5.1. Componentes del sistema fibrinolítico

5.1.1. Sistema Plasminógeno - Plasmina

El funcionamiento del sistema fibrinolítico tiene como base la conversión de un proenzima, el plasminógeno, en su enzima proteolíticamente activa, la plasmina, la cual es capaz de degradar fibrina y, así, eliminar el coágulo previamente formado (Collen y Lijnen, 1991) (Figura 17).

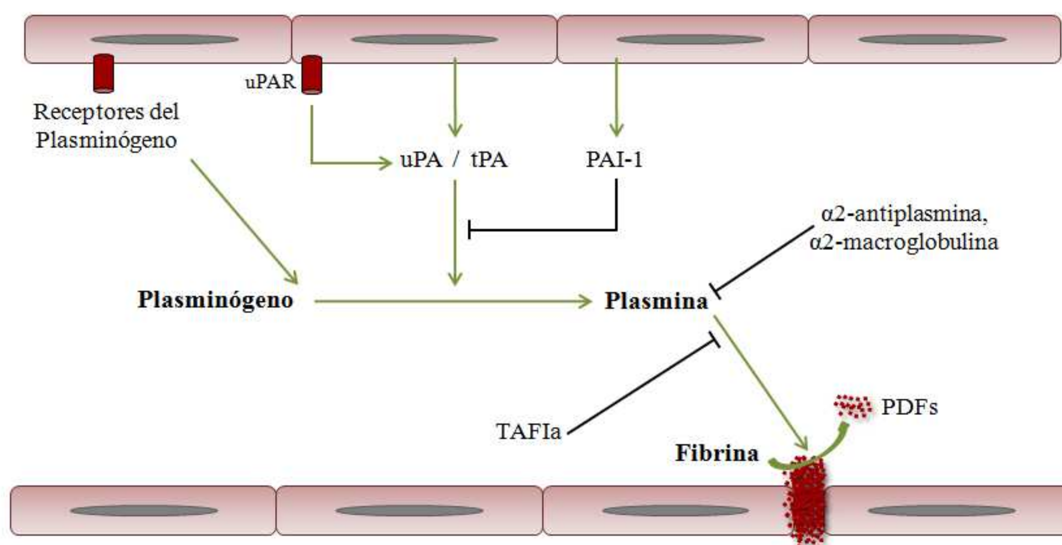

Figura 17. Esquema simplificado del sistema fibrinolítico. PAI-1, inhibidor del activador del plasminógeno-1; PDFs, productos de la degradación de la fibrina; TAFIa, inhibidor de la fibrinolisis activable por trombina activado; tPA, activador tisular del plasminógeno; uPA, activador del plasminógeno de tipo uroquinasa; uPAR, receptor del activador del plasminógeno de tipo uroquinasa.

El plasminógeno es una glicoproteína plasmática de 92 kDa sintetizada en el hígado y formada por 791 aminoácidos enlazados por 24 puentes disulfuro, 16 de los cuales dan lugar a 5 regiones homólogas de triple lazo llamadas *kringles* (Cesarman-Maus y Hajjar, 2005) (Figura 18). Al tratarse de un zimógeno, su conversión a plasmina depende de la escisión, por diferentes tipos de activadores, de un enlace peptídico Arg-Val en la posición 560-561 (Holvoet *et al*., 1985). Esto libera las cadenas pesada y ligera de la molécula de plasmina quedando unidas por dos puentes disulfuro. La cadena pesada de 65 kDa presenta los 5 dominios *kringle* y la cadena ligera de 25 kDa contiene el sitio activo de His_{602}, Asp_{645} y Ser_{740}, lo que conforma la característica tríada catalítica de las

proteasas de serina (Lähteenmäki *et al*., 2001). La plasmina como serina proteasa activa es capaz de degradar eficientemente el coágulo formado, generando productos solubles de degradación y logrando que la fibrina exponga sus residuos de lisina carboxi-terminales. Estos residuos son sitios de unión tanto para los dominios *kringle* 1 y 4 del plasminógeno como para los de sus activadores, lo que se traduce en un incremento significativo del proceso fibrinolítico (Cesarman-Maus y Hajjar, 2005). Además de con la fibrina, la plasmina puede realizar su actividad proteolítica sobre otros muchos sustratos, jugando un papel importante en procesos como la invasión celular, la quimiotaxis o la remodelación de los tejidos (Syrovets y Simmet, 2003).

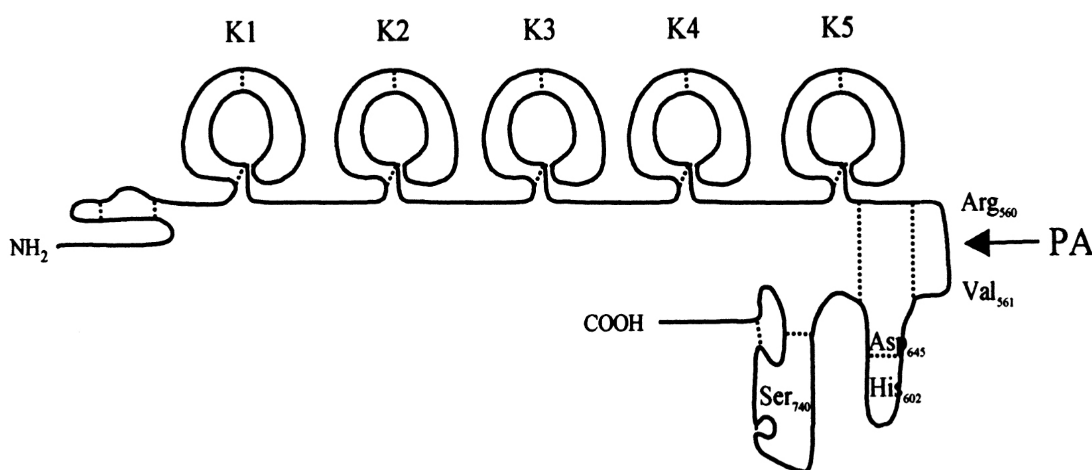

Figura 18. Estructura del plasminógeno. Los dominios *kringle* que contienen los sitios de unión a lisina están marcados como K1-K5. Se indican los aminoácidos que conforman la tríada catalítica (His_{602}, Asp_{645} y Ser_{740}) y el sitio de escisión para los activadores del plasminógeno entre los aminoácidos Arg_{560} y Val_{561}. Las líneas discontinuas señalan puentes disulfuro (Lähteenmäki *et al*., 2001). PA, activadores del plasminógeno.

El plasminógeno está presente en grandes cantidades tanto a nivel vascular como tisular. Debido al papel principal que cumple esta molécula, no solo en el sistema fibrinolítico, sino también en otros procesos celulares, es de suma importancia que esta gran reserva de actividad proteolítica se halle fuertemente regulada. Esto se logra gracias a la actividad de diferentes activadores, inhibidores y receptores.

5.1.2. Activadores del plasminógeno

Dos son los activadores fisiológicos del plasminógeno identificados hasta el momento, el activador tisular del plasminógeno (tPA) y el activador del plasminógeno de

tipo uroquinasa (uPA). La activación del plasminógeno mediada por tPA está relacionada principalmente con la disolución del coágulo de fibrina a nivel vascular, mientras que uPA se une a su receptor celular específico (uPAR) potenciando la activación del plasminógeno unido a células (Lijnen, 2006). Ambos son capaces de reconocer y escindir el puente peptídico Arg_{560}-Val_{561} del plasminógeno facilitando su conversión a plasmina.

Activador tisular del plasminógeno (tPA)

El tPA es una proteasa de serinas de 72 kDa sintetizada principalmente por las células endoteliales. Esta glicoproteína contiene cinco dominios estructurales entre los cuales se incluyen un *finger* tipo fibronectina, un dominio homólogo del factor de crecimiento epidérmico, dos estructuras *kringle* homólogas a las que contiene el plasminógeno y el dominio de proteasa de serinas (Pennica *et al*., 1983). El tPA es sintetizado como un polipéptido de cadena sencilla el cual, a diferencia de la mayoría de los precursores monocatenarios de la familia de las proteasas de serinas, es enzimáticamente activo. Por otra parte, su actividad en fase fluida puede aumentar mediante la hidrólisis del puente péptidico Arg_{275}-Ile_{276} por parte de la plasmina y su consiguiente transformación en proteasa de doble cadena (Tate *et al*., 1987). El principal papel del tPA es degradar la fibrina en los vasos sanguíneos. Pese a que la eficiencia catalítica del tPA es intrínsecamente baja, esta puede aumentar más de 100 veces en presencia de la propia fibrina. Esta estimulación ocurre a través de la formación de un complejo ternario mediante la unión del tPA con el plasminógeno y la fibrina. La fibrina no actúa simplemente como sustrato, sino también como cofactor estimulando su propia degradación. En un primer momento el tPA de cadena sencilla inicia la activación del plasminógeno en contacto con la red de fibrina intacta. A continuación, cuando la fibrina se encuentra parcialmente degradada por la acción de la plasmina, esta muestra nuevos sitios de unión al plasminógeno mediante la generación proteolítica de residuos carboxi-terminales de lisina. Estos residuos interaccionan con los dominios *kringle* presentes en el plasminógeno y en el tPA, aumentando notablemente el proceso fibrinolítico (Gebbink, 2011). Además de su papel principal en la disolución de coágulos sanguíneos, estudios recientes muestran al tPA como un importante modulador del sistema nervioso central, jugando un importante papel en los procesos de memoria, estrés, degeneración neuronal y Alzheimer (Zorio *et al*., 2008; Rijken y Lijnen, 2009).

Activador del plasminógeno de tipo uroquinasa (uPA)

El uPA es secretado como una proteína inactiva de cadena sencilla por una gran variedad de tipos celulares que incluyen células endoteliales vasculares, musculares lisas, monocitos y macrófagos, fibroblastos, células epiteliales, así como células tumorales malignas de diferente origen (Fuhrman, 2012). Se trata de una glicoproteína de 55 kDa de masa molecular, constituida por un dominio homólogo al factor de crecimiento epidérmico, una estructura *kringle* homóloga a la que contiene el plasminógeno y una tríada catalítica clásica del tipo de las proteasas de serina (Cesarman-Maus y Hajjar, 2005). Diferentes moléculas como la plasmina o la calicreína pueden llevar a cabo su activación mediante la rotura del puente peptídico Lys_{158}-Ile_{159}. El resultado es una proteasa de doble cadena unida por puente disulfuro cuya afinidad por el plasminógeno aumenta aproximadamente 300 veces. Además, en esta forma puede unirse a su receptor de membrana (uPAR), lo cual está considerado como un paso crítico en la función de uPA, permitiendo dicha unión focalizar la proteólisis llevada a cabo por uPA en el espacio pericelular inmediato (Nicholl *et al*., 2006). Pese a que su afinidad por la fibrina es mucho más baja que la que posee el tPA, uPA puede ser un activador efectivo del plasminógeno tanto en presencia como en ausencia de fibrina (Cesarman-Maus y Hajjar, 2005), lo que favorece la implicación del sistema uPA/uPAR en otros procesos como migración celular, diferenciación, proliferación y degradación de matrices. Por ello, uPA está considerada como una proteína crucial tanto en el crecimiento de la neoíntima como en el remodelado vascular, pudiendo ser otras moléculas, como las metaloproteasas de matriz o factores de crecimiento, susceptibles a la actividad de su dominio proteolítico (Nicholl *et al*., 2006; Fuhrman, 2012).

5.1.3. Inhibidores de la fibrinolisis

Inhibidores de los activadores del plasminógeno (PAIs)

El inhibidor del activador del plasminógeno-1 (PAI-1) está considerado como el principal regulador de la fibrinolisis *in vivo* (Loskutoff, 1991). Se trata de una glicoproteína de 47 KDa perteneciente a la superfamilia de las serpinas o inhibidores de proteasas de serina (Schleef *et al*., 1989). Después de iniciada la formación del trombo como resultado de un daño vascular, PAI-1 se libera principalmente a partir del endotelio y de las plaquetas, aunque puede ser sintetizado por otros tipos celulares. En condiciones

fisiológicas normales, la liberación de PAI-1 atenúa la activación del plasminógeno actuando a nivel de sus activadores tPA y uPA, contribuyendo así a la estabilización del trombo para el mantenimiento apropiado de la cicatrización de la herida y de la permeabilidad vascular (Iwaki *et al*., 2012). Con un menor grado de importancia, el inhibidor del activador del plasminógeno-2 (PAI-2) está relacionado con el mantenimiento de la hemostasia durante el embarazo y el parto, mientras que el inhibidor del activador del plasminógeno-3 (PAI-3) se encuentra en ciertos fluidos humanos, como los fluidos seminales y foliculares, presentando una baja actividad en plasma (Zorio *et al*., 2008).

Inhibidor de la fibrinolisis activable por trombina (TAFI)

La generación de residuos de lisina carboxi-terminales en la fibrina parcialmente degradada provoca una acumulación 30 veces mayor de plasminógeno en la superficie del coágulo y por tanto un aumento de la lisis en la segunda fase de la fibrinolisis. El TAFI es una glicoproteína de 60 KDa producida por el hígado, que tras su activación por trombina, tripsina o plasmina puede funcionar como una carboxipeptidasa eliminando los residuos de lisina carboxi-terminales de la red de fibrina. Al actuar solamente en la segunda fase de la lisis del coágulo, TAFI es considerado más bien un atenuador de la fibrinolisis (Nesheim *et al*., 1997; Sakharov *et al*., 1997; Rijken y Lijnen, 2009).

Inhibidores de la plasmina

La fibrinolisis también puede ser regulada a nivel de la plasmina por la serpina alfa 2-antiplasmina (Lijnen, 2006). Esta glicoproteína plasmática de 67 kDa es sintetizada en el hígado y realiza su función antifibrinolítica a tres niveles: 1) mediante la formación de un complejo con la plasmina, 2) inhibiendo la fijación del plasminógeno al coágulo de fibrina y 3) mediante su entrecruzamiento con la fibrina a través del factor XIIIa, lo que hace a esta última más resistente a la plasmina (Carpenter y Mathew, 2008). En menor medida, la plasmina puede ser también inhibida mediante la formación de complejos no covalentes con la alfa 2-macroglobulina (Cesarman-Maus y Hajjar, 2005).

5.1.4. Receptores del plasminógeno

Otro de los puntos clave en la regulación de la fibrinolisis es la llevada a cabo por los receptores celulares del plasminógeno. La unión del plasminógeno a la superficie celular permite, no solo dirigir la actividad proteolítica de la plasmina a aquellos sitios donde es requerida, sino también aumentar significativamente dicha actividad enzimática a nivel pericelular. Esto se debe a la existencia de residuos de lisina en sus extremos carboxi-terminales que permiten la unión a residuos *kringle*, lo que constituye la principal característica de los receptores fisiológicos del plasminógeno. Todo ello favorece, por una parte, la localización conjunta y simultánea del plasminógeno y sus activadores (tPA, uPA), y por otra la protección de la plasmina de nueva generación de la acción inhibitoria de la alfa 2-antiplasmina (Rijken y Lijnen, 2009; Madureira *et al*., 2011). Una gran variedad de células puede expresar un elevado número de receptores del plasminógeno. Entre ellos, los mejor caracterizados incluyen la α-enolasa, complejo glicoprotéico IIb-IIIa, antígeno de nefritis de Heymann, integrina $\alpha_M\beta_2$ y anexina A2 (Cesarman-Maus y Hajjar, 2005).

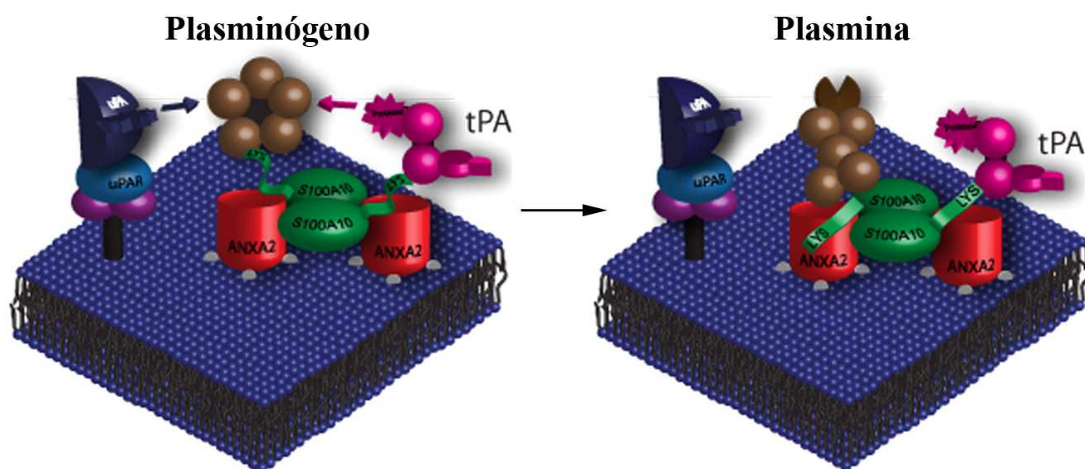

Figura 19. El tetrámero de anexina A2 se expresa en la superficie celular de numerosas células funcionando como receptor del plasminógeno. Consta de dos moléculas de anexina A2 unidas por un dímero de la proteína S100A10, el cual puede unir plasminógeno y tPA a través de sus residuos de lisina carboxi-terminales favoreciendo su localización conjunta en la membrana celular. Esto puede favorecer la acción del complejo uPA/uPAR con la consecuente generación de plasmina a nivel pericelular (Madureira *et al*., 2011).

La anexina A2 ha sido propuesta como el principal correceptor del plasminógeno y del tPA en las células endoteliales vasculares (Hajjar *et al*., 1994). Lleva a cabo su acción cuando se expresa en la superficie celular como un tetrámero compuesto de dos moléculas de anexina A2 unidas por un dímero de la proteína S100A10. La anexina A2 contiene sitios de unión a fosfolípidos con capacidad para anclarse a la superficie de la membrana celular, mientras que los residuos de lisina carboxi-terminales de S100A10 son los encargados de unir y hacer coincidir al plasminógeno con el tPA. El complejo de membrana uPA/uPAR también puede verse beneficiado de esta fijación del plasminógeno produciéndose, en cualquier caso, la localización conjunta del plasminógeno y sus activadores y la consecuente generación de plasmina a nivel pericelular (Flood y Hajjar, 2011; Madureira *et al*., 2011; Luo y Hajjar, 2013) (Figura 19).

5.2. Interacción de los patógenos con el sistema fibrinolítico de sus hospedadores

La plasmina es una proteasa de serinas de amplio espectro con una gran actividad proteolítica. Entre sus sustratos más comunes encontramos la fibrina, pero también diferentes componentes de la matriz extracelular y del tejido conectivo. El reclutamiento de esta enzima por parte de cualquier patógeno sanguíneo significaría una ventaja evolutiva, ya que no solo supondría un mecanismo efectivo para evitar su posible inmovilización por la red de coágulos de fibrina, sino también una ayuda para su diseminación y establecimiento en el hospedador mediante la degradación de los componentes de la matriz extracelular (Sun, 2006; Bhattacharya *et al*., 2012). Otras funciones como la degradación de inmunoglobulinas y de moléculas del complemento, la activación de metaloproteasas, la estimulación de la adherencia y de la invasión, así como la degradación de proteínas para la nutrición, han sido atribuidas a la interacción entre los patógenos y el sistema fibrinolítico (Kitt y Leigh, 1997; Yavlovich *et al*., 2004; Yavlovich y Rottem, 2007; Gong *et al*., 2008; Chung *et al*., 2011; Siemens *et al*., 2011; Bergmann *et al*., 2013).

La utilización del sistema fibrinolítico del hospedador como medio para obtener un beneficio ha sido ampliamente estudiada en organismos bacterianos desde hace un par de décadas (Lottenberg *et al*., 1994) (Figura 20). Un gran número de trabajos han permitido relacionar el empleo de la función proteolítica de la plasmina por parte de

diferentes especies de bacterias patógenas con el control de la hemostasia o con mecanismos para la mejora de su diseminación y de la evasión de los sistemas de vigilancia de la respuesta innata (Degen *et al*., 2007). Esta interacción es posible gracias al reclutamiento del plasminógeno en la superficie bacteriana mediante la expresión de proteínas que pueden actuar como receptores y a su transformación en plasmina mediante la acción de activadores del plasminógeno (Bhattacharya *et al*., 2012).

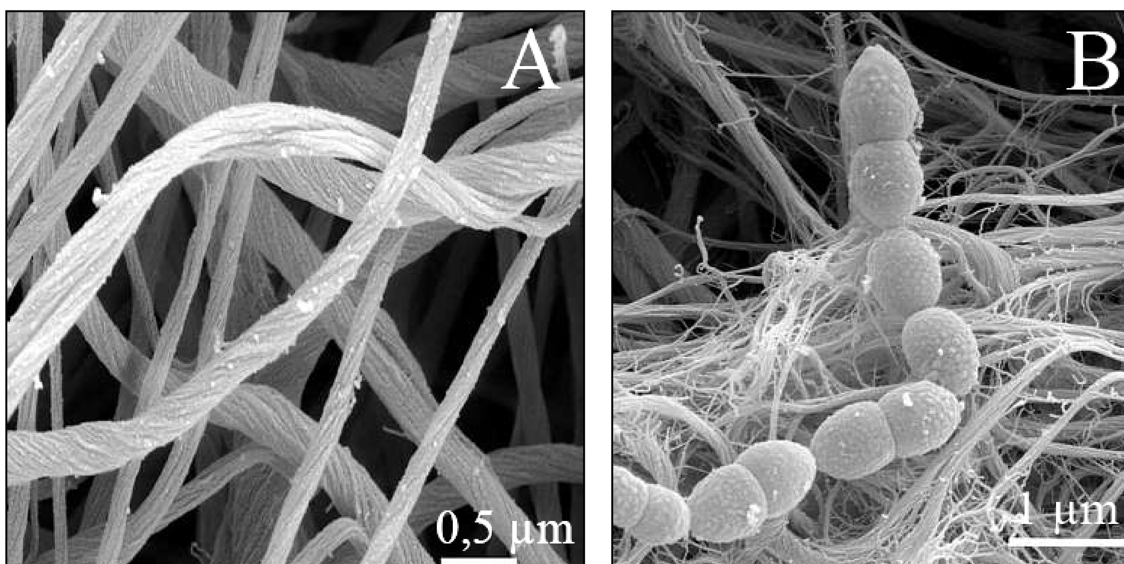

Figura 20. Imágenes captadas mediante microscopía electrónica de barrido de emisión de campo donde se puede apreciar la degradación de coágulos de fibrina provocada por *Streptococcus pneumoniae*. (A) Matriz de fibrina formada por gruesos haces formados a su vez por varias fibrillas de fibrina retorcidas sobre sí mismas. (B) Matriz de fibrina en degradación tras ser incubada con neumococos recubiertos con plasminógeno y su posterior activación a plasmina mediante uPA (Bergmann y Hammerschmidt, 2007).

La capacidad para expresar receptores de plasminógeno ha sido demostrada en un gran número de especies de bacterias (Sanderson-Smith *et al*., 2012). Estos receptores permiten inmovilizar el plasminógeno facilitando su conversión a plasmina por parte de los activadores, a la vez que evitan la función inhibitoria de la alfa 2-antiplasmina. Entre los mejor caracterizados se encuentran la enolasa y la GAPDH identificadas en los estreptococos de los grupos A y C. Estas enzimas, típicamente glicolíticas y con funciones básicas para el mantenimiento celular, pueden ser expresadas en la superficie bacteriana y llevar a cabo multitud de funciones (Lähteenmäki *et al*., 2001; Bhattacharya *et al*., 2012). Además, la secreción de receptores bacterianos de plasminógeno a través de vesículas de membrana externa también ha sido descrita, lo que permitiría la proteólisis

externa en el entorno pericelular como mecanismo para la nutrición y la mejora en la diseminación (Bergmann y Hammerschmidt, 2007; Toledo *et al*., 2012).

Tres son los principales activadores del plasminógeno que han sido descritos como proteínas secretadas o unidas a membrana en diferentes grupos bacterianos: la estreptoquinasa y la estafiloquinasa, producidas respectivamente por diferentes especies de los generos *Streptococcus* y *Staphylococcus*, y la proteasa Pla identificada en *Yersinia pestis*. Los dos primeros activan el plasminógeno mediante la formación de complejos catalíticamente activos tanto con el plasminógeno como con la plasmina, mientras que Pla es una aspartil proteasa que genera plasmina mediante su acción proteolítica (Degen *et al*., 2007).

Más recientemente, la capacidad para interaccionar con el sistema fibrinolítico del hospedador ha sido estudiada en organismos eucariotas causantes de enfermedades parasitarias (Figuera *et al*., 2013b). Un número limitado de trabajos ha permitido demostrar esta interacción en diferentes especies de protozoos y helmintos parásitos analizando antígenos excretados o de superficie. Las proteínas con capacidad para fijar plasminógeno identificadas hasta el momento en organismos parásitos aparecen en la tabla 2. La mayor parte de los estudios se han llevado a cabo con proteínas de las que su función como receptores del plasminógeno ya era conocida en especies de bacterias (enolasa, GAPDH, actina, FBAL…), mientras que otras proteínas como la LACK o la SMP-1 han sido relacionadas por primera vez con el sistema fibrinolítico en organismos parásitos (Gómez-Arreaza *et al*., 2011; Figuera *et al*., 2013a y b).

Al igual que en los trabajos llevados a cabo con bacterias, la interacción entre los parásitos y el sistema fibrinolítico del hospedador ha sido relacionada siempre con funciones *a priori* beneficiosas para el agente patógeno. Así, se ha vinculado la fijación de plasminógeno con la virulencia de los parásitos y con su éxito tanto en el proceso de infección como en su establecimiento en el hospedador. Esto ha sido recientemente sugerido en tripanosomas (Avilán *et al*., 2011). *Trypanosoma cruzi* puede fijar plasminógeno tanto en el hospedador vertebrado como en el vector y utilizarlo para atravesar tejidos (Almeida *et al*., 2004; Rojas *et al*., 2008). Además, se ha postulado que los parásitos del género *Leishmania* pueden interactuar con el plasminógeno en el momento de su inoculación en el hospedador definitivo como promastigotes, o como amastigotes cuando son liberados por los macrófagos para infectar otras células. La

generación de plasmina podría ayudar a los parásitos a reducir la matriz de fibrina asociada a la reacción inflamatoria y favorecer el encuentro entre *Leishmania* y nuevos macrófagos (Maldonado *et al*., 2006). Funciones relacionadas con la interacción célula-célula para la adherencia y penetración han sido propuestas en la utilización del plasminógeno por parte de los ooquinetos de *Plasmodium falciparum* y *P. berghei*. Tras su activación a plasmina podría ser utilizado para ayudar a la degradación de las proteínas de superficie de las células epiteliales del insecto vector para permitir la invasión parasitaria (Ghosh *et al*., 2011). Por su parte, se ha relacionado la fijación de plasminógeno por *Trichomonas vaginalis* con su penetración en la membrana basal lo que permitiría su asociación con la fibronectina y laminina. Esto garantizaría el acceso del parásito a factores de crecimiento y nutrientes contribuyendo al éxito de la infección (Mundodi *et al*., 2008). En el caso de los helmintos, funciones similares se han propuesto para *O. volvulus*, cuyas microfilarias podrían utilizar la actividad proteolítica de la plasmina unida a su superficie para facilitar su migración a través de los tejidos del hospedador (Jolodar *et al*., 2003). Además, en *Schistosoma bovis*, helminto parásito con localización intravascular, se ha relacionado la fijación de plasminógeno con una regulación del sistema hemostático por parte del parásito para evitar la formación de coágulos (Ramajo-Hernández *et al*., 2007; de la Torre-Escudero *et al*., 2010).

La mayor parte de los receptores de plasminógeno identificados tanto en bacterias como en parásitos comparten mecanismos similares de unión con los receptores fisiológicos de sus hospedadores (Miles *et al*., 2005; Figuera *et al*., 2013b). Se ha demostrado la participación de los residuos de lisina en la interacción de estos receptores con el plasminógeno mediante la realización de ensayos competitivos con análogos de este aminoácido, como el ácido ε-amino caproico. Los residuos de lisina que participan en la fijación del plasminógeno han sido identificados en los extremos carboxi-terminales de los receptores, pero también formando parte de motivos internos como el "FYDKERKVY" descrito en la enolasa de *S. pneumoniae* (Bergmann *et al*., 2003) y encontrado posteriormente con ligeras modificaciones en las enolasas de otras bacterias, hongos o parásitos (Figuera *et al*., 2013b).

Proteína	Especie	Referencia
Enolasa	*Leishmania mexicana*	Vanegas *et al*., 2007; Figuera *et al*., 2013a
	Trichomonas vaginalis	Mundodi *et al*., 2008
	Plasmodium falciparum	Ghosh *et al*., 2011
	Plasmodium berghei	Ghosh *et al*., 2011
	Fasciola hepatica	Bernal *et al*., 2004
	Echinostoma caproni	Marcilla *et al*., 2007
	Schistosoma bovis	Ramajo-Hernández *et al*., 2007; de la Torre-Escudero *et al*., 2010
	Schistosoma japonicum	Yang *et al*., 2010
	Clonorchis sinensis	Wang *et al*., 2011
	Taenia pisiformis	Zhang *et al*., 2015
	Onchocerca volvulus	Jolodar *et al*., 2003
GAPDH	*Trichomonas vaginalis*	Lama *et al*., 2009
	Schistosoma bovis	Ramajo-Hernández *et al*., 2007
	Clonorchis sinensis	Hu *et al*., 2014
	Onchocerca volvulus	Erttmann *et al*., 2005
LACK	*Leishmania mexicana*	Gómez-Arreaza *et al*., 2011
SMP-1	*Leishmania mexicana*	Figuera *et al*., 2013a
Anexina B30	*Clonorchis sinensis*	He *et al*., 2014
Actina	*Schistosoma bovis*	Ramajo-Hernández *et al*., 2007
FBAL	*Schistosoma bovis*	Ramajo-Hernández *et al*., 2007
ATP: guanidino quinasa	*Schistosoma bovis*	Ramajo-Hernández *et al*., 2007
Fosfoglicerato mutasa	*Schistosoma bovis*	Ramajo-Hernández *et al*., 2007
Triosafosfato isomerasa	*Schistosoma bovis*	Ramajo-Hernández *et al*., 2007
Adenilato quinasa	*Schistosoma bovis*	Ramajo-Hernández *et al*., 2007
Proteína hipotética AAW24823	*Schistosoma bovis*	Ramajo-Hernández *et al*., 2007
Proteína hipotética AAP06049	*Schistosoma bovis*	Ramajo-Hernández *et al*., 2007

Tabla 2. Proteínas fijadoras de plasminógeno identificadas en organismos parásitos. FBAL, fructosa-bifosfato aldolasa; GAPDH, gliceraldehído-3-fosfato deshidrogenasa.

5.3. Fisiopatología de la fibrinolisis

El gran número de sustratos sobre los que pueden realizar su función los componentes con actividad proteolítica del sistema fibrinolítico, requiere una estricta regulación del sistema para evitar una proteólisis indiscriminada (Draxler y Medcalf, 2015). En caso contrario podría producirse un escenario patológico, debido a que la plasmina y los activadores del plasminógeno están implicados en procesos como la proliferación, migración, inflamación y degradación de la matriz extracelular (Figura 21). Esto ha permitido relacionar la sobreactivación del sistema fibrinolítico con situaciones patológicas tan importantes como el crecimiento de la placa arterial, la aterosclerosis crónica, síndromes coronarios agudos, restenosis, la remodelación vascular e incluso con el cáncer, provocando que la plasmina sea considerada actualmente como una diana terapéutica potencialmente interesante desde diversos puntos de vista (Nicholl *et al*., 2006; Zorio *et al*., 2008).

Un gran número de trabajos llevados a cabo en investigación cardiovascular relacionada con humanos, algunos realizados mediante la utilización de ratones deficientes en componentes del sistema fibrinolítico, han permitido relacionar la activación del plasminógeno con la proliferación y migración de células vasculares humanas, así como con la degradación de matrices extracitoplasmáticas (Nicholl *et al*., 2005 y 2006; Yang *et al*., 2005; Roth *et al*., 2006; Hayashi *et al*., 2009). Del mismo modo se ha podido medir un incremento en los niveles de tPA, uPA y/o plasmina en diferentes situaciones patológicas, que tienen en común alguno o todos los mecanismos anteriormente citados (Syrovets y Simmet, 2004; Nicholl *et al*., 2006; Draxler y Medcalf, 2015). Incluso a altas concentraciones, se ha descrito que la plasmina puede provocar desorganización tanto del endotelio como del músculo liso perivascular e iniciación de la apoptosis celular si la estimulación persiste (Rossignol *et al*., 2006; Ho-Tin-Noe *et al*., 2009; Doeuvre *et al*., 2010).

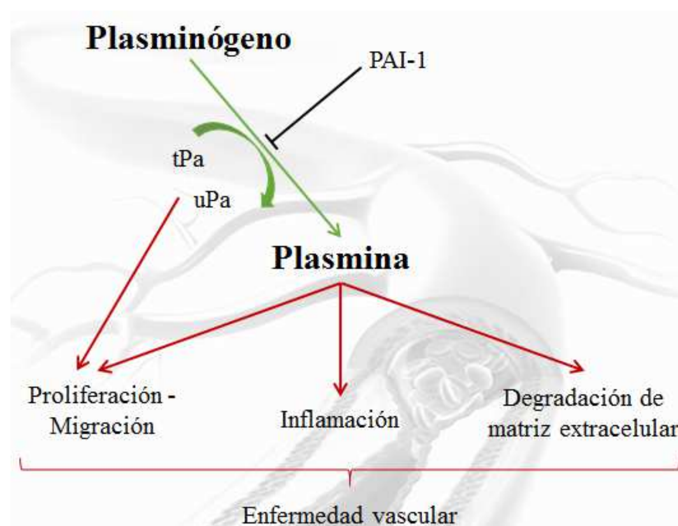

Figura 21. Esquema de la fisiopatología de la fibrinolisis.

Por otra parte, la plasmina puede unirse a una gran variedad de células, incluyendo monocitos, macrófagos, células dendríticas y otras, a través de receptores de baja afinidad provocando agregación de neutrófilos, degranulación plaquetaria y liberación de ácido araquidónico desde las células endoteliales. Esto indica una relación directa entre la plasmina y la activación proinflamatoria a gran escala, incluyendo la liberación de mediadores lipídicos y especies reactivas del oxígeno, quimiotaxis y expresión de citoquinas, así como la inducción de la expresión de otros genes proinflamatorios

(Syrovets y Simmet, 2004). La plasmina juega, por tanto, un papel central en la respuesta inmune celular, ayudando a la eliminación de organismos infecciosos. No obstante, su activación excesiva en enfermedades autoinmunes o inflamatorias de tipo crónico puede exacerbar la estimulación de células inflamatorias y por tanto, la patogénesis de la enfermedad (Syrovets *et al*., 2012).

Todos estos datos han permitido evidenciar la implicación de la activación fibrinolítica en enfermedades tan graves como el cáncer. Se ha demostrado que la sobreexpresión constitutiva del gen de uPA es una característica de la progresión maligna, lo que conduce a altos niveles de uroquinasa unida al receptor y por tanto, a una excesiva activación del plasminógeno. La plasmina unida a membrana, protegida de los inhibidores circulantes, es capaz de degradar las proteínas de la matriz extracelular tales como laminina y fibronectina, así como activar varias metaloproteasas de la matriz, lo que contribuye aún más a la degradación de la matriz extracelular. La plasmina generada en la superficie de las células tumorales es considerada, por lo tanto, como un evento clave en la invasión tumoral y la metástasis (Schmitt *et al*., 1997; Andreasen *et al*., 2000; Syrovets y Simmet, 2004).

6. DIAGNÓSTICO Y MANEJO DE LA DIROFILARIOSIS

El diagnóstico de la dirofilariosis es un aspecto fundamental dentro del cuadro general del manejo de la parasitosis. Dadas las implicaciones que tiene la enfermedad, tanto en su aspecto clínico veterinario y humano, como en los estudios epidemiológicos, el diagnóstico constituye el primer paso en el control de la zoonosis. Por otra parte, las complicaciones derivadas de la muerte masiva de los vermes adultos de *D. immitis* en el circuito vascular precisa de un número suficiente de técnicas diagnósticas que permita valorar la gravedad de cada situación y la intensidad de la parasitación, para seleccionar la pauta correcta de tratamiento que evite o disminuya el riesgo de tromboembolismos en el animal afectado (Knight, 1995).

6.1. Diagnóstico de la dirofilariosis cardiopulmonar canina

En el perro, los métodos diagnósticos se basan en la identificación de antígenos circulantes del parásito adulto en suero y en la detección de microfilarias en muestras de

sangre. Existen test comerciales basados en métodos inmunocromatográficos capaces de detectar proteínas secretadas por el tejido ovárico de las hembras adultas de *D. immitis*. Este diagnóstico presenta una alta especificidad al no observarse reacciones cruzadas con otras filarias caninas, como *D. repens* o *Acanthocheilonema reconditum*. Su sensibilidad también es grande pese a que pueden producirse falsos negativos en infecciones prepatentes (de menos de 5 meses), cuando las infecciones están producidas por un número muy escaso de vermes, o en infecciones causadas solo por machos (McCall, 1992). La detección de microfilarias está considerado como un método complementario pero necesario, ya que se estima que un 30% de las infecciones caninas por *D. immitis* en áreas endémicas son amicrofilarémicas (Rawlings *et al*., 1982). Este método se realiza habitualmente por examen microscópico después de proceder a la concentración de las microfilarias mediante el test de *Knott* o similares (Venco *et al*., 2011) (Figura 22). Debido a la existencia de otras especies de filarias que pueden aparecer en sangre periférica, esta técnica debe de ir acompañada de la determinación específica de las microfilarias mediante diferenciación morfológica (Carretón *et al*., 2012), tinción histoquímica de las zonas anatómicas con actividad fosfatasa (Chalifoux y Hunt, 1971; Peribáñez *et al*., 2001) o mediante amplificación del ADN por la reacción en cadena de la polimerasa (Favia *et al*., 1996). En cualquier caso, la positividad de una muestra a los test de microfilarias y de antígenos indicaría sin duda una infección microfilarémica por *D. immitis*. La forma amicrofilarémica de la infección se revela por un test negativo de microfilarias acompañado de un test positivo de antígenos, mientras que la negatividad en el test de antígenos y positividad en el de microfilarias, mostraría una infección por una especie distinta a *D. immitis* (Simón *et al*., 2012).

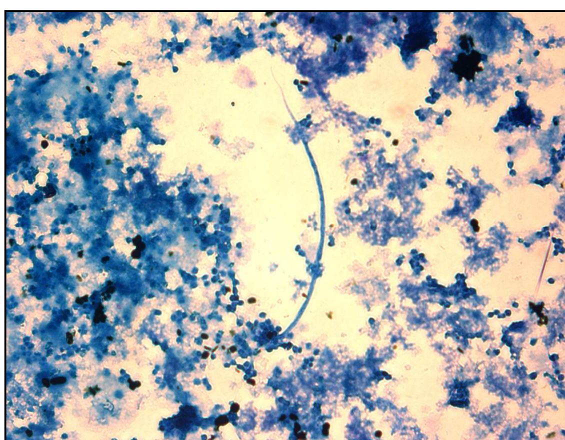

Figura 22. Observación de microfilarias mediante el test de *Knott*. En la imagen puede verse una microfilaria de *D. immitis* teñida con azul de metileno (40X) (Carretón et al., 2012).

Además de estos test, existen otros complementarios, como la radiografía torácica, la ecocardiografía o la electrocardiografía, que pueden aportar datos interesantes sobre la situación clínica de cada paciente y resultar de mucho interés a la hora de establecer un

correcto protocolo de tratamiento. El examen radiográfico de tórax no permite estimar la carga parasitaria (Venco *et al*., 2003), pero puede ser de utilidad en estadios avanzados de la enfermedad, poniendo de manifiesto ensanchamientos de las arterias pulmonares, anomalías del patrón pulmonar y en los casos más graves, cardiomegalia derecha (Venco *et al*., 2011). La ecocardiografía, en cambio, sí permite realizar una estimación aproximada del número y localización de las filarias, lo que le aporta cierto valor diagnóstico. Los vermes de *D. immitis* aparecen como dos líneas paralelas hiperecógenas, en el lumen de las cámaras del corazón y vasos pulmonares (Atkins *et al*., 1996; Venco *et al*., 1998). La ecocardiografía asociada a impulsos *Doppler* permite además evaluar con precisión la gravedad de la hipertensión pulmonar, por lo que esta prueba es altamente recomendable cuando las características clínicas y radiológicas sugieren una infección grave (Venco *et al*., 2011). En perros que se encuentran en la fase terminal de la enfermedad, el análisis electrocardiográfico permite revelar alteraciones tanto del eje como del ritmo eléctrico del corazón (Venco *et al*., 2011).

Recientemente, se ha investigado la utilización de moléculas liberadas a la sangre como consecuencia del daño celular en los vasos y el corazón, de una perfusión inadecuada o de la lisis de trombos, como biomarcadores tempranos en la dirofilariosis cardiopulmonar. Esto permitiría realizar una mejor evaluación del estado clínico del perro infectado, establecer un pronóstico y monitorizar el tratamiento elegido. Los resultados iniciales sugieren la posibilidad de usar la troponina I cardíaca y la mioglobina como marcadores de daño cardíaco y el dímero-D como herramienta de apoyo en el diagnóstico de tromboembolismos pulmonares (Carretón, 2013).

6.2. Tratamiento y prevención

Las reacciones secundarias resultantes de la destrucción masiva de los vermes adultos de *D. immitis* en el circuito sanguíneo complican en gran medida el tratamiento de la dirofilariosis cardiopulmonar, desaconsejándose en ciertos pacientes. En cualquier caso, se debe evaluar la situación de cada animal, considerando factores tales como el número de parásitos, edad y tamaño del perro, gravedad de la enfermedad pulmonar y el tipo de restricción de la actividad física al cual puede ser sometido el animal antes de establecer un correcto tratamiento (Venco *et al*., 2004). En función de estos factores la dirofilariosis se clasifica actualmente en dos categorías o niveles de gravedad (McCall *et*

al., 2008). El primer nivel comprende los perros con bajo riesgo de complicaciones tromboembólicas e incluye los animales asintomáticos con baja carga parasitaria, ausencia de lesiones de la vasculatura o parénquima pulmonar, que presenten radiografías torácicas normales, bajo nivel de antígenos circulantes, ausencia de parásitos en ecocardiografía, sin enfermedades concomitantes y con posibilidad de limitar su actividad física durante el tratamiento. En el grupo de alto riesgo de complicaciones tromboembólicas se incluyen los perros que cumplan, al menos, una de las siguientes condiciones: síntomas relacionados con la enfermedad (tos, lipotimias y ascitis), radiografías torácicas anormales compatibles con la dirofilariosis, elevado nivel de antígenos circulantes, visualización de los parásitos mediante ecocardiografía, enfermedades concomitantes o imposibilidad de limitar la actividad física del animal (Venco *et al*., 2011).

El tratamiento adulticida debe realizarse exclusivamente con clorhidrato de melarsomina. El protocolo clásico consiste en administrar dos inyecciones por vía intramuscular separadas por un intervalo de 24 horas en dosis de 2,5 mg/kg. No obstante, la *American Heartworm Society* recomienda un protocolo diferido añadiendo una tercera inyección con una dosis similar de melarsomina, al menos un mes antes de estas dos. Este protocolo resulta más eficaz al eliminar los vermes adultos de forma escalonada [50% de los adultos (90% machos y 10% hembras)] con la primera inyección y el resto con la segunda y tercera, y más seguro al dar suficiente tiempo a los pulmones para recuperarse del tromboembolismo causado por la muerte de los vermes en la primera inyección. Hay que tener en cuenta que el tromboembolismo es una consecuencia inevitable del tratamiento adulticida. Por ello, es esencial que este vaya acompañado de la restricción del ejercicio durante un mes desde la administración del fármaco adulticida para minimizar las complicaciones derivadas de la muerte de los parásitos. Además, para controlar los síntomas del tromboembolismo pulmonar se puede administrar prednisona a 0,5 mg/kg/12 h la primera semana y 0,5 mg/kg/24 h durante la segunda semana, seguido de 0,5 mg/kg/48 h durante 1 ó 2 semanas (Carretón *et al*., 2012; Simón *et al*., 2012). Puesto que la melarsomina no puede eliminar filarias menores de 4 meses de edad, el tratamiento debe comenzar con la administración de lactonas macrocíclicas a dosis preventivas durante los dos o tres meses previos para eliminar las larvas migratorias y las microfilarias. Por otra parte, la liberación masiva de *Wolbachia* durante el tratamiento adulticida y su implicación en la patogénesis de la enfermedad debe ser controlada. Por

ello, el tratamiento con tetraciclinas del tipo doxiciclina a dosis de 10 mg/kg/12 h durante 4 semanas antes de la administración del adulticida es recomendable, ya que elimina un 90% de la población de *Wolbachia* y provoca un debilitamiento y disminución en la fertilidad de las filarias adultas (Venco *et al*., 2011; Carretón *et al*., 2012; Simón *et al*., 2012).

En perros en los que no es recomendable la aplicación de una terapia causal, puede llevarse a cabo un tratamiento sintomático que incluye la administración de diversos fármacos (corticosteroides, digoxina, opiáceos…) y/o restricción de la actividad física por confinamiento en jaula (Dillon *et al*., 1995). La terapia quirúrgica puede aplicarse en perros con síndrome de vena cava, empleando *Flexible Alligator Forceps*©, introducidos a través de la vena yugular. Este instrumento permite extraer los vermes adultos de *D. immitis* localizados en el ventrículo derecho y arteria pulmonar con un riesgo de mortalidad intraoperatoria muy bajo y con una tasa de supervivencia y curación directamente proporcional al porcentaje de vermes extraídos (Ishihara *et al*., 1990) (Figura 23).

Teniendo en cuenta la gravedad de la enfermedad, la dificultad a la hora de clasificar a los perros infectados y los riesgos tromboembólicos derivados de la terapia elegida, la profilaxis constituye una alternativa de fundamental importancia. El tratamiento profiláctico de elección se basa en la administración mensual de lactonas macrocíclicas como ivermectina, milbemicina oxima, moxidectina o selamectina (McCall *et al*., 1986; McTier *et al*., 1992). Se recomienda que los cachorros de zonas endémicas comiencen la profilaxis cuanto antes, nunca más tarde de los dos meses de edad, iniciándose esta un mes antes del comienzo del período de transmisión y finalizando un mes después de que este termine (Atkins, 2011; American Heartworm Society, 2012).

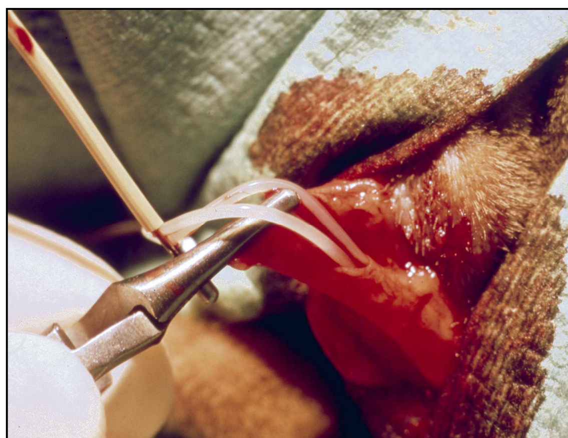

Figura 23. Verme adulto de *D. immitis* extraído de la cavidad cardíaca derecha de un gato con dirofilariosis a través de la vena yugular empleando *Flexible Alligator Forceps*© (Venco y Vezzoni, 2001).

BIBLIOGRAFÍA

Adcock, J.L., 1961. Pulmonary arterial lesions in canine dirofilariasis. Am. J. Vet. Res. 22, 655-662.

Almeida, L., Vanegas, G., Calcagno, M., Concepción, J.L., Avilán, L., 2004. Plasminogen interaction with *Trypanosoma cruzi*. Mem. Inst. Oswaldo Cruz 99, 63-67.

American Heartworm Society, 2012. Current canine guidelines for the diagnosis, prevention, and management of heartworm (*Dirofilaria immitis*) infection in dogs. American Heartworm Society, Wilmington, DE, USA. http://www.heartwormsociety.org/.

Andreasen, P.A., Egelund, R., Petersen, H.H., 2000. The plasminogen activation system in tumor growth, invasion and metastasis. Cell. Mol. Life Sci. 57, 25-40.

Araujo, A.M., 1996. Canine and human *Dirofilaria immitis* infections in Portugal. A review. Parassitologia 38, 366.

Atkins, C.E., De Francesco, T., Miller, M., Meurs, K., Keene, B.W., 1996. Prevalence of heartworm infection in cats with cardiorespiratory abnormalities, abstr 50. Abstr. 14th ACVIM Forum, San Antonio, TX.

Atkins, C., 2011. Heartworm disease in dogs: an update - 2011. En: Proceedings of the 36th World Small Animal Veterinary Congress WSAVA, October 14th-17th, 2011, Jeju, Korea, pp. 162-165.

Atwell, R.B., Buoro, I.B., Sutton, R.H., 1985. Experimental production of lesions in canine pulmonary arteries similar to those produced by *Dirofilaria immitis* infection. Vet. Rec. 116, 539-541.

Atwell, R.B., Sutton, R.H., Buoro, I.B., 1986. Early pulmonary lesions caused by dead *Dirofilaria immitis* in dogs exposed to homologous antigens. Br. J. Exp. Pathol. 67, 395-405.

Avilán, L., Gualdrón-López, M., Quiñones, W., González-González, L., Hannaert, V., Michels, P.A.M., Concepción, J.L., 2011. Enolase: a key player in the metabolism and a probable virulence factor of trypanosomatid parasites-perspectives for its use as a therapeutic target. Enzyme Res. 2011, 932549.

Bain, O., 2002. Evolutionary relationships among filarial nematodes. En: Klei, T., Rajan, T.V. (Eds.), The Filaria. Kluwer Academic Publishers, USA, pp. 21-30.

Bandi, C., Anderson, T.J., Genchi, C., Blaxter, M.L., 1998. Phylogeny of *Wolbachia* in filarial nematodes. Proc. Biol. Sci. 265, 2407-2413.

Barriga, O.O., 1982. Dirofilariasis. En: Steele, J.H., Schultz, M.G. (Eds.), Handbook series in zoonoses, vol 2. CRC Press Inc, Boca Raton, FL, USA, pp. 93-110.

Bazzocchi, C., Ceciliani, F., McCall, J.W., Ricci, I., Genchi, C., Bandi, C., 2000. Antigenic role of the endosymbionts of filarial nematodes: IgG response against the *Wolbachia* surface protein in cats infected with *Dirofilaria immitis*. Proc. Biol. Sci. 267, 1-6.

Bazzocchi, C., Genchi, C., Paltrinieri, S., Lecchi, C., Mortarino, M., Bandi, C., 2003. Immunological role of the endosymbionts of *Dirofilaria immitis*: the *Wolbachia* surface protein activates canine neutrophils with production of IL-8. Vet. Parasitol. 117, 73-83.

Bazzocchi, C., Comazzi, S., Santoni, R., Bandi, C., Genchi, C., Mortarino, M., 2007. *Wolbachia* surface protein (WSP) inhibits apoptosis in human neutrophils. Parasite Immunol. 29, 73-79.

Bergmann, S., Wild, D., Diekmann, O., Frank, R., Bracht, D., Chhatwal, G.S., Hammerschmidt, S., 2003. Identification of a novel plasmin(ogen)-binding motif in surface displayed alpha-enolase of *Streptococcus pneumoniae*. Mol. Microbiol. 49, 411-423.

Bergmann, S., Hammerschmidt, S., 2007. Fibrinolysis and host response in bacterial infections. Thromb. Haemost. 98, 512-520.

Bergmann, S., Schoenen, H., Hammerschmidt, S., 2013. The interaction between bacterial enolase and plasminogen promotes adherence of *Streptococcus pneumoniae* to epithelial and endothelial cells. Int. J. Med. Microbiol. 303, 452-462.

Bernal, D., de la Rubia, J.E., Carrasco-Abad, A.M., Toledo, R., Mas-Coma, S., Marcilla, A., 2004. Identification of enolase as a plasminogen-binding protein in excretory-secretory products of *Fasciola hepatica*. FEBS letters 563, 203-206.

Bhattacharya, S., Ploplis, V.A., Castellino, F.J., 2012. Bacterial plasminogen receptors utilize host plasminogen system for effective invasion and dissemination. J. Biomed. Biotechnol. 2012, 482096.

Bidgood, A., Collins, G.H., 1996. The prevalence of *Dirofilaria immitis* in dogs in Sydney. Aust. Vet. J. 73, 103-104.

Bilge, F.H., Kadipasaoglu, K.A., McCormick, C.M., Baier, R.E., 1989. Surface characterization of the cuticle of *Dirofilaria immitis*. J. Biomed. Mater. Res. 23, 1027-1047.

Bou-Gharios, G., Ponticos, M., Rajkumar, V., Abraham, D., 2004. Extra-cellular matrix in vascular networks. Cell Prolif. 37, 207-220.

Brattig, N.W., Bazzocchi, C., Kirsching, C.J., Reiling, N., Buttner, D.W., Ceciliani, F., Geisinger, F., Hochrein, H., Ernst, M., Wagner, H., Bandi, C., Hoerauf, A., 2004. The major Surface protein of *Wolbachia* endosymbionts in filarial nematodes elicits immune response Through TLR2 and TLR4. J. Immunol. 73, 437-445.

Brattig, N.W., Schwohl, A., Rickert, R., Büttner, D.W., 2006. The filarial parasite *Onchocerca volvulus* generates the lipid mediator prostaglandin E(2). Microbes Infect. 8, 873-879.

Cancrini, G., Kramer, L., 2001. Insect vectors of *Dirofilaria* spp. En: Simón, F., Genchi, C. (Eds.), Heartworm infection in humans and animals. Ediciones Universidad de Salamanca, España, pp. 63-82.

Cancrini, G., Magi, M., Gabrielli, S., Arispici, M., Tolari, F., Dell'Omodarme, M., Prati, M.C., 2006. Natural vectors of dirofilariasis in rural and urban areas of the Tuscan region, central Italy. J. Med. Entomol. 43, 574-579.

Carpenter, S.L., Mathew, P., 2008. α2-antiplasmin and its deficiency: Fibrinolysis out of balance. Haemophilia 14, 1250-1254.

Carretón, E., Morchón, R., Montoya-Alonso, J.A., 2012. Dirofilariosis cardiopulmonar canina. En: Montoya-Alonso, J.A., Carretón-Gómez, E. (Eds.), Dirofilariosis, pautas de manejo clínico. Multimédica Ediciones Veterinarias, España, pp. 1-130.

Carretón, E., 2013. Estudio de biomarcadores de daño cardiopulmonar en la dirofilariosis canina por *Dirofilaria immitis* (Tesis Doctoral Europea). Universidad de Las Palmas de Gran Canaria, Las Palmas de Gran Canaria.

Casiraghi, M., Bandi, C., Bazzocchi, C., Anderson, T., Genchi, C., 2001. A phylogenetic analysis of filarial nematodes: Comparison with the phylogeny of *Wolbachia* endosymbionts. Parasitology, 122, 93-103.

Cesarman-Maus, G., Hajjar, K.A., 2005. Molecular mechanisms of fibrinolysis. Br. J. Haematol. 129, 307-321.

Chakravarty, A., Chaudhuri, M.N., 1983. Studies on canine filariasis in West Bengal. Indian J. Anim. Health 22, 151-155.

Chalifoux, L., Hunt, R.D., 1971. Histochemical differentaion of *Dirofilaria immitis* and *Dipetalonema reconditum*. J. Am. Vet. Med. Assoc. 158, 602-605.

Chandrashekar, R., Tsuji, N., Morales, T.H., Carmody, A.B., Ozols, V.O., Welton, J., Tang, L., 2000. Removal of hydrogen peroxide by a 1-cysteine peroxiredoxin enzyme of the filarial parasite *Dirofilaria immitis*. Parasitol. Res. 86, 200-206.

Chung, M.C., Tonry, J.H., Narayanan, A., Manes, N.P., Mackie, R.S., Gutting, B., Mukherjee, D.V., Popova, T.G., Kashanchi, F., Bailey, C.L., Popov, S.G., 2011. *Bacillus anthracis* interacts with plasmin(ogen) to evade C3b-dependent innate immunity. PLoS One 6, e18119.

Clarke, L.L., Ballweber, L.R., Allen, K., Little, S.E., Lappin, M.R., 2014. Prevalence of select vector-borne disease agents in owned dogs of Ghana. J. S. Afr. Vet. Assoc. 85, 996.

Collen, D., Lijnen, H.R., 1991. Basic and clinical aspects of fibrinolysis and thrombolysis. Blood 78, 3114.

Crossgrove, K., Laudet, V., Maina, C.V., 2002. *Dirofilaria immitis* encodes Di-nhr-7, a putative orthologue of the *Drosophila* ecdysone-regulated E78 gene. Mol. Biochem. Parasitol. 119, 169-177.

Davis, G.E., Senger, D.R., 2005. Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. Circ. Res. 97, 1093-1107.

De la Torre-Escudero, E., Manzano-Román, R., Pérez-Sánchez, R., Siles-Lucas, M., Oleaga, A., 2010. Cloning and characterization of a plasminogen-binding surface-associated enolase from *Schistosoma bovis*. Vet. Parasitol. 173, 76-84.

Degen, J.L., Bugge, T.H., Goguen, J.D., 2007. Fibrin and fibrinolysis in infection and host defense. J. Thromb. Haemost. 5 Suppl 1, 24-31.

Dejana, E., Corada, M., Lampugnani, M.G., 1995. Endothelial cell-to-cell junctions. FASEB J. 9, 910-918.

Dillon, R.A., Warner, A.E., Molina, R.M., 1995. Pulmonary parenchymal changes in dogs and cats after experimental transplantation of dead *Dirofilaria immitis*. En: Soll, M.D. (Ed.), Proceedings of the Heartworm Symposium '92. American Heartworm Society, USA, pp. 97-101.

Doby, J.M., Guiguen, C., Lefeuvre, R., 1986. Présence de *Dirofilaria immitis* (Leidy, 1856) chez le chien en Bretagne. Bull. Soc. Fr. Parasitol. 4, 51-54.

Doeuvre, L., Plawinski, L., Goux, D., Vivien, D., Anglés-Cano, E., 2010. Plasmin on adherent cells: from microvesiculation to apoptosis. Biochem. J. 432, 365-373.

Draxler, D.F., Medcalf, R.L., 2015. The Fibrinolytic System-More Than Fibrinolysis? Transfus. Med. Rev. 29, 102-109.

Duménigo, B.E., Espino, A.M., Bouza, M., 1988. Prevalencia de filariasis canina en la Isla de la Juventud. Rev. Salud Anim. 10, 247-250.

Dupont, A., Corseaux, D., Dekeyzer, O., Drobecq, H., Guihot, A.L., Susen, S., Vincentelli, A., Amouyel, P., Jude, B., Pinet, F., 2005. The proteome and secretome of human arterial smooth muscle cells. Proteomics 5, 585-596.

Ehrenberg, J.P., Tamashiro, W.K., Scott, A.L., 1987. *Dirofilaria immitis*: identification and characterization of circulating parasite antigens. Exp. Parasitol. 63, 205-214.

Encyclopædia Britannica Online, Inc, 2015. http://global.britannica.com/.

Erttmann, K.D., Kleensang, A., Schneider, E., Hammerschmidt, S., Büttner, D.W., Gallin, M., 2005. Cloning, characterization and DNA immunization of an *Onchocerca volvulus* glyceraldehyde-3-phosphate dehydrogenase (Ov-GAPDH). Biochim. Biophys. Acta, 1741, 85-94.

Espinoza, E., Cordero, M., Muro, A., Lorente, F., Simón, F., 1993. Anti-*Dirofilaria immitis* IgE: seroepidemiology and seasonal variation in an exposed human population. Trop. Med. Parasitol. 44, 172-176.

Favia, G., Lanfrancotti, A., Della Torre, A., Cancrini, G., Coluzzi, M., 1996. Polymerase chain reaction-identification of *Dirofilaria repens* and *Dirofilaria immitis*. Parasitology 113, 567-571.

Fenn, K., Blaxter, M., 2006. *Wolbachia* genomes: revealing the biology of parasitism and mutualism. Trends Parasitol. 22, 60-65.

Ferri, E., Bain, O., Barbuto, M., Martin, C., Lo, N., Uni, S., Landmann, F., Baccei, S.G., Guerrero, R., de Souza Lima, S., Bandi, C., Wanji, S., Diagne, M., Casiraghi, M., 2011. New insights into the evolution of *Wolbachia* infections in filarial nematodes inferred from a large range of screened species. PLoS One 6, e20843.

Figuera, L., Acosta, H., Gómez-Arreaza, A., Dávila-Vera, D., Balza-Quintero, A., Quiñones, W., Mendoza-Briceño, R.V., Concepción, J.L., Avilán, L., 2013a. Plasminogen binding proteins in secreted membrane vesicles of *Leishmania mexicana*. Mol. Biochem. Parasitol. 187, 14-20.

Figuera, L., Gómez-Arreaza, A., Avilán, L., 2013b. Parasitism in optima forma: Exploiting the host fibrinolytic system for invasion. Acta Trop. 128, 116-123.

Flood, E.C., Hajjar, K.A., 2011. The annexin A2 system and vascular homeostasis. Vascul. Pharmacol. 54, 59-67.

**Foster, J., Ganatra, M., Kamal, I., Ware, J., Makarova, K., Ivanova, N., Bhattacharyya, A., Kapatral, V., Kumar, S., Posfai, J., Vincze, T., Ingram, J., Moran, L., Lapidus, A., Omelchenko, M., Kyrpides, N., Ghedin, E., Wang, S., Goltsman, E., Joukov, V., Ostrovskaya, O., Tsukerman, K., Mazur, M.,

Comb, D., Koonin, E., Slatko, B., 2005. The *Wolbachia* genome of *Brugia malayi*: endosymbiont evolution within a human pathogenic nematode. PLoS Biol. 3, e121.

Frank, G.R., Grieve, R.B., 1991. Metabolic labeling of *Dirofilaria immitis* third- and fourth-stage larvae and their excretory-secretory products. J. Parasitol. 77, 950-959.

Frank, G.R., Tripp, C.A., Grieve, R.B., 1996. Molecular cloning of a developmentally regulated protein isolated from excretory-secretory products of larval *Dirofilaria immitis*. Mol. Biochem. Parasitol. 75, 231-240.

Frank, G.R., Mondesire, R.R., Brandt, K.S., Wisnewski, N., 1998. Antibody to the *Dirofilaria immitis* aspartyl protease inhibitor homologue is a diagnostic marker for feline heartworm infections. J. Parasitol. 84, 1231-1236.

Frank, G.R., Wisnewsky, N., Brandt, K.S., Carter, C.R., Jennings, N.S., Selkirk, M.E., 1999. Molecular cloning of the 22-24 kDa excretory-secretory 22U protein of *Dirofilaria immitis* and other filarial nematode parasites. Mol. Biochem. Parasitol. 98, 297-302.

Fu, Y., Lan, J., Zhang, Z., Hou, R., Wu, X., Yang, D., Zhang, R., Zheng, W., Nie, H., Xie, Y., Yan, N., Yang, Z., Wang, C., Luo, L., Liu, L., Gu, X., Wang, S., Peng, X., Yang, G., 2012. Novel insights into the transcriptome of *Dirofilaria immitis*. PLoS One 7, e41639.

Fuhrman, B., 2012. The urokinase system in the pathogenesis of atherosclerosis. Atherosclerosis 222, 8-14.

Furlanello, T., Caldin, M., Vezzoni, A., Venco, L., Kitagawa, H., 1998. Patogenesi. En: Genchi, C., Venco, L., Vezzoni, A. (Eds.), La filariosi cardiopulmonare del cane e del gatto. Editorial Scivac, Italia, pp. 31-46.

Gaffney, P.J., Longstaff, C., 1994. An overiew of fibrinolysis. En: Bloom, A.L., Forbes, C.D., Thomas, D.P., Tuddenham, E.G.D. (Eds.), Hemostasis and Thrombosis, third edition. Churchill Livingstone, Edinburgh, pp. 549-571.

Galis, Z.S., Khatri, J.J., 2002. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. Circ. Res. 90, 251.

Geary, J., Satti, M., Moreno, Y., Madrill, N., Whitten, D., Headley, S.A., Agnew, D., Geary, T., Mackenzie, C., 2012. First analysis of the secretome of the canine heartworm, *Dirofilaria immitis*. Parasit. Vectors 5, 140.

Gebbink, M.F.B.G., 2011. Tissue-type plasminogen activator-mediated plasminogen activation and contact activation, implications in and beyond haemostasis. J. Thromb. Haemost. 9, 174-181.

Genchi, C., Kramer, L.H., Prieto, G., 2001. Epidemiology of canine and feline dirofilariasis: a global view. En: Simón, F., Genchi, C. (Eds.), Heartworm infection in humans and animals. Ediciones Universidad de Salamanca, España, pp. 121-134.

Genchi, C., Rinaldi, R., Mortarino, M., Genchi, M., Cringoli, G., 2009. Climate and *Dirofilaria* infection in Europe. Vet. Parasitol. 163, 286-292.

Genchi, C., Bowman, D., Drake, J., 2014. Canine heartworm disease (*Dirofilaria immitis*) in Western Europe: survey of veterinary awareness and perceptions. Parasit. Vectors 7, 206.

Ghosh, A.K., Coppens, I., Gårdsvoll, H., Ploug, M., Jacobs-Lorena, M., 2011. *Plasmodium* ookinetes coopt mammalian plasminogen to invade the mosquito midgut. PNAS 108, 17153-17158.

Godel, C., Kumar, S., Koutsovoulos, G., Ludin, P., Nilsson, D., Comandatore, F., Wrobel, N., Thompson, M., Schmid, C.D., Goto, S., Bringaud, F., Wolstenholme, A., Bandi, C., Epe, C., Kaminsky, R., Blaxter, M., Mäser, P., 2012. The genome of the heartworm, *Dirofilaria immitis*, reveals drug and vaccine targets. FASEB J. 26, 4650-4661.

Gómez-Arreaza, A., Acosta, H., Barros-Álvarez, X., Concepción, J.L., Albericio, F., Avilán, L., 2011. *Leishmania mexicana* LACK (*Leishmania* homolog of receptors for activated C-kinase) is a plasminogen binding protein. Exp. Parasitol. 127, 752-761.

Gong, Y., Hart, E., Shchurin, A., Hoover-Plow, J., 2008. Inflammatory macrophage migration requires MMP-9 activation by plasminogen in mice. J. Clin. Invest. 118, 3012-3024.

González-Miguel, J., Morchón, R., Siles-Lucas, M., Oleaga, A., Simón, F., 2010a. Identification of *Dirofilaria immitis* immunoreactive proteins recognized by sera from infected cats using two-dimensional electrophoresis and mass spectrometry. Mol. Biochem. Parasitol. 174, 78-82.

González-Miguel, J., Rosario, L., Rota-Nodari, E., Morchón, R., Simón, F., 2010b. Identification of immunoreactive proteins of *Dirofilaria immitis* and *D. repens* recognized by sera from patients with pulmonary and subcutaneous dirofilariosis. Parasitol. Int. 59, 248-256.

Grieve, R.B., Gebhardt, B.M., Bradley, R.E., Sr., 1979. *Dirofilaria immitis*: cell-mediated and humoral immune responses in experimentally-infected dogs. Int. J. Parasitol. 9, 275-279.

Grieve, R.B., Frank, G.R., Mika-Grieve, M., Culpepper, J.A., Mok, M., 1992. Identification of *Dirofilaria immitis* larval antigens with immunoprophylactic potential using sera from immune dogs. J. Immunol. 148, 2511-2515.

Guerrero, J., Rojo, F., Ródenas, A., 1989. Estudio de la incidencia de la enfermedad del gusano del corazón en la población canina española. Med. Vet. 6, 217-220.

Guerrero, J., Ducos de la Hitte, J., Genchi, C., Rojo, F., Gómez-Bautista, M., Varela, M.C., Labarthe, N., Bordini, E., Gonzales, G., Mancebo, O., Patino, F., Uribe, L.F., Samano, R., 1992. Update on the distribution of *Dirofilaria immitis* in dogs from Southern Europe and Latin America. En: Soll M.D. (Ed.), Proceedings Heartwom Symposium '92. American Heartworm Society, USA, pp. 31-37.

Guerrero, J., Nelson, C.T., Carithers, D.S., 2006. Results and realistic implications of the 2004 AHS-Merial heartworm survey. En: Proceedings of the AAVP 51st Annual Meeting. Honolulu, HI, USA, pp. 62-63.

Hajjar, K.A., Jacovina, A.T., Chacko, J., 1994. An endothelial cell receptor for plasminogen/tissue plasminogen activator. I. Identity with annexin II. J. Biol. Chem. 269, 21191-21197.

Hamel, D., Silaghi, C., Zapadynska, S., Kudrin, A., Pfister, K., 2013. Vector-borne pathogens in ticks and EDTA-blood samples collected from client-owned dogs, Kiev, Ukraine. Ticks Tick Borne Dis. 4, 152-155.

Haralampidis, S.T., 2003. Parasitic diseases of the animals and human. University Studio Press, Thessaloniki, Greece.

Harris, M.T., Fuhrman, J.A., 2002. Structure and expression of chitin synthase in the parasitic nematode *Dirofilaria immitis*. Mol. Biochem. Parasitol. 122, 231-234.

Hayashi, M., Matsuzaki, Y., Shimonaka, M., 2009. Impact of plasminogen on an in vitro wound healing model based on a perfusion cell culture system. Mol. Cell. Biochem. 322, 1-13.

He, L., Ren, M., Chen, X., Wang, X., Li, S., Lin, J., Liang, C., Liang, P., Hu, Y., Lei, H., Bian, M., Huang, Y., Wu, Z., Li, X., Yu, X., 2014. Biochemical and immunological characterization of annexin B30 from *Clonorchis sinensis* excretory/secretory products. Parasitol. Res. 113, 2743-2755.

Hidaka, Y., Hagio, M., Horii, Y., Murakami, T., Naganobu, K., Miyamoto, T., 2004. Histopathological and Enzyme Histochemical Observations on Mast Cells in Pulmonary Arterial Lesion of Dogs with *Dirofilaria immitis* Infestation. J. Vet. Med. Sci. 66, 1457-1462.

Ho-Tin-Noe, B., Enslen, H., Doeuvre, L., Corsi, J.M., Lijnen, H.R., Angles-Cano, E., 2009. Role of plasminogen activation in neuronal organization and survival. Mol. Cell. Neurosci. 42, 288-295.

Holvoet, P., Lijnen, H.R., Collen, D., 1985. A monoclonal antibody specific for lys-plasminogen. J. Biol. Chem. 260, 12106-12111.

Hong, X., Ma, D., Page, A.P., Kumar, S., Carlow, C.K., 1998. A highly conserved large molecular weight cyclophilin of filarial parasites. Exp. Parasitol. 88, 246-251.

Hu, Y., Zhang, E., Huang, L., Li, W., Liang, P., Wang, X., Xu, J., Huang, Y., Yu, X., 2014. Expression profiles of glyceraldehyde-3-phosphate dehydrogenase from *Clonorchis sinensis*: a glycolytic enzyme with plasminogen binding capacity. Parasitol. Res. 113, 4543-4553.

Huang, H., Wang, T., Yang, G., Zhang, Z., Wang, C., Yang, Z., Luo, L., Liu, L., Lan, J., Huang, X., 2009. Molecular characterization and phylogenetic analysis of *Dirofilaria immitis* of China based on COI and 12S rDNA genes. Vet. Parasitol. 160, 175-179.

Hungerford, J.E., Little, C.D., 1999. Developmental biology of the vascular smooth muscle cell: building a multilayered vessel wall. J. Vasc. Res. 36, 2.

Ibrahim, M.S., Tamashiro, W.K., Moraga, D.A., Scott, A.L., 1989. Antigen shedding from the surface of the infective stage larvae of *Dirofilaria immitis*. Parasitology 1, 89-97.

Ishihara, K., Kitagawa, H., Sasaky, Y., 1990. Efficacy of heartworm removal in dogs with dirofilarial hemoglobinuria using Flexible Alligator Forceps. Nihon Juigaku Zasshi 53, 591-599.

Iwaki, T., Urano, T., Umemura, K., 2012. PAI-1, progress in understanding the clinical problem and its aetiology. Br. J. Haematol. 157, 291-298.

Jolodar, A., Fischer, P., Bergmann, S., Büttner, D.W., Hammerschmidt, S., Brattig, N.W., 2003. Molecular cloning of an α-enolase from the human filarial parasite *Onchocerca volvulus* that binds human plasminogen. Biochim. Biophys. Acta 1627, 111-120.

Kadispasaoglu, A.K., Bilge, F.H., 1989. Partial characterization of the adsorbed protein layer on *Dirofilaria immitis* (nematode) cuticle. Parasitol. Res. 75, 554-558.

Kaiser, L., Spickard, R.C., Sparks, H.V., Jr., Williams, J.F., 1989. *Dirofilaria immitis*: Alteration of endothelium-dependent relaxation in the "in vivo" canine femoral artery. Exp. Parasitol. 69, 9-15.

Kaiser, L., Lamb, V.L., Tithof, P.K., Gage, D.A., Chamberlin, B.A., Watson, J.T., Williams, J.F., 1992. *Dirofilaria immitis*: do filarial cyclooxygenase products depress endothelium-dependent relaxation in the in vitro rat aorta? Exp. Parasitol. 75, 159-167.

Kaiser, L., Williams, J.F., 1998. *Dirofilaria immitis*: heartworm infection converts histamine-induced constriction to endothelium-dependent relaxation in canine pulmonary artery. Exp. Parasitol. 88, 146-153.

Kartashev, V., Batashova, I., Kartashov, S., Ermakov, A., Mironova, A., Kuleshova, Y., Ilyasov, B., Kolodiy, I., Klyuchnikov, A., Ryabikina, E., Babicheva, M., Levchenko, Y., Pavlova, R., Pantchev, N., Morchón, R., Simón, F., 2011. Canine and human dirofilariosis in the Rostov Region (Southern Russia). Vet. Med. Int. 2011, 685713.

Kawabata, A., Nakagaki, K., Yoshida, M., Shirota, K., 2008. Histopathological comparison of pulmonary artery lesions between raccoon dogs (*Nyctereutes procyonoides*) and domestic dogs experimentally infected with *Dirofilaria immitis*. J. Vet. Med. Sci. 70, 301-303.

Kendall, K., Collins, G.H., Pope, S.E., 1991. *Dirofilaria immitis* in cats from inner Sydney. Aust. Vet. J. 68, 356-357.

Khedri, J., Radfar, M.H., Borji, H., Azizzadeh, M., Akhtardanesh, B., 2014. Canine Heartworm in Southeastern of Iran with Review of disease distribution. Iran. J. Parasitol. 9, 560-567.

Kitagawa, H., Sasaky, Y., Ishiara, K., 1987. Canine dirofilarial hemoglobinuria: changes in right heart hemodynamics and heartworm migration from pulmonary artery towards right atrium following beta 1-blocker administration. Nihon Juigaku Zasshi 49, 1081-1086.

Kitt, A.J., Leigh, J.A., 1997. The auxotrophic nature of *Streptococcus uberis*: the acquisition of essential acids from plasmin derived casein peptides. Adv. Exp. Med. Biol. 418, 647-650.

Kittleson, M.D., 1998. Heartworm infestation and disease (dirofilariasis). En: Kittleson, M.D., Kienle, R.D. (Eds.), Small Animal Cardiovascular Medicine, 1st ed. Mosby, St Louis, USA, pp. 370-401.

Knight, D.H., 1995. Guidelines for diagnosis and management of heartworm (*Dirofilaria immitis*) infection. En: Bonagura, J.D. (Ed.), Kirk's Current Veterinary Therapy XII, Small Animal Practice. WB Saunders Co, USA, pp. 879-887.

Kozek, W.J., Figueroa, H.F., 1977. Intracytoplasmatic bacteria on *Onchocerca volvulus*. Am. J. Trop. Med. Hyg. 26, 663-678.

Kozek, W.J., Gonzalez, J.A., Jr., Amigo, L.A., 2007. *Wolbachia* of *Dirofilaria immitis*: an historical perspective and morphological characteristics. En: Genchi, C., Rinaldi, L., Cringoli, G. (Eds.), Mappe Parassitologiche 8. Rolando Editore, Naples, pp. 209-210.

Kramer, L.H., Tamarozzi, F., Morchón, R., López-Belmonte, J., Marcos-Atxutegi, C., Martín-Pacho, R., Simón F., 2005. Immune response to and tissue localization of the *Wolbachia* surface protein (WSP) in dogs with natural heartworm (*Dirofilaria immitis*) infection. Vet. Immunol. Immunopathol. 106, 303-308.

Labarthe, N., Guerrero, J., 2005. Epidemiology of heartworm: what is happening in South America and Mexico? Vet. Parasitol. 133, 149-156.

Lähteenmäki, K., Kuusela, P., Korhonen, T.K., 2001. Bacterial plasminogen activators and receptors. FEMS Microbiol. Rev. 25, 531-552.

Lama, A., Kucknoor, A., Mundodi, V., Alderete, J.F., 2009. Glyceraldehyde-3-phosphate dehydrogenase is a surface-associated, fibronectin-binding protein of *Trichomonas vaginalis*. Infect. Immun. 77, 2703-2711.

Lamb, T.J., Le Goff, L., Kurniawan, A., Guiliano, D.B., Fenn, K., Blaxter, M.L., Read, A.F., Allen, J.E., 2004. Most of the response elicited against *Wolbachia* surface protein in filarial nematode infection is due to the infective larval stage. J. Infect. Dis. 189, 120-127.

Lee, A.C., Montgomery, S.P., Theis, J.H., Blagburn, B.L., Eberhard, M.L., 2010. Public health issues concerning the widespread distribution of canine heartworm disease. Trends Parasitol. 26, 168-173.

Levy, J.K., Lappin, M.R., Glaser, A.L., Birkenheuer, A.J., Anderson, T.C., Edinboro, C.H., 2011. Prevalence of infectious diseases in cats and dogs rescued following Hurricane Katrina. J. Am. Vet. Med. Assoc. 238, 311-317.

Lijnen, H.R., 2006. Elements of the Fibrinolytic System. Ann. N. Y. Acad. Sci. 936, 226-236.

Lillibridge, C.D., Rudin, W., Philipp, M.T., 1996. *Dirofilaria immitis*: ultrastructural localization, molecular characterization, and analysis of the expression of p27, a small heat shock protein homolog of nematodes. Exp. Parasitol. 83, 30-45.

Limberger, R.J., McReynolds, L.A., 1990. Filarial paramyosin: cDNA sequences from *Dirofilaria immitis* and *Onchocerca volvulus*. Mol. Biochem. Parasitol. 38, 271-280.

Liu, L.X., Serhan, C.N., Weller, P.F., 1990. Intravascular filarial parasites elaborate cyclooxygenase-derived eicosanoids. J. Exp. Med. 172, 993-996.

Liu, L.X., Weller, P.F., 1990. Arachidonic acid metabolism in filarial parasites. Exp. Parasitol. 71, 496-501.

Lok, J.B., 1988. *Dirofilaria* spp.: Taxonomy and distribution. En: Boreham, P.F.L, Atwell, R.B. (Eds.), Dirofilariasis. CRC Press, Inc., Boca Raton, Florida, USA, pp. 1-28.

Loskutoff, D.J. 1991. Regulation of PAI-1 gene expression. Fibrinolysis 5, 197-206.

Lottenberg, R., Minning-Wenz, D., Boyle, M.D., 1994. Capturing host plasmin(ogen): a common mechanism for invasive pathogens? Trends Microbiol. 2, 20-24.

Luo, M., Hajjar, K.A., 2013. Annexin A2 system in human biology: cell surface and beyond. Semin. Thromb. Hemost. 39, 338-346.

Madureira, P.A., Surette, A.P., Phipps, K.D., Taboski, M.A.S., Miller, V.A., Waisman, D.M., 2011. The role of the annexin A2 heterotetramer in vascular fibrinolysis. Blood 118, 4789-4797.

Maldonado, J., Calcagno, M., Puig, J., Maizo, Z., Avilán, L.A., 2006. A study of cutaneous lesions caused by *Leishmania mexicana* in plasminogen-deficient mice. Exp. Mol. Pathol. 80, 289-294.

Manfredi, M.T., Di Cerbo, A., Genchi, M., 2007. Biology of filarial worms parasitizing dogs and cats. En: Genchi, C., Rinaldi, L., Cringoli, G. (Eds.), Mappe Parassitologiche 8. Rolando Editore, Naples, pp. 39-47.

Marcilla, A., Pérez-García, A., Espert, A., Bernal, D., Muñoz-Antolí, C., Esteban, J.G., Toledo, R., 2007. *Echinostoma caproni*: identification of enolase in excretory/secretory products, molecular cloning, and functional expression. Exp. Parasitol. 117, 57-64.

Marcos-Atxutegi, C., Kramer, L.H., Fernández, I., Simoncini, L., Genchi, M., Prieto, G., Simón F., 2003. Th_1 response in BALB/c mice immunized with *Dirofilaria immitis* soluble antigens: a possible role for *Wolbachia*? Vet. Parasitol. 112, 117-130.

Marcos-Atxutegi, C., Gabrielli, S., Kramer, L.H., Cancrini, G., Simón, F., 2004. Antibody response against *Dirofilaria immitis* and the *Wolbachia* endosymbiont in naturally infected canine and human hosts. En: Mas-Coma S. (Ed.), Proceedings of the IX European Multicolloquium of Parasitology (EMOP IX). Editorial Medimond, Italia, pp. 297-302.

Marquardt, W.C., Demaree, R.S., Grieve, R.B., 2000. Parasitology and Vector Biology. Harcourt Academic Press, USA, 702 pp.

Martinez-Lemus, L.A., 2012. The dynamic structure of arterioles. Basic Clin. Pharmacol. Toxicol. 110, 5-11.

McCall, J.W., Dzimiansky, T.M., Plue, R.E., Seward, L., Blair, L.S., 1986. Ivermectine in heartworm prophylaxis: study with experimentally induced and naturally acquired infections. En: Otto, G.F. (Ed.), Proceedings of the Heartworm Symposium ‘86. American Heartworm Society, Batavia, IL, USA, pp. 9-14.

McCall, J.W., 1992. A parallel between experimentally induced canine and feline heartworm disease. En: Proceedings of XVII WSAVA World Congress. Delfino Publisher, Rome, pp. 255-261.

McCall, J.W., Genchi, C., Kramer, L.H., Guerrero, J., Venco, L., 2008. Heartworm disease in animals and humans. Adv. Parasitol. 66, 193-285.

McCracken, M.D., Patton, S., 1993. Pulmonary Arterial Changes in Feline Dirofilariasis. Vet. Pathol. 30, 64-69.

McGarry, H.F., Egerton, G.L., Taylor, M.J., 2004. Population dynamics of *Wolbachia* bacterial endosymbionts in *Brugia malayi*. Mol. Biochem. Parasitol. 135, 57-67.

McLaren, D.J., Worms, M.J., Laurence, B.R., Simpson, M.G., 1975. Microorganisms in filarial larvae (Nematoda). Trans. R. Soc. Trop. Med. Hyg. 69, 509-514.

McTier, T.L., McCall, J.W., Dzimianski, M.T., Aguilar, R., Wood, I., 1992. Prevention of heartworm infection in cats by treatment with ivermectin at one month post-infection. En: Soll, M.D. (Ed.), Proceedings of the Heartworm Symposium ‘92. American Heartworm Society, Batavia, IL, USA, pp. 111–116.

Michiels, C., 2003. Endothelial cell functions. J. Cell. Physiol. 196, 430-443.

Miles, L.A., Hawley, S.B., Baik, N., Andronicos, N.M., Castellino, F.J., Parmer, R.J., 2005. Plasminogen receptors: the sine qua non of cell surface plasminogen activation. Front. Biosci. 10, 1754-1762.

Montoya, J.A., Morales, M., Juste, M.C., Corbera, J.A., 2007. Heartworm (*Dirofilaria immitis*) infection in dogs: current update in Spain. En: Genchi, C., Rinaldi, L., Cringoli, G. (Eds.), *Dirofilaria immitis* and *D. repens* in dog and cat and human infections. Rolando Editore, Naples, Italy, pp. 175-180.

Morchón, R., López-Belmonte, J., Rodríguez-Barbero, A., Simón, F., 2006. High levels of serum thromboxane B2 are generated during human pulmonary dirofilariosis. Clin. Vaccine Immunol. 13, 1175-1176.

Morchón, R., Bazzocchi, C., López-Belmonte, J., Martín-Pacho, J.R., Kramer, L.H., Grandi, G., Simón, F., 2007a. iNOs expression is stimulated by the major surface protein (WSP) from *Wolbachia* symbiont bacteria of *Dirofilaria immitis* in a murine model of immunization. Parasitol. Int. 56, 71-75.

Morchón, R., López-Belmonte, J., Bazzocchi, C., Grandi, G., Kramer, L., Simón, F., 2007b. Dogs with patent *Dirofilaria immitis* infection have higher expression of

circulating IL-4, IL-10 and iNOs mRNA than those with occult infection. Vet. Immunol. Immunopathol. 115, 184-188.

Morchón, R., Roca, F., López-Belmonte, J., Genchi, M., Venco, L., Rodríguez-Barbero, A., Simón, F., 2007c. Changes in the levels of eicosanoids in cats naturally and experimentally infected with *Dirofilaria immitis*. Vet. Parasitol. 147, 271-275.

Morchón, R., Rodríguez-Barbero, A., Velasco, S., López-Belmonte, J., Simón, F., 2008. Vascular endothelial cell activation by adult *Dirofilaria immitis* antigens. Parasitol. Int. 57, 441-446.

Morchón, R., Juste, M.C., Montoya, J.A., González-Miguel, J., Rodríguez-Barbero, A., Simón, F., 2009. Are microfilariae related to the production of eicosanoids in dogs naturally infected with *Dirofilaria immitis*? Rev. Iber. Parasitol. 1, 21-24.

Morchón, R., González-Miguel, J., Mellado, I., Velasco, S., Rodríguez-Barbero, A., Simón, F., 2010. Adult *Dirofilaria immitis* excretory/secretory antigens upregulate the production of prostaglandin E2 and downregulate monocyte transmigration in an "in vitro" model of vascular endothelial cell cultures. Vet. Parasitol. 170, 331-335.

Morchón, R., Carretón, E., González-Miguel, J., Mellado-Hernández, I., 2012a. Heartworm Disease (*Dirofilaria immitis*) and Their Vectors in Europe - New Distribution Trends. Front. Physiol. 3, 196.

Morchón, R., Carretón, E., Grandi, G., González-Miguel, J., Montoya-Alonso, J.A., Simón, F., Genchi, C., Kramer, L.H., 2012b. Anti-*Wolbachia* surface protein antibodies are present in the urine of dogs naturally infected with *Dirofilaria immitis* with circulating microfilariae but not in dogs with occult infections. Vector Borne Zoonotic Dis. 12, 17-20.

Morchón, R., González-Miguel, J., Carretón, E., Kramer, L.H., Valero, L., Montoya-Alonso, J.A., Simón, F., Siles-Lucas, M., 2014. Proteomic analysis of the somatic and surface compartments from *Dirofilaria immitis* adult worms. Vet. Parasitol. 203, 144-52.

Moreau, E., Chauvin, A., 2010. Immunity against helminths: interactions with the host and the intercurrent infections. J. Biomed. Biotechnol. 2010, 428593.

Mosher, D.F., 1990. Blood coagulation and fibrinolysis: an overview. Clin. Cardiol. 13, VI5-11.

Mundodi, V., Kucknoor, A.S., Alderete, J.F., 2008. Immunogenic and plasminogen-binding surface-associated α-enolase of *Trichomonas vaginalis*. Infect. Immun. 76, 523-531.

Mupanomunda, M., Williams, J.F., Mackenzie, C.D., Kaiser, L., 1997. *Dirofilaria immitis*: heartworm infection alters pulmonary artery endothelial cell behavior. J. Appl. Physiol. 82, 389-398.

Nagase, H., Visse, R., Murphy, G., 2006. Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc. Res. 69, 562-573.

Nelson, C.T., McCall, J.W., Rubin, S.B., Buzhardt, L.F., Dorion, D.W., Graham, W., Longhofer, S.L., Guerrero, J., Robertson-Plouch, C., Paul, A.; Executive Board of the American Heartworm Society, 2005. 2005 guidelines for the diagnosis, prevention and management of heartworm (*Dirofilaria immitis*) infection in dogs. Vet. Parasitol. 133, 255-266.

Nesheim, M., Wang, W., Boffa, M., Nagashima, M., Morser, J., Bajzar, L., 1997. Thrombin, thrombomodulin and TAFI in the molecular link between coagulation and fibrinolysis. Thromb. Haemost. 78, 386-391.

Newcombe, K.M., Ryan, W.G., 2002. Prevalence of feline heartworm disease—a global review. En: Proceedings of the WSAVA, Granada, Spain.

Nicholl, S.M., Roztocil, E., Galaria, I.I., Davies, M.G., 2005. Plasmin induces smooth muscle cell proliferation. J. Surg. Res. 127, 39-45.

Nicholl, S.M., Roztocil, E., Davies, M.G., 2006. Plasminogen Activator System and Vascular Disease. Curr. Vasc. Pharmacol. 4, 101-116.

Oleaga, A., Pérez-Sanchéz, R., Pagés, E., Marcos-Atxutegi, C., Simón, F., 2009. Identification of immunoreactive proteins from the dog heartworm (*Dirofilaria immitis*) differentially recognized by the sera from dogs with patent or occult infections. Mol. Biochem. Parasitol. 166, 134-141.

Owhashi, M., Futaki, S., Kitagawa, K., Horii, Y., Maruyama, H., Hayashi, H., Nawa, Y., 1993. Molecular cloning and characterization of a novel neutrophil chemotactic factor from a filarial parasite. Mol. Immunol. 30, 1315-1320.

Patnaik, M.M., 1989. On filarial nematodes in domestic animals in Orissa. Indian Vet. J. 66, 573-574.

Pennica, D., Holmes, W.E., Kohr, W.J., Harkins, R.N., Vehar, G.A., Ward, C.A., Bennet, W.F., Yelverton, E., Seeburg, P.H., Heyneker, H.L., Goeddel, D.V., Collen, D., 1983. Cloning and expression of human tissue-type plasminogen activator cDNA in *E. coli*. Nature 301, 214-221.

Peribáñez, M.A., Lucientes, J., Arce, S., Morales, M., Castillo, J.A., Gracia, M.J., 2001. Histochemical differentiation of *Dirofilaria immitis*, *Dirofilaria repens* and *Acanthocheilonema dracunculoides* microfilariae by staining with a commercial kit, Leucognost-SP. Vet. Parasitol. 102, 173-175.

Philipp, M., Davis, T.B., 1986. Biochemical and immunologic characterization of a major surface antigen of *Dirofilaria immitis* infective larvae. J. Immunol. 136, 2621-2627.

Prieto, G., McCall, J.W., Venco, L., Genchi, M., Simón, F., Genchi, C., 2001. IgG response against infective larvae of *Dirofilaria immitis* in experimentally infected cats. Vet. Res. 32, 93-96.

Prieto, G., Ceciliani, F., Venco, L., Morchón, R., Simón, F., 2002. Feline dirofilariosis: antibody response to antigenic fractions containing specific 20 to 30 kDa polypeptides from the adult *Dirofilaria immitis* somatic antigen. Vet. Parasitol. 103, 341-353.

Quinn, R., Williams, J., 2011. Hipertensión pulmonar canina, Parte 1: una revisión a fondo de su fisiopatología y clasificaciones. Vet. Med. 6, 18-25.

Ramajo-Hernández, A., Pérez-Sánchez, R., Ramajo-Martín, V., Oleaga, A., 2007. *Schistosoma bovis*: plasminogen binding in adults and the identification of plasminogen-binding proteins from the worm tegument. Exp. Parasitol. 115, 83-91.

Rawlings, C.A., Dawe, D.L., McCall, J.W., Keith, J.C., Prestwood, A.K., 1982. Four types of occult *Dirofilaria immitis* infection in dogs. J. Am. Vet. Med. Assoc. 180, 1323- 1326.

Rawlings, C.A., 1986. Heartworm disease in dogs and cats. WB Saunders Co, Philadelphia, PA, USA, 329 pp.

Richer, J.K., Sakanari, J.A., Frank, G.R., Grieve, R.B., 1992. *Dirofilaria immitis*: proteases product by third-and fourth-stage larvae. Exp. Parasitol. 75, 213-222.

Rijken, D.C., Lijnen, H.R., 2009. New insights into the molecular mechanisms of the fibrinolytic system. J. Thromb. Haemost. 7, 4-13.

Rojas, M., Labrador, I., Concepción, J.L., Aldana, E., Avilán, L., 2008. Characteristics of plasminogen binding to *Trypanosoma cruzi* epimastigotes. Acta Trop. 107, 54-58.

Rossignol, P., Angles-Cano, E., Lijnen, H.R., 2006. Plasminogen activator inhibitor–1 impairs plasminogen activation–mediated vascular smooth muscle cell apoptosis. Thromb. Haemost. 96, 665-670.

Roth, D., Piekarek, M., Paulsson, M., Christ, H., Bloch, W., Krieg, T., Davidson, J.M., Eming, S.A., 2006. Plasmin modulates vascular endothelial growth factor-A-mediated angiogenesis during wound repair. Am. J. Pathol. 168, 670-684.

Sakharov, D.V., Plow, E.F., Rijken, D.C., 1997. On the mechanism of the antifibrinolytic activity of plasma carboxypeptidase B. J. Biol. Chem. 272, 14477-14482.

Sanderson-Smith, M.L., De Oliveira, D.M., Ranson, M., McArthur, J.D., 2012. Bacterial plasminogen receptors: mediators of a multifaceted relationship. J. Biomed. Biotechnol. 2012, 272148.

Sato, M., Koyama, A., Iwai, K., Kawabata, Y., Kojima, S., 1985. Human pulmonary dirofilariasis with special reference to the ELISA for the diagnosis and follow-up study. Z. Parasitenkd. 71, 561-563.

Schleef, R.R., Higgins, D.L., Pillemer, E., Levitt, L.J., 1989. Bleeding diathesis due to decreased functional activity of type 1 plasminogen activator inhibitor. J. Clin. Invest. 83, 1747-1752.

Schmitt, M., Harbeck, N., Thomssen, C., Wilhelm, O., Magdolen, V., Reuning, U., Ulm, K., Höfler, H., Jänicke, F., Graeff, H., 1997. Clinical impact of the plasminogen activation system in tumor invasion and metastasis: prognostic relevance and target for therapy. Thromb. Haemost. 78, 285-296.

Schwartz, S.M., 1997. Smooth muscle migration in vascular development and pathogenesis. Transpl. Immunol. 5, 255-260.

Scott, A.L., Diala, C., Moraga, D.A., Ibrahim, M.S., Redding, L., Tamashiro, W.K., 1988. *Dirofilaria immitis*: biochemical and immunological characterization of the surface antigens from adult parasites. Exp. Parasitol. 67, 307-323.

Scott, A.L., Ibrahim, M.S., Tamashiro, W.K., 1990. Surface-associated antigens of second, third and fourth stage larvae of *Dirofilaria immitis*. Acta Trop. 47, 339-353.

Shea, C., Hough, D., Xiao, J., Tzertzinis, G., Maina, C.V., 2004. An rxr/usp homolog from the parasitic nematode, *Dirofilaria immitis*. Gene 324, 171-182.

Siemens, N., Patenge, N., Otto, J., Fiedler, T., Kreikemeyer, B., 2011. *Streptococcus pyogenes* M49 plasminogen/plasmin binding facilitates keratinocyte invasion via integrin–integrin-linked kinase (ILK) pathways and protects from macrophage killing. J. Biol. Chem. 286, 21612-21622.

Simón, F., Muro-Alvarez, A., Cordero-Sánchez, M., Martín-Martín, J., 1991. A seroepidemiologic survey of human dirofilariosis in Western Spain. Trop. Med. Parasitol. 42, 106-108.

Simón, F., Genchi, C., 2000. Dirofilariasis and other zoonotic filariases: an emerging public health problem in developed countries. Res. Rev. Parasitol. 60, 1-16.

Simón, F., Genchi, C., Prieto, G., Allende, E., 2001. Immunity in the vertebrate hosts. En: Simón, F., Genchi, C. (Eds.), Heartworm infection in humans and animals. Ediciones Universidad de Salamanca, Salamanca, Spain, p. 218.

Simón, F., Prieto, G., Morchón, R., Bazzocchi, C., Bandi, C., Genchi, C., 2003. Immunoglobulin G antibodies against the endosymbionts of filarial nematodes (*Wolbachia*) in patients with pulmonary dirofilariasis. Clin. Diagn. Lab. Immunol. 10, 180-181.

Simón, F., López-Belmonte, J., Marcos-Atxutegi, C., Morchón, R., Martín-Pacho, J.R., 2005. What is happening outside North America regarding human dirofilariasis? Vet. Parasitol. 133, 181-189.

Simón, F., Kramer, L.H., Román, A., Blasini, W., Morchón, R., Marcos-Atxutegi, C., Grandi, G., Genchi, C., 2007. Immunopathology of *Dirofilaria immitis* infection. Vet. Res. Commun. 31, 161-171.

Simón, F., Morchón, R., Rodríguez-Barbero, A., López-Belmonte, J., Grandi, G., Genchi, C., 2008. *Dirofilaria immitis* and *Wolbachia*-derived antigens: its effect on endothelial mammal cells. Vet. Parasitol. 158, 223-231.

Simón, F., Morchón, R., González-Miguel, J., Marcos-Atxutegi, C., Siles-Lucas, M., 2009. What is new about animal and human dirofilariosis? Trends Parasitol. 25, 404-409.

Simón, F., Siles-Lucas, M., Morchón, R., González-Miguel, J., Mellado, I., Carretón, E., Montoya-Alonso, J.A., 2012. Human and animal dirofilariasis: the emergence of a zoonotic mosaic. Clin. Microbiol. Rev. 25, 507-544.

Simón, L., Afonin, A., López-Díez, L.I., González-Miguel, J., Morchón, R., Carretón, E., Montoya-Alonso, J.A., Kartashev, V., Simón, F., 2014. Geo-environmental model for the prediction of potential transmission risk of *Dirofilaria* in an area with dry climate and extensive irrigated crops. The case of Spain. Vet. Parasitol. 200, 257-264.

Sironi, M., Bandi, C., Sacchi, L., Di Sacco, B., Damiani, G., Genchi, C., 1995. Molecular evidence of close relative of the arthropod endosymbiont *Wolbachia* in a filarial worm. Mol. Biochem. Parasitol. 74, 223-227.

Slocombe, J.O., Villeneuve, A., 1993. Heartworm in dogs in Canada in 1991. Can. Vet. J. 34, 630-633.

Soares, H.S., Camargo, L.M., Gennari, S.M., Labruna, M.B., 2014. Survey of canine tick-borne diseases in Lábrea, Brazilian Amazon: 'accidental' findings of *Dirofilaria immitis* infection. Rev. Bras. Parasitol. Vet. 23, 473-80.

Song, K.H., Lee, S.E., Hayasaki, M., Shiramizu, K., Kim, D.H., Cho, K.W., 2003. Seroprevalence of canine dirofilariosis in South Korea. Vet. Parasitol. 114, 231-236.

Sorci, G., Cornet, S., Faivre, B., 2013. Immune evasion, immunopathology and the regulation of the immune system. Pathogens 2, 71-91.

Sun, H., 2006. The interaction between pathogens and the host coagulation system. Physiology 21, 281-288.

Sun, M., Zhuo, W., Guo, S., Liao, S., Shi, D., Liu, J., Cheng, Z., Liu, Y., Niu, X., Wang, S., Yang, D., 2012. Serological survey of canine dirofilariosis in Chongqing, Kunming, Nanchang, Fuzhou, Guangzhou, Shenzhen, and Nanning in Southern China. Vet. Parasitol. 185, 225-228.

Syrovets, T., Simmet, T., 2004. Novel aspects and new roles for the serine protease plasmin. Cell. Mol. Life Sci. 61, 873-885.

Syrovets, T., Lunov, O., Simmet, T., 2012. Plasmin as a proinflammatory cell activator. J. Leukoc. Biol. 92, 509-519.

Tamashiro, W.K., Rao, M., Scott, A.L., 1987. Proteolytic cleavage of IgG and other protein substrates by *Dirofilaria immitis* microfilarial enzymes. J. Parasitol. 73, 149-154.

Tanaka, H., Watanabe, M., Ogawa, Y., 1985. Parasites of stray dogs and cats in the Kanto region, Honshu, Japan. J. Vet. Med. Jap. 771, 657-661.

Tate, K.M., Higgins, D.L., Holmes, W.E., Winkler, M.E., Heyneker, H.L., Vehar, G.L., 1987. Functional role of proteolytic cleavage at arginine-275 of human tissue plasminogen activator as assessed by site-directed mutagenesis. Biochemistry 26, 338-343.

Toledo, A., Coleman, J.L., Kuhlow, C.J., Crowley, J.T., Benach, J.L., 2012. The enolase of *Borrelia burgdorferi* is a plasminogen receptor released in outer membrane vesicles. Infect. Immun. 80, 359-368.

Townsley, M.I., 2012. Structure and composition of pulmonary arteries, capillaries and veins. Compr. Physiol. 2, 675-709.

Tripp, C., Frank, R.S., Selkirk, M.E., Tang, L., Grieve, M.M., Frank, G.R., Grieve, R.B., 1998. *Dirofilaria immitis*: molecular cloning and expression of a cDNA encoding a selenium-independent secreted glutathione peroxidase. Exp. Parasitol. 88, 43-50.

Turner, J.D., Langley, R.S., Johnston, K.L., Gentil, K., Ford, L., Wu, B., Graham, M., Sharpley, F., Slatko, B., Pearlman, E., Taylor, M.J., 2009. *Wolbachia* lipoprotein stimulates innate and adaptive immunity through Toll-like receptors 2

and 6 to induce disease manifestations of filariasis. J. Biol. Chem. 284, 22364-22378.

Vanegas, G., Quiñones, W., Carrasco-López, C., Concepción, J.L., Albericio, F., Avilán, L., 2007. Enolase as a plasminogen binding protein in *Leishmania mexicana*. Parasitol. Res. 101, 1511-1516.

Venco, L., Furlanello, F., Vezzoni, A., 1998. Orientamenti terapeutici. En: Genchi, C., Venco, L., Vezzoni, A. (Eds.), La filariosi cardiopulmonare del cane e del gatto. Editorial Scivac, Cremona, Italy, p. 198.

Venco, L., Vezzoni, A., 2001. Heartworm (*Dirofilaria immitis*) disease in dogs and cats. En: Simón, F., Genchi, C. (Eds.), Heartworm infection in humans and animals. Ediciones Universidad de Salamanca, Salamanca, Spain, pp. 161-177.

Venco, L., Genchi, C., Vigevani Colson, P., Kramer, L., 2003. Relative utility of echocardiography, radiography, serologic testing and microfilariae counts to predict adult worm burden in dogs naturally infected with heartworms. En: Seward, R.L., Knight, D.H. (Eds.), Recent advances in heartworm disease: American Heartworm Society Symposium '01. American Heartworm Society, Batavia, IL, USA, pp. 111-124.

Venco, L., McCall, J.W., Guerrero, J., Genchi, C., 2004. Efficacy of long-term monthly administration of ivermectin on the progress of naturally acquired heartworm infection in dogs. Vet. Parasitol. 124, 259-268.

Venco, L., 2007. Heartworm (*Dirofilaria immitis*) disease in dogs. En: Genchi, C., Rinaldi, L., Cringoli, G. (Eds.), *Dirofilaria immitis* and *D. repens* in dog and cat and human infections. Rolando Editore, Naples, Italy, pp. 117-125.

Venco, L., Genchi, C., Simón, F., 2011. La filariosis cardiopulmonar (*Dirofilaria immitis*) en el perro. En: Simón, F., Genchi, C., Venco, L., Montoya, M.N. (Eds.), La filariosis en las especies domésticas y en el hombre. Merial Laboratorios, Barcelona, Spain, pp. 19-60.

Vezzani, D., Eiras, F.D., Wisnivesky, C., 2006. Dirofilariasis in Argentina: historical review and first report of *Dirofilaria immitis* in a natural mosquito population. Vet. Parasitol. 136, 259-273.

Vieira, L., Silvestre-Ferreira, A.C., Fontes-Sousa, A.P., Balreira, A.C., Morchón, R., Carretón, E., Vilhena, H., Simón, F., Montoya-Alonso, J.A., 2014a. Seroprevalence of heartworm (*Dirofilaria immitis*) in feline and canine hosts from central and northern Portugal. J. Helminthol. 14, 1-5.

Vieira, L., Vieira, M.J., Oliveira, J.M., Simões, A.R., Diez-Baños, P., Gestal, J., 2014b. Prevalence of canine heartworm (*Dirofilaria immitis*) disease in dogs of central Portugal. Parasite 21, 5.

Vincent, A.L., Partono, J.K., Ash, L.R., 1975. A comparison of the body wall ultrastructure of *Brugia pahangi* with that of *Brugia malayi*. J. Parasitol. 63, 567-570.

Wagenseil, J.E., Mecham, R.P., 2009. Vascular extracellular matrix and arterial mechanics. Physiol. Rev. 89, 957-989.

Wang, J.S., Tung, K.C., Huang, C.C., Lai, C.H., 2005. Alteration of extracellular collagen matrix in the myocardium of canines infected with *Dirofilaria immitis*. Vet. Parasitol. 131, 261-265.

Wang, X., Chen, W., Hu, F., Deng, C., Zhou, C., Lv, X., Fan, Y., Men, J., Huang, Y., Sun, J., Hu, D., Chen, J., Yang, Y., Liang, C., Zheng, H., Hu, X., Xu, J., Wu, Z., Yu, X., 2011. *Clonorchis sinensis* enolase: identification and biochemical characterization of a glycolytic enzyme from excretory/secretory products. Mol. Biochem. Parasitol. 177, 135-142.

Wu, C.C., Fan, P.C., 2003. Prevalence of canine dirofilariasis in Taiwan. J. Helminthol. 77, 83-88.

Xie, X., Bain, O., Williams, S.A., 1994. Molecular phylogenetic studies on filarial parasite based on 5S ribosomal spacer sequences. Parasite 1, 141-151.

Yang, J., Qiu, C., Xia, Y., Yao, L., Fu, Z., Yuan, C., Feng, X., Lin, J., 2010. Molecular cloning and functional characterization of *Schistosoma japonicum* enolase which is highly expressed at the schistosomulum stage. Parasitol. Res. 107, 667-677.

Yang, Z., Eton, D., Zheng, F., Livingstone, A.S., Yu, H., 2005. Effect of tissue plasminogen activator on vascular smooth muscle cells. J. Vasc. Surg. 42, 532-538.

Yates, D.M., Wolstenholme, A.J., 2004. An ivermectin-sensitive glutamate-gated chloride channel subunit from *Dirofilaria immitis*. Int. J. Parasitol. 34, 1075-1081.

Yavlovich, A., Katzenell, A., Tarshis, M., Higazi, A.A., Rottem, S., 2004. *Mycoplasma fermentans* binds to and invades HeLa cells: involvement of plasminogen and urokinase. Infect. Immun. 72, 5004-5011.

Yavlovich, A., Rottem, S., 2007. Binding of host extracellular matrix proteins to *Mycoplasma fermentans* and its effect on adherence to, and invasion of HeLa cells. FEMS Microbiol. Lett. 266, 158-162.

Zhang, S., Guo, A., Zhu, X., You, Y., Hou, J., Wang, Q., Luo, X., Cai, X., 2015. Identification and functional characterization of alpha-enolase from *Taenia pisiformis* metacestode. Acta Trop. 144, 31-40.

Zipfel, P.F., Schrum, S., Bialonski, A., Büttner, D.W., 1998. The peroxidoxin 2 protein of the human parasite *Onchocerca volvulus*: recombinant expression,

immunolocalization, and demonstration of homologous molecules in other species. Parasitol. Res. 84, 623-631.

Zorio, E., Gilabert-Estellés, J., España, F., Ramón, L.A., Cosín, R., Estellés, A., 2008. Fibrinolysis: the key to new pathogenetic mechanisms. Curr. Med. Chem. 15, 923-929.

HIPÓTESIS Y OBJETIVOS

Uno de los hechos clave de la dirofilariosis cardiopulmonar es el desarrollo de endarteritis proliferativa, un proceso hiperplásico de la pared arterial, de gran importancia para el desarrollo posterior de la patología pulmonar y cardíaca. Pueden producirse, además, graves tromboembolismos causados por la muerte de los vermes adultos de *Dirofilaria immitis*, que ocurren, de forma natural o como consecuencia de un tratamiento filaricida. Puesto que los mecanismos de estas alteraciones no son bien conocidos, es necesario estudiarlos, ya que su entendimiento puede facilitar el manejo de estas situaciones por los clínicos veterinarios, contribuyendo a mejorar la calidad de vida de los animales afectados. Dada la capacidad de supervivencia de *D. immitis* en sus hospedadores y el hecho de que los tromboembolismos aparecen cuando mueren los vermes, nuestra primera hipótesis fue que el parásito controla y modifica el hábitat sanguíneo para facilitar su supervivencia a través de moléculas presentes en sus productos antigénicos, generando un estado neto antitrombótico mediante la utilización de productos profibrinolíticos.

Por otra parte, la supuesta activación del sistema fibrinolítico a largo plazo por parte del parásito podría generar una sobreproducción de plasmina, producto final de esta ruta. Este fenómeno ha sido relacionado en otros contextos con situaciones patológicas graves que incluyen proliferación y migración de las células de la pared arterial, así como destrucción de la matriz extracelular. Dada la aparente similitud entre estos procesos patológicos y los que se producen durante el desarrollo de la endarteritis proliferativa en la dirofilariosis cardiopulmonar, nuestra segunda hipótesis fue que la sobreactivación de la ruta fibrinolítica por parte de *D. immitis* estaría directamente relacionada con la aparición de dichos procesos patológicos en la pared vascular de los animales afectados. Para demostrar ambas hipótesis propusimos los siguientes objetivos dentro de la presente Tesis Doctoral:

1. Analizar la interacción de los antígenos de *D. immitis* con el sistema fibrinolítico de su hospedador en relación con los mecanismos de supervivencia a nivel vascular.
2. Estudiar si la activación del sistema fibrinolítico por parte del parásito tiene influencia en los procesos patológicos descritos en el desarrollo de la endarteritis proliferativa en la dirofilariosis cardiopulmonar.

PRIMER CAPÍTULO

"Excretory/secretory antigens from *Dirofilaria immitis* adult worms interact with the host fibrinolytic system involving the vascular endothelium"

Los antígenos excretores/secretores de los vermes adultos de *Dirofilaria immitis* interaccionan con el sistema fibrinolítico del hospedador implicando al endotelio vascular

Javier González-Miguel, Rodrigo Morchón, Isabel Mellado, Elena Carretón, José Alberto Montoya-Alonso, Fernando Simón

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Resumen

Dirofilaria immitis es el agente causal de la dirofilariosis cardiopulmonar canina y felina. El parásito puede sobrevivir durante largos períodos de tiempo (7 años o más) en el sistema circulatorio de los reservorios inmunocompetentes, produciendo generalmente una enfermedad vascular inflamatoria crónica. Además, la muerte simultánea de grupos de vermes adultos puede desencadenar una patología aguda caracterizada por la exacerbación de las reacciones inflamatorias y la aparición de tromboembolismos graves. En el contexto de las relaciones *D. immitis*/hospedador, el objetivo de este trabajo fue investigar la interacción entre los antígenos excretores/secretores de los vermes adultos de *D. immitis* (DiES) y el sistema fibrinolítico del hospedador. Mediante el empleo de un enzimoinmunoensayo demostramos que el extracto DiES es capaz de fijar plasminógeno y generar plasmina, aunque este hecho requiere la presencia del activador tisular del plasminógeno (tPA). Por otra parte, establecemos que el extracto DiES aumenta la expresión de tPA en cultivos de células endoteliales vasculares. Adicionalmente, 10 proteínas fijadoras de plasminógeno del extracto DiES fueron identificadas por espectrometría de masas (HSP60, actina-1/3, actina, actina 4, transglutaminasa, GAPDH, Ov87, LOAG_14743, galectina y P22U). Los datos sugieren que los antígenos del extracto DiES interaccionan con el entorno del parásito regulando la activación del sistema fibrinolítico del hospedador e implicando al endotelio vascular en el proceso.

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Excretory/secretory antigens from *Dirofilaria immitis* adult worms interact with the host fibrinolytic system involving the vascular endothelium

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Abstract

Dirofilaria immitis is the causative agent of canine and feline heartworm disease. The parasite can survive for long periods of time (7 years or more) in the circulatory system of immunocompetent reservoirs, producing usually a chronic inflammatory vascular disease. In addition, the simultaneous death of groups of adult worms can trigger an acute disease characterized by the exacerbation of inflammatory reactions and the emergence of serious thromboembolic events. In the context of the *D. immitis*/host relationships, the aim of this study was to investigate the interaction between the excretory/secretory antigens from *D. immitis* adult worms (DiES) and the fibrinolytic system of the host. Using an enzyme-linked immunosorbent assay we showed that DiES extract is able to bind plasminogen and generate plasmin, although this fact requires the presence of the tissue plasminogen activator (t-PA). Moreover, we established that DiES extract enhances t-PA expression in cultured vascular endothelial cells. Additionally, 10 plasminogen-binding proteins from DiES extract were identified by mass spectrometry (HSP60, actin-1/3, actin, actin 4, transglutaminase, GAPDH, Ov87, LOAG_14743, galectin and P22U). The data suggest that DiES antigens interact with the environment of the parasite regulating the activation of the fibrinolytic system of the host with involvement of the vascular endothelium in the process.

Keywords: *Dirofilaria immitis,* excretory/secretory antigens, plasminogen binding, t-PA, endothelial cells, mass spectrometry.

Highlights

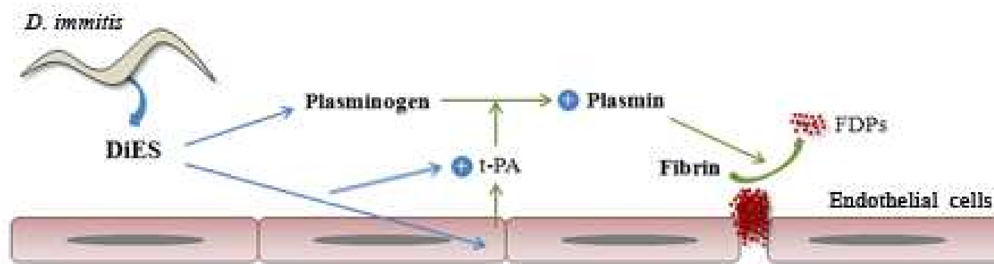
> Excretory/secretory antigens from *D. immitis* adult worms (DiES) bind plasminogen.

> Plasminogen is activated by this extract and plasmin is generated.

> DiES extract stimulate t-PA expression in vascular endothelial cell cultures.

> We identify 10 plasminogen-binding proteins of DiES extract.

Graphical Abstract



1. Introduction

Heartworm disease (HD) is a serious and potentially fatal disease caused by the filaroid nematode *Dirofilaria immitis* that affects dogs and cats all over the world [1]. The adult worms lodge in the pulmonary arteries and the right ventricle of infected hosts where they can live for years [2], causing a chronic inflammatory pathology. Initially the damages affect the arteries (endarteritis and perivascular inflammation), spreading later to the lung parenchyma and the right heart chambers [3]. In addition, when groups of worms die naturally or as a consequence of filaricide treatment, very serious alterations occur, with the exacerbation of inflammatory reactions and the formation of massive thromboembolisms [4] that put the life of the infected animals in immediate risk.

Since *D. immitis* can survive in the long term in the vascular system of immunocompetent hosts, it is reasonable to assume that adult worms interact with their intravascular environment, modulating the immune response and the associated pathology by means of the action of their metabolic products [excretory/secretory (ES) antigens], as it occurs in other parasitic infections [5,6]. A key point of vantage in blood parasites is the hemostasis, which is closely associated to fibrinolysis, inflammatory reactions and angiogenesis [7]. During the fibrinolysis, plasminogen binds to specific receptors together with activators of the process from which the most important is the t-PA that is mainly synthesized and secreted by endothelial cells. This binding determines

the transformation of plasminogen into serin protease plasmin, which is the enzyme that lyses the fibrin [8], degrading it into soluble products including the D-dimer [9]. Moreover, the activation of the plasminogen–plasmin system plays a key role in the degradation of extracellular matrices [10] that has been related to cell invasion and intra-organic migration of different pathogens [11,12]. Plasminogen and t-PA bind to receptors present on cells in the fibrin clots, to annexin-A2 of vascular endothelial cells and integrin αMβ2 of leukocytes [8]. Activation of plasminogen by binding to molecules secreted by bacteria with which it forms complexes has also been described [13]. Related to parasites, several molecules associated to the surface of protozoa and helminths [14-19] as well as molecules of the ES products [12,20], that bind plasminogen, have been identified. Additionally, it has been described that such interactions are mediated by carboxyl-terminal lysine residues of the plasminogen receptors [21].

We have previously observed some facts that suggest the interaction of *D. immitis* with its vascular environment: (i) DiES promote vasodilation, stimulating the expression of prostaglandin E2 by vascular endothelial cells, also limiting the transmigration of monocytes to the perivascular tissue [22]. (ii) In another study we found that serum levels of D-dimer are significantly higher in the 47% of dogs with HD analyzed than in the healthy dogs used as controls, indicating the presence of thromboembolisms and their degradation [23].

Although *D. immitis* is able to survive in the circulatory system of its hosts for years, there are no data on the interaction of adult worms with the fibrinolytic system of their hosts. The aim of this study was to demonstrate that the DiES antigens can bind plasminogen, generate plasmin, and stimulate the increase of t-PA synthesis by vascular endothelial cells. Additionally, we identified some plasminogen binding proteins of DiES extract using immunoproteomic techniques and mass spectrometry (MS).

2. Materials and Methods

2.1. Collection of ES extract of proteins from *D. immitis* adult worms

DiES were prepared as previously described [22] with minor modifications and stored at −80 °C. In brief, live worms (25) obtained from a naturally infected dog were washed in sterile phosphate-buffered saline solution (PBS) pH 7.2 and incubated for

24 h in 50 ml of Eagle's minimum essential medium (EMEM) supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin at 37 °C. A cocktail of protease inhibitors was added to the medium following the methodology described by Maizels et al. [24]. The medium was dialyzed against water for 24 h and filtered through an Amicon YC05 membrane (Millipore). The protein concentration of DiES was measured by DC protein assay commercial kit (Bio-Rad). DiES was tested for the presence of endotoxin contamination using a quantitative *Limulus* amebocyte lysate test (BioWhittaker). The endotoxin quantity was under the sensitivity level of cell stimulation (<0.4 U/mg protein).

2.2. Plasminogen binding assay

To determine whether plasminogen would bind components of the DiES extract, an enzyme-linked immunosorbent assay (ELISA) was performed. Multiwell microplates (Costar) were coated with 1 µg/well of DiES extract diluted in carbonate buffer, pH 9.6, overnight at 4 °C. The wells were blocked with 1% BSA in PBS and incubated successively with increasing amounts (from 0 µg to 3 µg) of human plasminogen (Acris Antibodies), with a sheep anti-human plasminogen IgG (Acris Antibodies) at 1:2000 dilution and then with a peroxidase-conjugated donkey anti-sheep IgG (Sigma) at 1:4000 dilution. All incubations were performed for 1 h at 37 °C and between each step washed three times with PBS wash buffer (PBS containing 0.05% $Tween_{20}$). Ortho-phenylene-diamine was used as a chromogen. Optical densities (OD) were measured at 492 nm in an Easy Reader (Bio-Rad). In parallel, competition assays were performed by including 50 mM of the lysine analogue ε-aminocaproic acid (εACA) during plasminogen incubation. Some wells coated with BSA only were used as negative controls.

2.3. Plasminogen activation assay

Plasminogen activation assay was performed in a test volume of 100 µl by measuring the amidolytic activity of generated plasmin [15]. In each well 2 µg of human plasminogen (Acris Antibodies) were incubated in PBS with 3 µg of the chromogenic substrate S-2251 (Sigma) in the presence of 1 µg of DiES. Activation of plasminogen was initiated by addition of 15 ng of t-PA (Sigma). In parallel, plasmin generation was also measured in the absence of t-PA. Plates were incubated at 37 °C for

2 h and the hydrolysis of the chromogenic substrate was monitored by measuring absorbance at 405 nm every 30 min. Each sample was analyzed in triplicate.

2.4. Cell culture and stimulation of endothelial cells

Vascular endothelial cells HAAE-1 from ATCC (LGC Promochem) were grown and treated as previously described [22]. In brief, endothelial cells were grown in Ham's F12k medium (ATCC) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS) (ATCC), 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 mg/ml heparin (Sigma) and 0.03 mg/ml endothelial cell growth supplement (ECGS) (Sigma). Plates were precoated with 0.1% pig gelatine (Sigma). Cells were cultured at 37 °C in a humidified atmosphere in the presence of 5% CO_2–95% air. Medium was changed every 3 days. Endothelial cells (10^6 cells/plate) were plated on 100 mm culture plates and grown for 4 days to obtain confluent cultures and treated with 1 μg/ml of DiES for 24 h. Non-stimulated cells were used as controls under the same conditions.

2.5. Two-dimensional electrophoresis (2-DE) of DiES extract

The 2-DE of DiES was performed as described before by us for the somatic antigen of adult worms of *D. immitis* [25]. Briefly, DiES extract was purified with the ReadyPrep 2-D Cleanup Kit (Bio-Rad) and resuspended in rehydration buffer 2-D (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)). The samples were divided into 125 μl aliquots (containing 60 μg of protein) and stored at −20 °C until use. When they were used DiES aliquots were supplemented with ampholytes and DTT, incubated and centrifuged to remove all particulate material, and then applied to 7-cm IPG strips (Bio-Rad) with linear pH ranges of 3–10, 5–8 and 7–10, using a Protean IEF Cell (Bio-Rad) for isoelectric focusing (IEF). After IEF, strips were reduced and alkylated, and second dimension separation was done in 12% acrylamide gels. Gels were then silver stained with the PlusOne Silver Staining Kit, Protein (GE Healthcare) or transferred to nitrocellulose membranes for their immunoblot analysis. The 2-D images were scanned with the GS-800 Densitometer (Bio-Rad) and analyzed with the Quantity One Software v.4.6.5 (Bio-Rad).

2.6. Immunoblot assays

To determine which proteins of DiES extract bind plasminogen, they were electrotransferred from 2D gels to nitrocellulose membranes at 20 V for 30 min using a

Trans-Blot SD Semi-Dry Transfer cell (Bio-Rad). Blots were blocked with 2% BSA in PBS wash buffer, for 1 h at room temperature. DiES membranes were incubated overnight at 4 °C with 10 µg/ml of human plasminogen. Then, the blots were incubated with a sheep anti-human plasminogen IgG (Acris Antibodies) at 1:1000 dilution and with a peroxidase-conjugated donkey anti-sheep IgG (Sigma) at 1:2000 dilution, each incubation for 90 min. All incubations were performed at 37 °C with shaking and between each step washed three times with washing buffer for 5 min per wash. Protein bands were revealed with 4-chloro naphthol. Negative controls were also used in which the plasminogen had been omitted. In addition, competition assays were performed by including 50 mM εACA during plasminogen incubation. Membranes were digitized with the scanner GS-800 Densitometer (Bio-Rad) using the Quantity One Software v.4.6.5 (Bio-Rad). Matching of 2-D gels with the homologous Western blot to identify plasminogen-binding proteins, the assignment of molecular weights (MW) and isoelectric points (pI) of each protein were analyzed using the PDQuest Software v.8.0.1 (Bio-Rad). All assays were performed in triplicate to assess the reproducibility of the spot pattern.

Western blot analysis for the t-PA expression was performed as previously described [22]. Treated and non-treated vascular endothelial cells were lysed in ice-cold lysis buffer. Protein samples (20 µg) were separated by SDS–PAGE under reducing conditions and blotted onto polyvinylidine difluoride membranes. Membranes were blocked before incubation with the primary antibody rabbit anti-t-PA (Santa Cruz Biotechnology) at 1:1000. After incubation with HRP-conjugated anti-rabbit secondary antibody at 1:20,000 dilution, bands were visualized by a luminol-based detection system with p-iodophenol enhancement. Anti-α-tubulin antibody (Oncogene Research Products) was used to confirm loading of comparable amount of protein in each lane. Protein expression was quantified by densitometry using Scion Image Software.

2.7. MS and protein identification

In gel digestion of proteins and MS analysis were done as described before by us [25]. The spots containing plasminogen-binding proteins were excised manually from the gels and sent to the Unit of Proteomics of the Centro Nacional de Investigaciones Cardiovasculares (Madrid, Spain) for MS analysis. For peptide mass fingerprinting and the acquisition of LIFT TOF/TOF spectra, an aliquot of the digestion of each spot was

deposited onto a 600 μm AnchorChip MALDI probe (Bruker-Daltonics). Peptide mass fingerprint spectra were measured on a Bruker Ultraflex TOF/TOF MALDI mass spectrometer (Bruker-Daltonics) [26] in positive-ion reflector mode. The measured tryptic peptide masses were transferred through the MS BioTools program (Bruker-Daltonics) as inputs to search the National Centre for Biotechnology Information non-redundant database (NCBInr) using Mascot software (Matrix Science). When necessary, MS/MS data from the LIFT TOF/TOF spectra were combined with MS peptide mass fingerprint (PMF) data for database searches.

2.8. Statistical analysis

The results from the plasminogen binding assay, plasminogen activation assay and Western blots for the t-PA expression were analyzed with the Student's *t*-test. The results were expressed as the mean ± SD of at least 3 independent experiments. In all experiments, a significant difference was defined as a *p*-value of <0.05 for a confidence level of 95%.

3. Results

3.1. Proteins of DiES extract bind plasminogen

The binding level of plasminogen to DiES extract was studied by ELISA. This test showed that the DiES extract binds plasminogen and that this binding is directly proportional to the amount of plasminogen (Fig. 1). The negative control consisting of wells coated only with BSA showed some non-specific binding activity, but always in a value significative lower than that shown by the DiES extract ($p < 0.05$). To determine whether or not lysine residues are involved in binding, a competition experiment including 50 mM εACA was carried out. In this case the binding between DiES extract and plasminogen was inhibited about 70%, resulting in slightly higher optical densities than the negative control (Fig. 1).

3.2. Plasminogen is activated by proteins of DiES extract and plasmin is generated

The ability to activate plasminogen by DiES extract and to generate plasmin was assessed by measuring the amidolytic activity of plasmin generated in the presence of the antigenic extract and plasminogen. This effect was measured in the presence or absence of a physiological activator of the process, t-PA, to observe the ability of the DiES extract proteins of activating plasminogen on their own. Negative controls

replacing DiES for BSA or t-PA were also used. As shown in Fig. 2, the generation of plasmin by t-PA is enhanced by DiES reaching optical density values significative higher ($p < 0.05$) than the negative controls in the presence of t-PA. However, DiES extract is unable to generate plasmin without t-PA resulting in optical density values identical to the negative control.

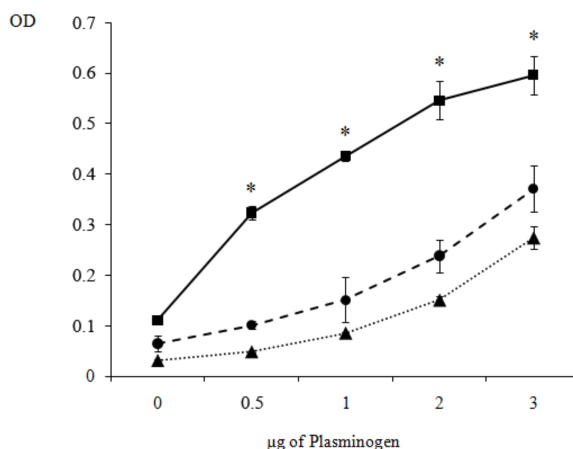


Figure 1. Plasminogen binding to 1 µg of DiES extract of *D. immitis* measured over a range of plasminogen amounts per cavity using a microtiter plate method. (■) Incubation with increasing amounts of plasminogen, 0–3 µg. (●) Competition assay with 50 mM εACA included during plasminogen incubation. (▲) Negative control consisted of wells coated only with BSA. Each point is the mean of three replicates ± SD. The asterisk (*) designates significant ($p < 0.05$) differences.

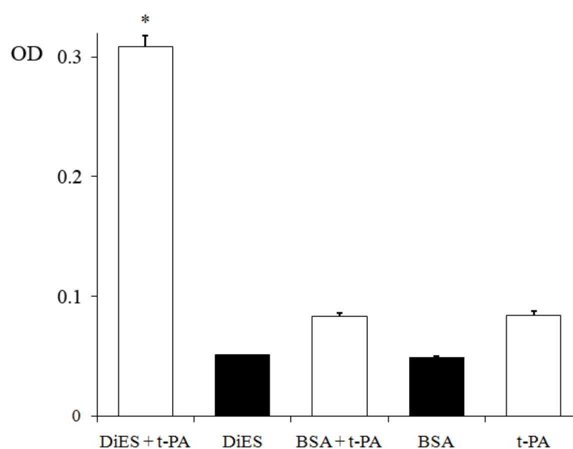


Figure 2. Plasminogen activation and plasmin generation by DiES extract of *D. immitis*. (□) 15 ng of t-PA was added to mixtures containing 2 µg of human plasminogen and 3 µg of the chromogenic substrate S-2251 (Sigma) in the presence or absence of 1 µg of DiES (or BSA as negative control) in a test volume of 100 µl. (■) No t-PA were added to reaction mixtures. Each point is the mean of three replicates ± SD. The asterisk (*) designates significant ($p < 0.05$) differences.

3.3. DiES extract stimulate t-PA expression in vascular endothelial cell cultures

D. immitis is an intravascular parasite for which we previously observed interactions of DiES antigens with the vascular endothelium. Additionally, the DiES extract activates the transformation of plasminogen to plasmin in a t-PA-dependent manner. Thus, the objective of this experiment was to determine whether or not DiES enhances the synthesis of t-PA in vascular endothelial cells (HAAE-1). Proteins from DiES-treated vascular endothelial cell extracts were separated by SDS–PAGE and analyzed by Western blotting using anti-t-PA antibody. As shown in Fig. 3, DiES induced a significative increase in t-PA ($p < 0.05$) protein expression after 24 h of stimulation.

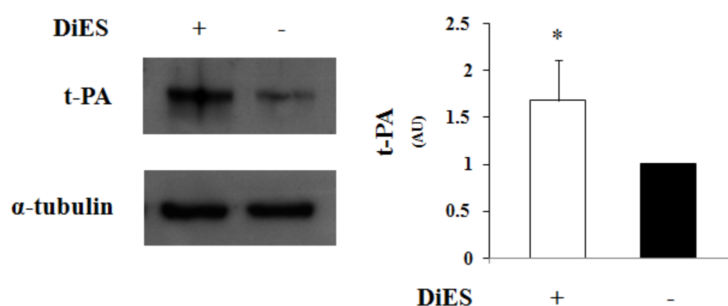


Figure 3. Effect of DiES on the expression of t-PA in human vascular endothelial cells. Protein extracts from lysed DiES treated or untreated confluent cell cultures were analyzed by Western blot for t-PA. α-tubulin served as a protein control. Results were expressed as the mean ± SEM of at least 3 independent experiments. The asterisk (*) designates significant ($p < 0.05$) differences from control cells. (□) Stimulated endothelial cells. (■) Non-treated control cells. AU, arbitrary units.

3.4. Two-dimensional analysis of DiES extract

To obtain an overall view of all the proteins of the DiES, this extract were first electrofocused using 3–10 linear immobilized pH gradient strips. Silver nitrate staining of these 2-D gels revealed about 570 spots in the excretome of *D. immitis* with pIs between 5 and 9.8, and a broad range of MWs (10–150 kDa). Only 24 spots were observed with pI < 5 (not shown).

In order to improve spot resolution and detection, once the spot MW and pI ranges were determined, the DiES extract were electrofocused in 5–8 and 7–10 IPG strips. With these new conditions, silver staining revealed a total of 636 spots, most of

them (594) located between pH 5 and 8. The remaining 96 spots had pIs between 8 and 9.8 (Fig. 4A and B).

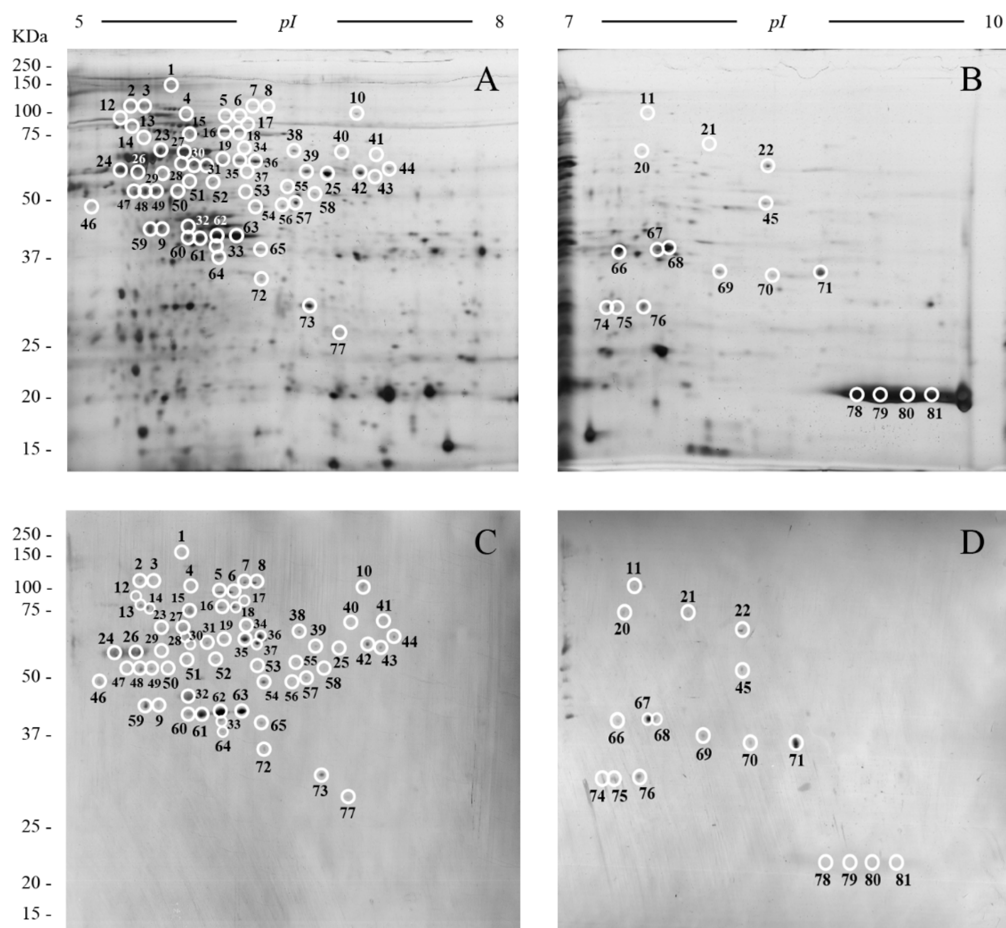


Figure 4. Representative 2-DE of 60 µg of the DiES extract from adult *D. immitis* worms. The gels were in the 5–8 and 7–10 pH ranges, 12% polyacrylamide and silver-stained (A and B). Plasminogen-binding spots revealed on ligand blots from gels A and B (C and D). Reference molecular masses are indicated on the left. The plasminogen-binding spots analyzed by MS are circled and numbered.

3.5. Identification of plasminogen-binding proteins

To identify plasminogen-binding proteins, ligand blotting of 2D gels of 5–8 and 7–10 pH with plasminogen was performed, after electrotransfering them to nitrocellulose membranes.

As shown in Fig. 4C and D, 81 plasminogen-binding spots were revealed. This represents a binding rate of 12.73% of total spots revealed in the excretome of *D. immitis*. Most of them ($n = 60$) were resolved in a narrow range of MWs and pIs

(between 37 and 150 KDa, and 5.2 and 7.2, respectively). In the control blots, in which plasminogen incubation was omitted, the anti-plasminogen antibody did not reveal any spots (not shown). In the competition experiments, the inclusion of 50 mM εACA inhibited plasminogen binding to DiES demonstrating the specificity of the reaction (Fig. S1).

Spot number	Accesion code	Protein definition	Species	MW (kDa) theor/exp	pI theor/exp	Sequence coverage (%)	Mascot score
23	ACY25666	Chaperonin-like protein HSP60	*Brugia malayi*	61.4/67.1	5.7/5.6	11	130
27	AF121264_1	Chaperonin protein HSP60	*Onchocerca volvulus*	64.5/67.0	5.7/5.8	17	145
28	AF121264_1	Chaperonin protein HSP60	*Onchocerca volvulus*	64.5/65.2	5.7/5.8	16	130
31	ACT1_CAEEL	Actin-1/3	*Caenorhabditis elegans*	42.1/65.2	5.3/5.9	11	41
32	XP_001894819	Actin	*Brugia malayi*	42.1/43.3	5.3/5.8	4	64
33	NP_508842	ACTin family member (act-4)	*Caenorhabditis elegans*	37.5/39.4	5.4/6.0	17	142
37	AAC24752	Transglutaminase precursor	*Dirofilaria immitis*	57.6/61.0	5.7/6.3	19	91
66	XP_001899850	Glyceraldehyde 3-phosphate dehydrogenase	*Brugia malayi*	32.1/40.8	8.5/7.5	20	207
67	XP_001899850	Glyceraldehyde 3-phosphate dehydrogenase	*Brugia malayi*	32.1/40.7	8.5/7.8	25	292
69	AAD00843	Ov87	*Onchocerca volvulus*	36.7/36.4	8.9/8.2	24	161
71	AAD00843	Ov87	*Onchocerca volvulus*	36.7/36.5	8.9/9.0	16	157
72	XP_003150284	Hypothetical protein LOAG_14743	*Loa loa*	13.3/33.7	6.7/6.3	11	94
73	AAF37720	Galectin	*Dirofilaria immitis*	32.2/30.1	6.0/6.6	11	118
78	AAD11968	P22U	*Dirofilaria immitis*	24.4/22.0	8.9/9.2	66	499
79	AAD11968	P22U	*Dirofilaria immitis*	24.4/22.0	8.9/9.4	62	458
80	AAD11968	P22U	*Dirofilaria immitis*	24.4/22.0	8.9/9.6	62	489
81	AAD11968	P22U	*Dirofilaria immitis*	24.4/22.0	8.9/9.8	54	201

Table 1. Plasminogen-binding protein spots of DiES extract identified by MALDI-TOF MS. Exp, experimental; theo, theoretical.

The matching of spots revealed by ligand-blotting with their homologous spots in the silver-stained 2-D gels allowed to select a total of 81 plasminogen-binding spots of *D. immitis*, which were manually excised from 2-D gels and submitted to analysis by

MS. Table 1 shows the identity of these proteins and their MWs and pIs (theoretical and experimental), the NCBI accession number, the sequence coverage and the Mascot score. Seventeen of 81 spots were identified (21%) and corresponded to 10 different proteins. The proteins identified were chaperonine protein HSP60, actin-1/3, actin, actin 4, transglutaminase precursor, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Ov87, hypothetical protein LOAG_14743, galectin and P22U. Between 1 and 4 isoforms of each protein were identified. Most proteins were identified by their similarity to homologous proteins from other species of filarial worms. Thus of the 17 spots identified 9 corresponded to other filarial proteins (*Brugia malayi*, *Onchocerca volvulus* and *Loa loa*), while 6 spots corresponded to 3 proteins of *D. immitis* deposited in databases (transglutaminase precursor, galectin and P22U). The 2 remaining spots corresponded to proteins from the nematode *Caenorhabditis elegans*.

4. Discussion

D. immitis infections are typically characterized by the persistence of adult worms in vascular location for years in which they are exposed to multiple aggression mechanisms from the host and where their presence causes severe and sometimes fatal pathological changes. One of these mechanisms is the generation of thromboembolisms. This process is physiologically regulated by the fibrinolytic system which is able to lyse fibrin clots. Its activation by molecules of *D. immitis* could have beneficial effects for the survival of the parasite in the circulatory system.

In this study we demonstrate in an in vitro system that DiES antigens bind plasminogen. This binding is dependent on the presence of lysine residues, as it is inhibited by ε-aminocaproic acid. We also demonstrate that DiES extract activates plasminogen and generate plasmin in a t-PA-dependent manner. All this is consistent with experimental studies carried out in bacteria, protozoa and helminths [17,19,27,28].

We also demonstrated that DiES antigens significantly stimulate the basal production of t-PA by vascular endothelial cells cultured in vitro. This is consistent also with the key role of vascular endothelium in the regulation of haemostasis [7] and with the intravascular niche of *D. immitis*. We have previously shown that DiES antigens interact with the vascular endothelium, stimulating vasodilatation and reducing leukocyte transmigration, which highlights the importance of the endothelium in the

activation of mechanisms that likely promote parasite survival, also limiting the damage to the host [22].

On the other hand, plasmin produced by plasminogen activation is also involved in the lysis of extra-cytoplasmic matrices [29], which is interpreted as a mechanism related to cell invasion and intra-organic migration of different parasites [12,14]. As many molecules involved in the binding of plasminogen are multifunctional, we cannot rule out that though the activation of the fibrinolytic system has predictable beneficial effects, both for the parasite and for the host, some of the molecules involved in the process can exert other activities, contributing to the generation of damage on the host. A consequence of the arrival of the *D. immitis* adult worms into the pulmonary arteries is the appearance of proliferative endarteritis caused by the proliferation and migration of smooth muscle cells of the vascular wall into the lumen [3]. It has been shown that the over-expression of t-PA in damaged endothelium induces proliferation and migration of smooth muscle cells in humans [30]. Since we have observed an over-expression of t-PA by vascular endothelial cells stimulated by DiES, it will be necessary to study in the future if the proliferative endarteritis is associated with over-expression of t-PA by endothelial cells and therefore the activation of the fibrinolytic system of the host by *D. immitis* adult worms.

The proteomic analysis of DiES extract also allowed us to identify 17 plasminogen-binding spots by MS, which corresponded to 10 proteins. Their identification was possible by the existence of a significant amount of available information on the filarial worms. Of the proteins identified in the DiES, HSP 60, different proteins of the family of actins and GAPDH are among the best characterized binding-plasminogen molecules. The HSP 60 is a binding protein belonging to the family of heat shock proteins. Its plasminogen-binding activity has been demonstrated in bacteria [31,32], in which this activity has been associated with disruption of the extracellular matrix of tissues and invasion [32].

Three proteins of the actin family binding plasminogen (actin-1/3, actin and actin-4) have been identified in the DiES extract. The interaction between actin and plasminogen is well known, as well as the fact that specific binding occurs through lysine residues which stimulate the tPA-dependent plasmin generation [33]. In addition,

its function as plasminogen receptor has been demonstrated on the surface of endothelial cells [34] and in the tegument of *Schistosoma bovis* [17]. The glycolytic enzyme GAPDH is a multifunctional molecule whose plasminogen-binding activity has been observed in different pathogens such as bacteria and fungi [35,36], blood helminths such as *S. bovis* [17] and tissue helminths like *O. volvulus* [16].

The other proteins identified in our study (transglutaminase precursor, Ov87, galectin, P22U and LOAG_14743) have been related to plasminogen binding for the first time. However, there is evidence that some of them are associated with nearby or related fibrinolysis processes. It has been demonstrated that galectin-1, which belongs to the family of galectins (like galectin and Ov87 proteins identified here) acts as receptor of t-PA and is the responsible for the increase of catalytic activity that occurs in the pancreatic cancer [37]. Moreover, the LOAG_14743 is a hypothetical protein of the annexin family. Within this family, annexin-A2 is one of the best-characterized plasminogen receptors on endothelial cells [38].

To conclude, we have demonstrated that DiES antigens in vitro activate the plasminogen binding and plasmin production, involving the vascular endothelium in the regulation of this process through the stimulus of the expression of t-PA by vascular endothelial cells. These facts demonstrate the interaction of *D. immitis* with its vascular environment through his metabolic products, promoting mechanisms for its own survival. Ten plasminogen binding molecules of the DiES extract have been identified by proteomic analysis and MS, suggesting that *D. immitis* adult worms use different molecules to maintain the balance of the vascular environment. Future studies are needed to obtain a complete understanding of this process during HD and to elucidate if molecules of the plasminogen binding process are involved in other mechanisms related to the occurrence of pathological changes in the pulmonary arteries.

Acknowledgements

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References

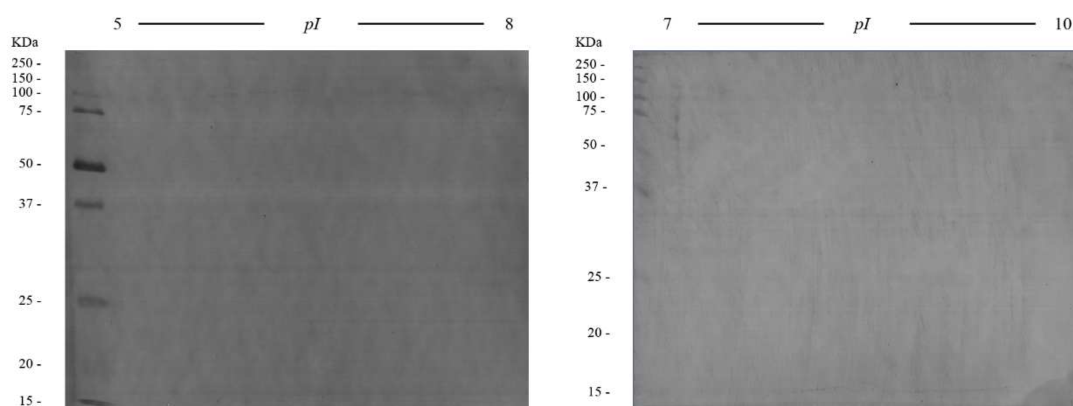
1. Genchi C, Kramer LH, Prieto G. Epidemiology of canine and feline dirofilariasis: a global view. In: Simón F, Genchi C, editors. Heartworm infection in humans and animals, España: Ediciones Universidad de Salamanca; 2001, p. 121-34.
2. Quiroz-Romero H. Parasitología y enfermedades parasitarias de los animales domésticos. México: Dimusa S.A.; 1984, p. 620-27.
3. Venco L. Heartworm (*Dirofilaria immitis*) disease in dogs. In: Genchi C, Ronaldi L, Cringoli G, editors. *Dirofilaria immitis* and *D. repens* in dog and cat and human infections, Italia: Rolando Editore; 2007, p. 117-25.
4. McCall JW, Genchi C, Kramer LH, Guerrero J, Venco L. Heartworm disease in animals and humans. Adv Parasitol 2008;66:193-285.
5. Dzik JM. Molecules released by helminth parasites involved in host colonization. Acta Biochim Pol 2006;53(1):33-64.
6. Bennuru S, Semnani R, Meng Z, Ribeiro JM, Veenstra TD, Nutman TB. *Brugia malayi* excreted/secreted proteins at the host/parasite interface: stage- and genderspecific proteomic profiling. PLoS Negl Trop Dis 2009;3:e410.
7. Becker BF, Heindl B, Kupatt C, Zahler S. Endothelial function and hemostasis. Z Kardiol 2000;89(3):160-7.
8. Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. Br J Haematol 2005;129(3):307-21.
9. Goggs R, Benigni L, Fuentes VL, Chan DL. Pulmonary thromboembolism. J Vet Emerg Crit Care (San Antonio) 2009;19(1):30-52.
10. Stephens RW, Vaheri A. In: Kreis T, Vale R, editors. Guidebook to the Extracellular Matrix and Adhesion Proteins, New York: Oxford University Press; 1995, p. 81–82.
11. Jong AY, Chen SH, Stins MF, Kim KS, Tuan TL, Huang SH. Binding of *Candida albicans* enolase to plasmin(ogen) results in enhanced invasion of human brain microvascular endothelial cells. J Med Microbiol 2003;52:615-22.
12. Bernal D, de la Rubia JE, Carrasco-Abad AM, Toledo R, Mas-Coma S, Marcilla A. Identification of enolase as a plasminogen-binding protein in excretory-secretory products of *Fasciola hepatica*. FEBS Lett 2004;563(1-3):203-6.
13. Bergmann S, Hammerschmidt S. Fibrinolysis and host response in bacterial infections. Thromb Haemost 2007;98(3):512-20.

14. Jolodar A, Fischer P, Bergmann S, Büttner DW, Hammerschmidt S, Brattig NW. Molecular cloning of an alpha-enolase from the human filarial parasite *Onchocerca volvulus* that binds human plasminogen. Biochim Biophys Acta 2003;1627(2-3):111-20.
15. Almeida L, Vanegas G, Calcagno M, Concepción JL, Avilan L. Plasminogen interaction with *Trypanosoma cruzi*. Mem Inst Oswaldo Cruz 2004;99(1):63-7.
16. Erttmann KD, Kleensang A, Schneider E, Hammerschmidt S, Büttner DW, Gallin M. Cloning, characterization and DNA immunization of an *Onchocerca volvulus* glyceraldehyde-3-phosphate dehydrogenase (Ov-GAPDH). Biochim Biophys Acta 2005;1741(1-2):85-94.
17. Ramajo-Hernández A, Pérez-Sánchez R, Ramajo-Martín V, Oleaga A. *Schistosoma bovis*: plasminogen binding in adults and the identification of plasminogen-binding proteins from the worm tegument. Exp Parasitol 2007;115(1):83-91.
18. Vanegas G, Quiñones W, Carrasco-López C, Concepción JL, Albericio F, Avilán L. Enolase as a plasminogen binding protein in *Leishmania mexicana*. Parasitol Res 2007;101(6):1511-6.
19. Mundodi V, Kucknoor AS, Alderete JF. Immunogenic and plasminogen-binding surface-associated alpha-enolase of *Trichomonas vaginalis*. Infect Immun 2008;76(2):523-31.
20. Marcilla A, Pérez-García A, Espert A, Bernal D, Muñoz-Antolí C, Esteban JG, et al. *Echinostoma caproni*: identification of enolase in excretory/secretory products, molecular cloning, and functional expression. Exp Parasitol 2007;117(1):57-64.
21. Plow EF, Herren T, Redlitz A, Miles LA, Hoover-Plow JL. The cell biology of the plasminogen system. FASEB J 1995;9(10):939-45.
22. Morchón R, González-Miguel J, Mellado I, Velasco S, Rodríguez-Barbero A, Simón F. Adult *Dirofilaria immitis* excretory/secretory antigens upregulate the production of prostaglandin E2 and downregulate monocyte transmigration in an "in vitro" model of vascular endothelial cell cultures. Vet Parasitol 2010;170(3-4):331-5.
23. Carretón E, Corbera JA, Juste MC, Morchón R, Simón F, Montoya-Alonso JA. *Dirofilaria immitis* infection in dogs: Cardiopulmonary biomarker levels. Vet Parasitol 2011;176(4):313-6.

24. Maizels RM, Blaxter ML, Robertson BD, Selkirk ME. Parasite antigen parasite genes: a laboratory manual for molecular parasitology. Cambridge: Cambridge University Press; 1991.
25. González-Miguel J, Rosario L, Rota-Nodari E, Morchón R, Simón F. Identification of immunoreactive proteins of *Dirofilaria immitis* and *D. repens* recognized by sera from patients with pulmonary and subcutaneous dirofilariosis. Parasitol Int 2010;59(2):248-56.
26. Suckau D, Resemann A, Schuerenberg M, Hufnagel P, Franzen J, Holle A. A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. Anal Bioanal Chem 2003;376:952–65.
27. Avilan L, Calcagno M, Figuera M, Lemus L, Puig J, Rodriguez AM. Interaction of *Leishmania mexicana* promastigotes with the plasminogen-plasmin system. Mol Biochem Parasitol 2000;110(2):183-93.
28. Xolalpa W, Vallecillo AJ, Lara M, Mendoza-Hernandez G, Comini M, Spallek R, Singh M, Espitia C. Identification of novel bacterial plasminogen-binding proteins in the human pathogen *Mycobacterium tuberculosis*. Proteomics 2007;7(18):3332-41.
29. Vassalli JD, Sappino AP, Belin D. The plasminogen activator/plasmin system. J Clin Invest 1991;88(4):1067-72.
30. Yang Z, Eton D, Zheng F, Livingstone AS, Yu H. Effect of tissue plasminogen activator on vascular smooth muscle cells. J Vasc Surg 2005;42(3):532-8.
31. Schaumburg J, Diekmann O, Hagendorff P, Bergmann S, Rohde M, Hammerschmidt S, et al. The cell wall subproteome of *Listeria* monocytogenes. Proteomics 2004;4(10):2991-3006.
32. Dudani AK, Mehic J, Martyres A. Plasminogen and angiostatin interact with heat shock proteins. Mol Cell Biochem 2007;300(1-2):197-205.
33. Lind SE, Smith CJ. Actin accelerates plasmin generation by tissue plasminogen activator. J Biol Chem 1991;266(26):17673-8.
34. Dudani AK, Ben-Tchavtchavadze M, Porter S, Tackaberry E. Angiostatin and plasminogen share binding to endothelial cell surface actin. Biochem Cell Biol 2005;83(1):28-35.

35. Crowe JD, Sievwright IK, Auld GC, Moore NR, Gow NA, Booth NA. *Candida albicans* binds human plasminogen: identification of eight plasminogen-binding proteins. Mol Microbiol 2003;47(6):1637-51.
36. Bergmann S, Rohde M, Hammerschmidt S. Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus pneumoniae* is a surface-displayed plasminogen-binding protein. Infect Immun 2004;72(4):2416-9.
37. Roda O, Ortiz-Zapater E, Martínez-Bosch N, Gutiérrez-Gallego R, Vila-Perelló M, Ampurdanés C, et al. Galectin-1 is a novel functional receptor for tissue plasminogen activator in pancreatic cancer. Gastroenterology 2009;136(4):1379-90.
38. Dassah M, Deora AB, He K, Hajjar KA. The endothelial cell annexin A2 system and vascular fibrinolysis. Gen Physiol Biophys 2009;28:20-8.

Supplementary material



Supplemental Figure S1. Competition assay of DiES-plasminogen immunoblot analysis. The assay was performed by including 50 mM εACA during plasminogen incubation. The membranes were in the 5-8 and 7-10 pH ranges. Reference molecular masses are indicated on the left. As shown in Figure S1 the inclusion of 50 mM εACA inhibit plasminogen binding to DiES.

SEGVNDO CAPÍTVLO

"Surface associated antigens of *Dirofilaria immitis* adult worms activate the host fibrinolytic system"

Los antígenos asociados a la superficie de los vermes adultos de *Dirofilaria immitis* activan el sistema fibrinolítico del hospedador

Javier González-Miguel, Rodrigo Morchón, Elena Carretón, José Alberto Montoya-Alonso, Fernando Simón

Veterinary Parasitology 196 (2013) 235-240.

Factor de impacto (2013): 2,545.

Resumen

La dirofilariosis cardiopulmonar (*Dirofilaria immitis*) se caracteriza por eventos aparentemente contradictorios, como la supervivencia a largo plazo de los vermes adultos en el sistema circulatorio de los hospedadores infectados y el desarrollo de procesos potencialmente mortales como la aparición de tromboembolismos. Por lo tanto, mecanismos desarrollados por los parásitos como la activación del sistema fibrinolítico, son un punto clave para la supervivencia tanto de los vermes como del hospedador. El objetivo de este trabajo fue investigar la interacción entre los antígenos asociados a la superficie de los vermes adultos de *D. immitis* (DiSAA) y el sistema fibrinolítico del hospedador. Se demostró que el extracto DiSAA es capaz de fijar plasminógeno y generar plasmina, ocurriendo esto último de un modo dependiente del activador tisular del plasminógeno (tPA). Adicionalmente, 11 proteínas fijadoras de plasminógeno del extracto DiSAA fueron identificadas mediante proteómica y espectrometría de masas (MS): (actina-5C, actina-1, enolasa, fructosa-bifosfato aldolasa, GAPDH, dominio proteico MSP, MSP 2, lectina de unión a beta-galactosidasa, galectina, proteína contenedora del dominio inmunoglobulina I y ciclofilina Ovcyp-2). Debido a que en un trabajo previo hemos demostrado la interacción positiva entre los antígenos excretores/secretores de *D. immitis* (DiES) y el sistema fibrinolítico del hospedador y a que muchas de las moléculas identificadas aquí son compartidas por ambos compartimentos antigénicos, se propone que DiSAA coopera en la activación del sistema fibrinolítico promoviendo la lisis de los coágulos de fibrina.

Veterinary Parasitology 196 (2013) 235–240

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Short communication

Surface associated antigens of *Dirofilaria immitis* adult worms activate the host fibrinolytic system

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Abstract

Cardiopulmonary dirofilariosis (*Dirofilaria immitis*) is characterized by apparent contradictory events, like the long-term survival of adult worms in the circulatory system of the infected hosts and the development of life-threatening events like thromboembolisms and others. Thus parasite mechanisms, like the activation of fibrinolytic system, are key to the survival of both the worms and the host. The aim of this study was to investigate the interaction between *D. immitis* adult worms surface-associated antigens (DiSAA) and the fibrinolytic system of the host. We demonstrate that DiSAA extract is able to bind plasminogen and generate plasmin, with the latter occurring in a tissue plasminogen activator (t-PA) dependent manner. Additionally, 11 plasminogen-binding proteins from DiSAA extract were identified by proteomics and mass spectrometry (MS) (actin-5C, actin-1, enolase, fructose-bisphosphate aldolase, GAPDH, MSP domain protein, MSP 2, beta-galactosidase-binding-lectin, galectin, immunoglobulin I-set domain-containing protein and cyclophilin Ovcyp-2). Because in a previous work we have shown the positive interaction between the excretory/secretory antigens of *D. immitis* (DiES) and the host fibrinolytic system and many of the molecules identified here are shared by both antigens, we hypothesize that DiSAA cooperate in host fibrinolytic system activation promoting the fibrin clot lysis.

Key words: *Dirofilaria immitis*; surface associated antigens; fibrinolysis; plasminogen binding.

1. Introduction

Dirofilaria immitis is the causative agent of canine and feline cardiopulmonary dirofilariosis and human pulmonary dirofilariosis. It is a vector-borne transmitted disease with a cosmopolitan distribution (Genchi et al., 2001). *D. immitis* adult worms can survive for years (7 or more) in the pulmonary arteries and right ventricle of dogs (Quiroz-Romero, 1984), causing a chronic vascular disease mainly associated with inflammatory reactions. Moreover, the simultaneous death of groups of adult worms can trigger an acute pathology characterized by the exacerbation of the inflammatory reactions and the occurrence of serious thromboembolisms (Venco, 2007) that poses an immediate risk for the life of the affected hosts.

The fibrinolytic system activity is based on the conversion of plasminogen into plasmin, the enzyme responsible of fibrin clots lysis (Cesarman-Maus and Hajjar, 2005). This process is regulated by the t-PA, mainly synthesized by vascular endothelial cells. It has been recently demonstrated that the metabolic products excreted by *D. immitis* "in vitro" stimulate the host fibrinolytic system. Furthermore, this stimulation causes an over-expression of t-PA in vascular endothelial cells (González-Miguel et al., 2012), suggesting the existence of regulatory mechanisms of the thromboembolisms by this parasite in its intravascular habitat. On the other hand, parasite-surface molecules have been widely linked to key roles related to the host/parasite relationships (Fetterer and Rhoads, 1993) and some of them have been related to the plasminogen-binding activity in protozoa (Almeida et al., 2004, Vanegas et al., 2007 and Mundodi et al., 2008) and helminth parasites (Jolodar et al., 2003, Erttmann et al., 2005 and Ramajo-Hernández et al., 2007). In this work we demonstrate that different surface-associated molecules of *D. immitis* adult worms bind plasminogen and generate plasmin, activating the host fibrinolytic system.

2. Materials and Methods

2.1. Collection of surface associated antigens from *D. immitis* adult worms (DiSAA)

DiSAA extract was obtained following the methodology described by Wedrychowicz et al. (1994) with minor modifications. In brief, live worms (7) obtained from a naturally infected dog were washed and then incubated in saline solution containing 0.25% CTAB with a cocktail of protease inhibitors (Maizels et al., 1991) at 37 °C for 4 h. The worms were separated from detergent and extracted proteins were

precipitated with sodium acetate 0.002 M with nine volumes of 96% ethanol, at –20 °C for 48 h followed by centrifugation (10,000 × *g*, 10 min). The resulting pellets were re-suspended in PBS pH 7.2 and stored at –80 °C until use. Previously, protein concentration of DiSAA was measured by DC protein assay commercial kit (Bio-Rad).

2.2. Plasminogen binding assay

To determine whether the plasminogen would bind surface components of *D. immitis* an ELISA test was carried out as described previously (González-Miguel et al., 2012). In brief, multiwell microplates (Costar) were coated with 1 µg/well of DiSAA extract, blocked and then incubated with increasing amounts (from 0 µg to 3 µg) of human plasminogen (acris antibodies). After incubation with the corresponding antibodies and with a chromogen, the optical density was measured at 492 nm in an easy reader (Bio-Rad). In parallel, competition assays were performed by including 50 mM of the lysine analogue ε-aminocaproic acid (εACA) during plasminogen incubation.

2.3. Plasminogen activation assay

Plasminogen activation assay was performed in a test volume of 100 µl by measuring the amidolytic activity of generated plasmin (González-Miguel et al., 2012). In each well 2 µg of human plasminogen (acris antibodies) were incubated in PBS with 3 µg of the chromogenic substrate S-2251 (Sigma) in the presence of 1 µg of DiSAA extract. Activation of plasminogen was initiated by addition of 15 ng of t-PA (Sigma). In parallel, plasmin generation was also measured in the absence of t-PA. Plates were incubated at 37 °C for 2 h and the hydrolysis of the chromogenic substrate was monitored by measuring absorbance at 405 nm every 30 min. Each sample was analyzed in triplicate.

2.4. Two-dimensional electrophoresis (2-DE) of DiSAA extract and immunoblot assay

The 2-DE of DiSAA extract was performed as described before by us (González-Miguel et al., 2012). Briefly, DiSAA extract aliquots were supplemented with ampholytes, incubated and centrifugated, and then applied to 7 cm IPG strips (Bio-Rad) with linear pH ranges of 3–10, 5–8 and 7–10, using a Protean IEF Cell (Bio-Rad) for isoelectric focusing (IEF). After IEF, strips were reduced and alkilated, and second dimension was done in 12% acrylamide gels. Gels were then silver stained with the PlusOne silver staining kit, protein (GE Healthcare).

To determine which proteins of DiSAA extract bind plasminogen an immunoblot was performed (González-Miguel et al., 2012). The 2-D gels were transferred to nitrocellulose membranes which were blocked and then incubated overnight at 4 °C with 10 µg/ml of human plasminogen. After incubating the membranes with the corresponding antibodies, proteins were revealed with 4-chloro naphthol.

The 2-D gels and membranes were scanned and analyzed with the quantity one software v.4.6.5 (Bio-Rad). Matching of 2-D gels with the homologous Western blot to identify plasminogen-binding proteins was analyzed using the PDQuest Software v.8.0.1 (Bio-Rad).

2.6. MS and protein identification

In gel digestion of proteins and MS analysis were done as described before by us (González-Miguel et al., 2012). The spots containing plasminogen-binding proteins were excised manually from the gels and sent to the unit of Proteomics of the Centro de Investigación Príncipe Felipe (Valencia, Spain) for MS analysis. For peptide mass fingerprinting and the acquisition of LIFT TOF/TOF spectra, an aliquot of the digestion of each spot was deposited onto a 600 µm AnchorChip MALDI probe (Bruker-Daltonics). Peptide mass fingerprint spectra were measured on a Bruker Ultraflex TOF/TOF MALDI mass spectrometer (Bruker-Daltonics) in positive-ion reflector mode. The measured tryptic peptide masses were transferred through the MS BioTools software (Bruker-Daltonics) as inputs to search the National Centre for Biotechnology Information non-redundant database (NCBInr) using Mascot software (Matrix Science). When necessary, MS/MS data from the LIFT TOF/TOF spectra were combined with MS peptide mass fingerprint data for database searches.

2.7. Statistical analysis

The results from the plasminogen binding assay and plasminogen activation assay were analyzed with the Student's *t*-test. The results were expressed as the mean ± SD of at least 3 independent experiments. In all experiments, a significant difference was defined as a p-value < 0.05 for a confidence level of 95%.

Results

3.1. Proteins of DiSAA extract bind plasminogen

The binding capacity of plasminogen to DiSAA extract was measured by ELISA. The test showed that DiSAA binds plasminogen obtaining optical densities statistically higher ($p < 0.05$) than those of the control wells (coated only with BSA) (Fig. 1). This binding is also directly proportional to the amount of plasminogen. The competition assay showed that the inclusion of 50 mM εACA inhibits the plasminogen-binding (Fig. 1), demonstrating that this union is dependent on lysine residues.

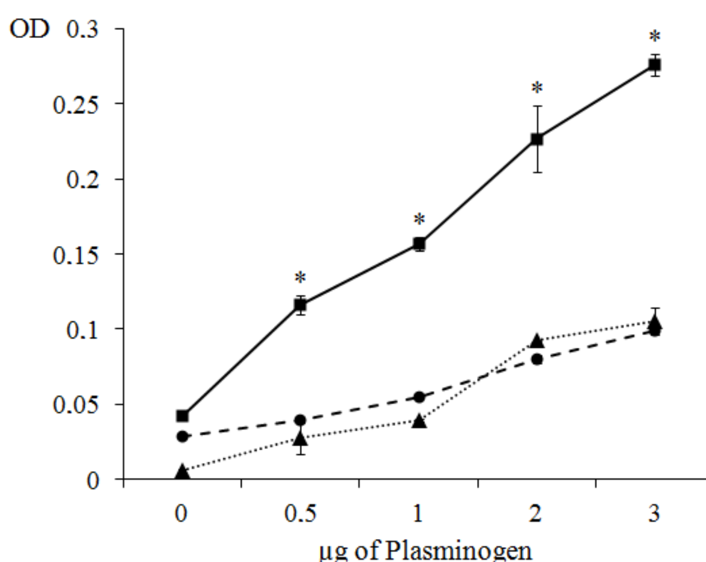

Figure 1. Plasminogen binding to 1 µg of DiSAA extract of *D. immitis* measured over a range of plasminogen amounts using a microtiter plate method. (■) Incubation with increasing amounts of plasminogen, 0–3 µg. (●) Competition assay with 50 mM εACA included during plasminogen incubation. (▲) Negative control consisted of wells coated only with BSA. Each point is the mean of three replicates ±SD. The asterisk (*) designates significant ($p < 0.05$) differences.

3.2. The plasminogen-binding activity of DiSAA extract generates plasmin

The ability to activate plasminogen by DiSAA extract and to generate plasmin was assessed by measuring the amidolytic activity of plasmin generated in the presence of the antigenic extract and plasminogen. This effect was measured in the presence or absence of t-PA, to observe the ability of the DiSAA extract proteins of activating plasminogen on their own. Negative controls replacing DiSAA by BSA or t-PA were also used. As shown in Fig. 2, the generation of plasmin by t-PA is enhanced by DiSAA reaching optical density values significative higher ($p < 0.05$) than the negative controls.

Furthermore this effect is inhibited by 50 mM εACA, indicating the involvement of lysine residues in the process. However, DiSAA extract is unable to generate plasmin without t-PA resulting in optical density values identical to the negative control.

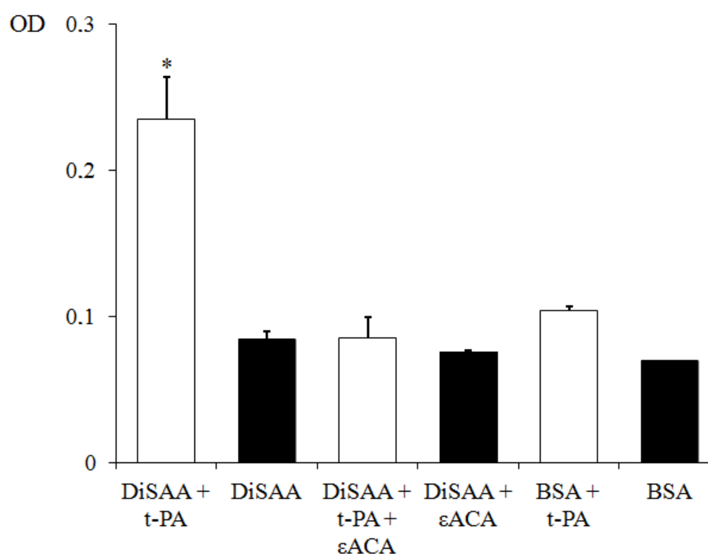

Figure 2. Plasminogen activation and plasmin generation by DiSAA extract of *D. immitis*. (□) 15 ng of t-PA was added to mixtures containing 2 µg of human plasminogen, 3 µg of S-2251 (Sigma) and 1 µg of DiSAA extract (or BSA as negative control) in the presence or absence of 50 mM of εACA in a test volume of 100 µl. (■) No t-PA was added to reaction mixtures. Each point is the mean of three replicates ±SD. The asterisk (*) designates significant ($p < 0.05$) differences.

3.3. Two-dimensional analysis of DiSAA extract

To obtain an overall view of all the proteins of the DiSAA, this extract were first electrofocused using 3–10 linear immobilized pH gradient strips. Silver nitrate staining of these 2-D gels revealed about 315 spots in the *D. immitis* surface proteome, many sparsely settled, with isoelectric points (pIs) between 5 and 9.7, and a broad range of molecular weights (MWs) (10–145 kDa). Only 4 spots were observed with pI‹5 (not shown). In order to improve spot resolution and detection, once the spot MW and pI ranges were determined, the DiSAA extract were electrofocused in 5–8 and 7–10 IPG strips. With these new conditions, silver staining revealed a total of 347 spots, most of them (318) located between pIs 5 and 8. The remaining 29 spots had pIs between 8 and 9.8 (Fig. 3A and B).

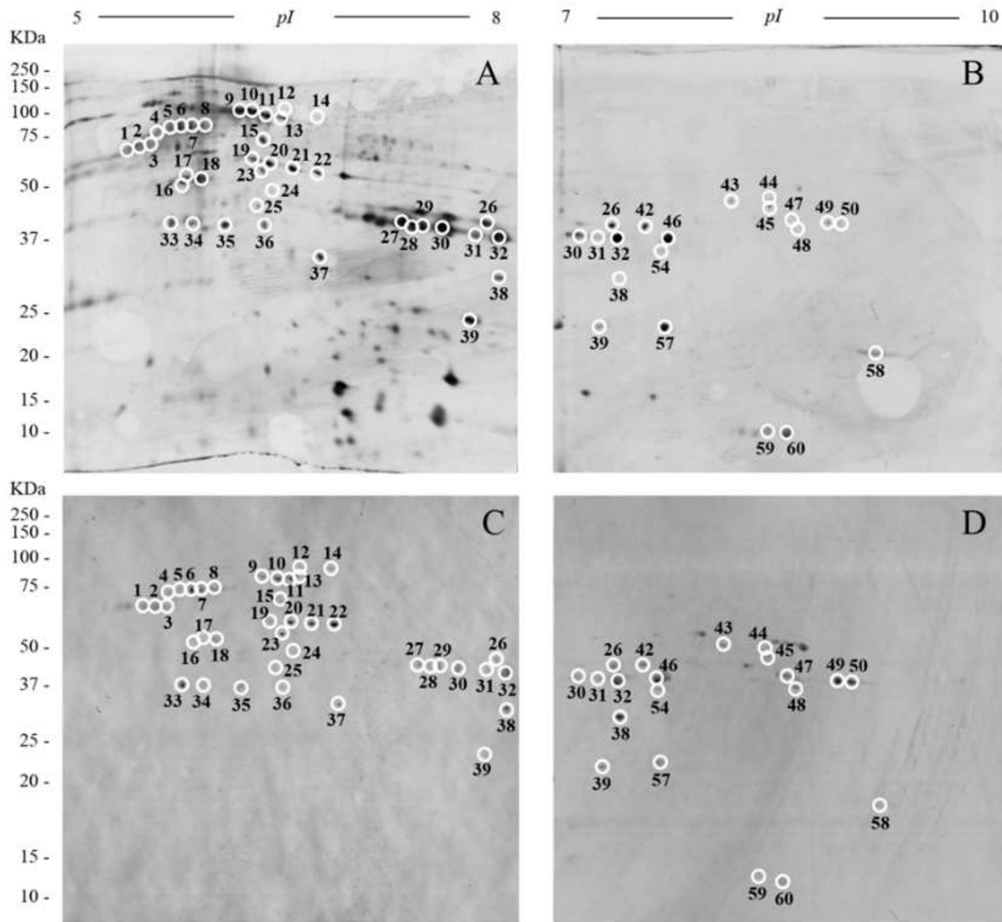

Figure 3. Representative 2-DE of 60 µg of the DiSAA extract from adult *D. immitis* worms. The gels were in the 5–8 and 7–10 pH ranges, 12% polyacrylamide and silver-stained (A and B). Plasminogen-binding spots revealed on ligand blots from gels A and B (C and D). Reference molecular masses are indicated on the left. The plasminogen-binding spots analyzed by MS are circled and numbered.

3.4. Identification of plasminogen-binding proteins

To identify plasminogen-binding proteins, a ligand blotting with plasminogen of 2D gels of 5–8 and 7–10 pH was performed. As shown in Fig. 3C and D, 61 plasminogen-binding spots were revealed (17.58% for total spots revealed in the surface proteome). Most of them ($n = 42$) were resolved in a narrow range of MWs and pIs (between 40 and 100 kDa, and 5.4 and 8, respectively). In the control blots, in which plasminogen incubation was omitted, the anti-plasminogen antibody did not reveal any spots (not shown).

The matching of spots revealed by ligand-blotting with their homologous in the silver-stained 2-D gels allowed us to select a total of 53 plasminogen-binding spots of *D. immitis*, which were manually excised from 2-D gels and submitted to analysis by MS.

Table 1 shows the identity of these proteins and their MWs and pIs (theoretical and experimental), the number of access to similar information available in the NCBI database, the sequence coverage and the Mascot score. Sixteen of 53 spots were identified (30.18%) and corresponded to 11 different proteins. Between 1 and 4 isoforms of each protein were identified. Most proteins were identified by their similarity to homologous proteins from other species of filarial worms. Thus of the 16 spots identified 14 corresponded to other filarial proteins (*Brugia malayi*, *Onchocerca volvulus* and *Loa loa*). The 2 remaining spots corresponded to a protein from the nematode *Trichinella spiralis* (actin-5C) and to a protein from the free-living protist parasite *Acanthamoeba castellanii* (actin-1).

Spot number	Accesion code	Protein definition	Species	MW (kDa) theor/exp	pI theor/exp	Queries matched	Mascot score
17	EFV54220	Actin-5C	*Trichinella spiralis*	41.8/54.1	5.3/5.7	4	154
18	P02578	Actin-1	*Acanthamoeba castellanii*	41.7/52.8	5.4/5.8	6	190
20	XP_001896281	Enolase	*Brugia malayi*	47.5/59.6	6.0/6.3	6	248
22	Q7YZX3	Enolase	*Onchocerca volvulus*	47.1/55.5	6.0/6.6	8	64
26	AAB52600	Fructose-bisphosphate aldolase	*Onchocerca volvulus*	39.2/40.5	7.7/7.7	8	247
42	AAB52600	Fructose-bisphosphate aldolase	*Onchocerca volvulus*	39.2/39.0	7.7/7.9	2	59
28	P48812	GAPDH	*Brugia malayi*	36.1/40.0	7.7/7.2	13	128
30	P48812	GAPDH	*Brugia malayi*	36.1/39.8	7.7/7.4	17	259
32	P48812	GAPDH	*Brugia malayi*	36.1/37.4	7.7/7.8	11	84
46	P48812	GAPDH	*Brugia malayi*	36.1/36.0	7.7/8.0	9	71
35	XP_001900868	MSP domain protein with Glu-rich domain	*Brugia malayi*	18.1/40.1	5.5/6.0	2	64
60	P13263	Major sperm protein 2	*Onchocerca volvulus*	14.3/15.7	7.8/8.8	18	265
37	AAA20541	Beta-galactosidase-binding-lectin	*Onchocerca volvulus*	32.0/33.6	6.0/6.6	20	143
38	XP_001900812	Galectin	*Brugia malayi*	31.8/30.3	6.4/7.8	3	68
39	XP_003139445	Immunoglobulin I-set domain-containing protein	*Loa loa*	22.5/24.2	6.6/7.6	8	307
58	AAC47233	Cyclophilin Ovcyp-2	*Onchocerca volvulus*	18.5/20.9	8.3/9.4	1	71

Table 1. Plasminogen-binding protein spots of DiSAA extract identified by MALDI-TOF MS. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; Exp, experimental; theo, theoretical.

4. Discussion

Cardiopulmonary dirofilariosis is caused by the long-term presence of *D. immitis* adult worms in the pulmonary arteries and right ventricle of their definitive host. In this location, worms are exposed to the effector mechanisms of the host immune system. Moreover, the death of the adult worms triggers some pathological processes that immediately threat the host's life, the thromboembolisms being one of the most serious. However, *D. immitis* can survive through different mechanisms of both immune evasion and modulation of its vascular environment (Simón et al., 2012). In the live worms there are two antigenic compartments participating in the parasite/host relationships, the excreted metabolic products (DiES antigens) and the surface-associated antigens, many of which are also excreted (Smith, 1991), taking part in the excretory/secretory antigens. In this study we demonstrate that the DiSAA bind plasminogen and stimulate plasmin generation. Surface-associated antigens have also been related to the plasminogen recruitment in both bacterial (Bergmann and Hammerschmidt, 2007) and parasitic infections (Jolodar et al., 2003 and Ghosh and Jacobs-Lorena, 2011). It has also been postulated that plasmin produced by plasminogen activation plays a key role in the degradation of extracellular matrices, migration through the tissues (Bergmann and Hammerschmidt, 2007) and evasion of the immune response (Barthel et al., 2012). For the conversion of plasminogen into plasmin t-PA is necessary, being mainly synthesized and secreted by the vascular endothelium (Cesarman-Maus and Hajjar, 2005). Given that it has been previously shown that the DiES causes an over-expression of t-PA in cultured vascular endothelial cells (González-Miguel et al., 2012), it is possible that a combined action of both compartments (DiES and DiSAA) could exist. Thus *D. immitis* worms are not only capable of activating the fibrinolytic system in order to avoid clot formation in the systemic level by action of the DiES, but also in their immediate environment by the action of the DiSAA. This combined action would be of great importance as a parasite survival mechanism.

The combination of proteomic, immunomic and MS techniques allowed us to identify a total of 16 spots of the DiSAA extract that corresponded to 11 plasminogen-binding proteins. These were identified using the available information of filarial proteins in databases. Among them, various enzymes from the group of actins (Dudani et al., 2005 and Ramajo-Hernández et al., 2007), enolase (Jolodar et al., 2003, Marcilla et al., 2007 and Mundodi et al., 2008), fructose-bisphosphate aldolase (Ramajo-Hernández et

al., 2007 and de la Paz Santangelo et al., 2011) and GAPDH (Erttmann et al., 2005 and Ramajo-Hernández et al., 2007) have been extensively studied due to their interaction with the fibrinolytic system in different types of pathogens. Furthermore, 3 isoforms of actin, 2 of GAPDH and the galectin were identified as plasminogen-binding proteins on the DiES antigens (González-Miguel et al., 2012). The other proteins identified in our study (MSP domain protein, major sperm protein 2, beta-galactosidase-binding-lectin, immunoglobulin I-set domain-containing protein and cyclophilin Ovcyp-2) have been identified as plasminogen binding proteins for the first time. These proteins are involved in different biological processes such as structural activity (MSP domain protein and MSP 2), carbohydrate binding (beta-galactosidase-binding-lectin) or protein folding (cyclophilin Ovcyp-2). Further studies are needed to know the real effect of the identified plasminogen-binding proteins on the survival mechanisms of *D. immitis*.

In summary, we have demonstrated the "in vitro" interaction between the surface-associated antigens of *D. immitis* and the host fibrinolytic system, supplementing the already demonstrated fibrinolytic activity of the excretory/secretory antigens of this species.

Acknowledgements

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References

Almeida, L., Vanegas, G., Calcagno, M., Concepción, J.L., Avilan, L., 2004. Plasminogen interaction with *Trypanosoma cruzi*. Mem. Inst. Oswaldo Cruz. 99, 63–67.

Barthel, D., Singh, B., Riesbeck, K., Zipfel, P.F., 2012. *Haemophilus influenzae* uses the surface protein E to acquire human plasminogen and to evade innate immunity. J. Immunol. 188, 379-385.

Bergmann, S., Hammerschmidt, S., 2007. Fibrinolysis and host response in bacterial infections. Thromb. Haemost. 98, 512–520.

Cesarman-Maus, G., Hajjar, K.A., 2005. Molecular mechanisms of fibrinolysis. Br. J. Haematol. 129, 307–321.

de la Paz Santangelo, M., Gest, P.M., Guerin, M.E., Coinçon, M., Pham, H., Ryan, G., Puckett, S.E., Spencer, J.S., Gonzalez-Juarrero, M., Daher, R., Lenaerts, A.J., Schnappinger, D., Therisod, M., Ehrt, S., Sygusch, J., Jackson, M., 2011. Glycolytic and non-glycolytic functions of *Mycobacterium tuberculosis* fructose-1,6-bisphosphate aldolase, an essential enzyme produced by replicating and non-replicating bacilli. J. Biol. Chem. 286, 40219-40231.

Dudani, A.K., Ben-Tchavtchavadze, M., Porter, S., Tackaberry, E., 2005. Angiostatin and plasminogen share binding to endothelial cell surface actin. Biochem. Cell. Biol. 83, 28–35.

Erttmann, K.D., Kleensang, A., Schneider, E., Hammerschmidt, S., Büttner, D.W., Gallin, M., 2005. Cloning, characterization and DNA immunization of an *Onchocerca volvulus* glyceraldehyde-3-phosphate dehydrogenase (Ov-GAPDH). Biochim. Biophys. Acta 1741, 85–94.

Fetterer, R.H., Rhoads, M.L., 1993. Biochemistry of the nematode cuticle: relevance to parasitic nematodes of livestock. Vet. Parasitol. 46, 103-111.

Genchi, C., Kramer, L.H., Prieto, G., 2001. Epidemiology of canine and feline dirofilariasis: a global view. In: Simón, F., Genchi, C., (Eds.), Heartworm infection in humans and animals. Ediciones Universidad de Salamanca, España, pp. 121–134.

Ghosh, A.K., Jacobs-Lorena, M., 2011. Surface-expressed enolases of *Plasmodium* and other pathogens. Mem. Inst. Oswaldo Cruz. 106, 85-90.

González-Miguel, J., Morchón, R., Mellado, I., Carretón, E., Montoya-Alonso, J.A., Simón, F., 2012. Excretory/secretory antigens from *Dirofilaria immitis* adult worms interact with the host fibrinolytic system involving the vascular endothelium. Mol. Biochem. Parasitol. 181, 134-140.

Jolodar, A., Fischer, P., Bergmann, S., Büttner, D.W., Hammerschmidt, S., Brattig, N.W., 2003. Molecular cloning of an alpha-enolase from the human filarial parasite *Onchocerca volvulus* that binds human plasminogen. Biochim. Biophys. Acta. 1627, 111–120.

Maizels, R.M., Blaxter, M.L., Robertson, B.D., Selkirk, M.E., 1991. Parasite antigen parasite genes: a laboratory manual for molecular parasitology. Cambridge University Press, Cambridge, UK.

Marcilla, A., Pérez-García, A., Espert, A., Bernal, D., Muñoz-Antolí, C., Esteban, J.G., Toledo, R., 2007. *Echinostoma caproni*: identification of enolase in excretory/secretory products, molecular cloning, and functional expression. Exp. Parasitol. 117, 57–64.

Mundodi, V., Kucknoor, A.S., Alderete, J.F., 2008. Immunogenic and plasminogen-binding surface-associated alpha-enolase of *Trichomonas vaginalis*. Infect. Immun. 76, 523–531.

Quiroz-Romero, H., 1984. Parasitología y enfermedades parasitarias de los animales domésticos. Dimusa S.A., México, pp. 620–627.

Ramajo-Hernández, A., Pérez-Sánchez, R., Ramajo-Martín, V., Oleaga, A., 2007. *Schistosoma bovis*: plasminogen binding in adults and the identification of plasminogen-binding proteins from the worm tegument. Exp. Parasitol. 115, 83–91.

Simón, F., Siles-Lucas, M., Morchón, R., González-Miguel, J., Mellado, I., Carretón, E., Montoya-Alonso, J.A., 2012. Human and animal dirofilariasis: the emergence of a zoonotic mosaic. Clin. Microbiol. Rev. 25, 507-544.

Smith, H.V., 1991. Immune evasión and immunopathology in *Toxocara canis* infection. In: Kennedy, M.W. (Ed.), Parasitic nematodes – antigens, membranes and genes. Taylor & Francis, UK, pp. 116-139.

Vanegas, G., Quiñones, W., Carrasco-López, C., Concepción, J.L., Albericio, F., Avilán, L., 2007. Enolase as a plasminogen binding protein in *Leishmania mexicana*. Parasitol. Res. 101, 1511–1516.

Venco, L., 2007. Heartworm (*Dirofilaria immitis*) disease in dogs. In: Genchi, C., Ronaldi, L., Cringoli, G. (Eds.), *Dirofilaria immitis* and *D. repens* in dog and cat and human infections. Rolando Editore, Italia, pp. 117–125.

Wedrychowicz, H., Holmes, P.H., Bairden, K., Tait, A., 1994. Surface and excretory/secretory antigens of fourth-stage larvae and adult *Ostertagia circumcincta*. Vet. Parasitol. 53, 117-132.

TERCER CAPÍTULO

"Surface-displayed glyceraldehyde 3-phosphate dehydrogenase and galectin from *Dirofilaria immitis* enhance the activation of the fibrinolytic system of the host"

La gliceraldehído-3-fosfato deshidrogenasa y la galectina asociadas a la superficie de *Dirofilaria immitis* potencian la activación del sistema fibrinolítico del hospedador

Javier González-Miguel, Rodrigo Morchón, Mar Siles-Lucas, Ana Oleaga, Fernando Simón

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Resumen

La dirofilariosis cardiopulmonar es una enfermedad cosmopolita causada por *Dirofilaria immitis*, un parásito filaroideo cuyos vermes adultos viven durante años en el sistema vascular de su hospedador. Estudios previos han demostrado que *D. immitis* puede utilizar sus antígenos excretores/secretores (ES) y de superficie para potenciar la fibrinolisis, lo que podría limitar la formación de coágulos en su entorno. Además, varias isoformas de la gliceraldehído-3-fosfato deshidrogenasa (GAPDH) y la galectina (GAL) fueron identificadas en ambos extractos antigénicos como proteínas fijadoras de plasminógeno. El objetivo de este trabajo es estudiar la interacción de la GAPDH y la GAL de *D. immitis* con el sistema fibrinolítico del hospedador. Este estudio incluye la clonación, secuenciación y expresión de las formas recombinantes de la GAPDH y la GAL de *D. immitis* (rDiGAPDH y rDiGAL) y el análisis de sus capacidades como proteínas fijadoras de plasminógeno. Los resultados indican que rDiGAPDH y rDiGAL son capaces de fijar plasminógeno y estimular la generación de plasmina mediada por el activador tisular del plasminógeno (tPA). Esta interacción necesita la implicación de residuos de lisina, muchos de los cuales se encuentran localizados externamente en ambas proteínas como se demuestra con el modelado molecular de sus estructuras secundarias. Además, mostramos que rDiGAPDH y rDiGAL aumentan la expresión del activador del plasminógeno de tipo uroquinasa (uPA) en cultivos de células endoteliales caninas y que ambas proteínas se expresan en la superficie de *D. immitis* en contacto directo con la sangre del hospedador. Estos datos sugieren que *D. immitis* podría utilizar la GAPDH y la GAL asociadas a la superficie como receptores fisiológicos del plasminógeno para modificar el equilibrio fibrinolítico hacia la generación de plasmina, lo que podría

constituir un mecanismo de supervivencia para evitar la formación de coágulos en su hábitat intravascular.

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Surface-displayed glyceraldehyde 3-phosphate dehydrogenase and galectin from *Dirofilaria immitis* enhance the activation of the fibrinolytic system of the host

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Abstract

Cardiopulmonary dirofilariosis is a cosmopolitan disease caused by *Dirofilaria immitis*, a filaroid parasite whose adult worms live for years in the vascular system of its host. Previous studies have shown that *D. immitis* can use their excretory/secretory (ES) and surface antigens to enhance fibrinolysis, which could limit the formation of clots in its surrounding environment. Moreover, several isoforms of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and galectin (GAL) were identified in both antigenic extracts as plasminogen-binding proteins. The aim of this work is to study the interaction of the GAPDH and GAL of *D. immitis* with the fibrinolytic system of the host. This study includes the cloning, sequencing and expression of the recombinant forms of the GAPDH and GAL of *D. immitis* (rDiGAPDH and rDiGAL) and the analysis of their capacity as plasminogen-binding proteins. The results indicate that rDiGAPDH and rDiGAL are able to bind plasminogen and stimulate plasmin generation by tissue plasminogen activator (tPA). This interaction needs the involvement of lysine residues, many of which are located externally in both proteins as have been shown by the molecular modeling of their secondary structures. In addition, we show that rDiGAPDH and rDiGAL enhance the expression of the urokinase-type plasminogen activator (uPA) on canine endothelial cells in culture and that both proteins are expressed on the surface of *D. immitis* in close contact with the blood of the host. These data suggest that *D. immitis* could use the associated surface GAPDH and GAL as physiological plasminogen receptors to shift the fibrinolytic balance towards the

generation of plasmin, which might constitute a survival mechanism to avoid the clot formation in its intravascular habitat.

Key words: *Dirofilaria immitis*; glyceraldehyde 3-phosphate dehydrogenase; galectin; plasminogen; fibrinolysis.

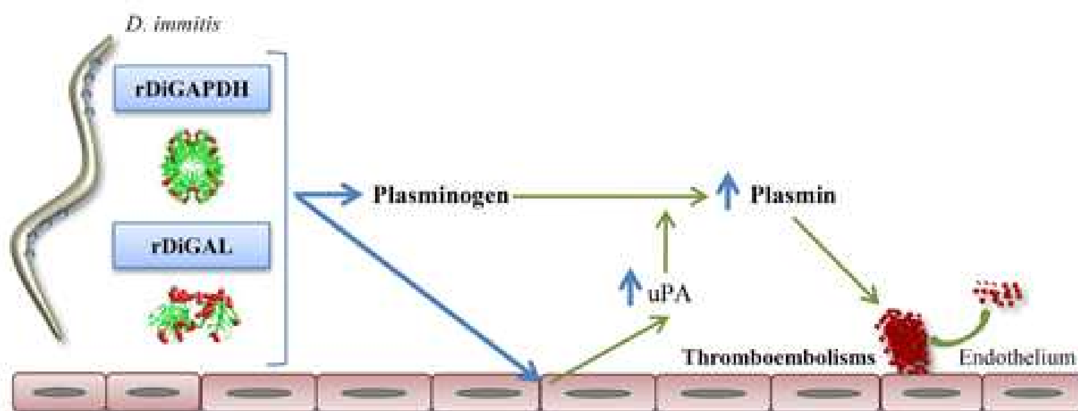
Abbreviations

ES, excretory/secretory; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GAL, galectin; rDiGAPDH, recombinant form of the GAPDH of *D. immitis*; rDiGAL, recombinant form of the GAL of *D. immitis*; tPA, tissue plasminogen activator; uPA, urokinase-type plasminogen activator; OP, optical density; εACA, lysine analogue ε-aminocaproic acid; CnAOEC, canine aortic endothelial cells; DiES, excretory/secretory antigens from *D. immitis* adult worms.

Highlights

- ✓ The *Dirofilaria immitis* GAPDH and GAL were cloned and sequenced.
- ✓ rDiGAPDH and rDiGAL bind plasminogen and generate plasmin activating fibrinolysis.
- ✓ Plasminogen activation by rDiGAPDH and rDiGAL requires tPA and lysine residues.
- ✓ rDiGAPDH and rDiGAL enhance the expression of uPA in canine endothelial cells.
- ✓ rDiGAPDH and rDiGAL are located exposed to the host on the surface of *D. immitis.*

Graphical Abstract



1. Introduction

Fibrinolysis is one of the main anticlotting mechanisms of the hemostatic system. Its key molecule is plasminogen, an abundant component of blood and zymogen of serine protease plasmin, enzyme responsible for degrading fibrin clots. The conversion of plasminogen into plasmin is regulated by binding to receptors via its five kringle domains, which have affinity for lysine residues and plasminogen activators (tPA and uPA) (Cesarman-Maus and Hajjar, 2005).

In order to maintain and propagate in the circulatory system, many bloodborne pathogens not only require adaptations to evade the activity of the host immune system, but also need to prevent blood clotting through interaction with the fibrinolytic system (Mebius et al., 2013). Cardiopulmonary dirofilariosis is a chronic and potentially fatal parasitic disease that affects dogs and cats around the world (Genchi et al., 2001). It is characterized by the presence of *D. immitis* adult worms in the pulmonary arteries and right ventricle of the infected hosts, where they can live for years causing a chronic inflammatory pathology (Venco, 2007). In previous studies, we have demonstrated the ability of *D. immitis* to bind plasminogen, enhancing plasmin generation by tPA by using two antigenic compartments (ES and surface) in an in vitro system. We have also observed that the ES antigens are able to induce an overexpression of the fibrinolytic activator tPA in vascular endothelial cells in culture. Additionally, we have respectively identified a total of 10 and 11 plasminogen-binding proteins in the ES and surface extracts of the parasite, which included different isoforms of GAPDH and GAL (González-Miguel et al., 2012 and González-Miguel et al., 2013).

GAPDH has historically been regarded as a "housekeeping" protein. However, its involvement in numerous cellular processes in addition to glycolysis has been recently demonstrated. These include DNA repair, tRNA export, membrane fusion and transport, cytoskeletal dynamics and cell death (Tristan et al., 2011). Moreover its relationship with the fibrinolytic system has been widely studied being identified as plasminogen-binding protein in bacteria (Bhattacharya et al., 2012), fungi (Crowe et al., 2003) and parasites (Erttmann et al., 2005, Ramajo-Hernández et al., 2007 and Lama et al., 2009). GAPDH is one of the most studied plasminogen receptors in parasites together with enolase, which has been reported as plasminogen-binding protein for the

related filaria *Onchocerca volvulus* (Jolodar et al., 2003) or *Schistosoma bovis* (Ramajo-Hernández et al., 2007 and de la Torre-Escudero et al., 2010) among others.

Galectins are β-galactoside-binding proteins characterized by its high level of evolutionary conservation, having been identified in many species from nematodes to mammals. Galectins have a wide range of biological functions in different processes including homeostasis, apoptosis, and vascular embryogenesis and in pathological conditions such as pre-eclampsia, inflammation, diabetes, atherosclerosis and cancer (Astorgues-Xerri et al., 2014). Related to filarial worms, onchocercal molting L3 strongly express GAL, being this protein proposed as good target for protective responses (Joseph et al., 2000). The interaction between this molecule and plasminogen has not yet demonstrated. However, the link between GAL-1 expression and cancer cell invasion with the demonstration of a direct interaction between tPA and GAL-1 in pancreatic cancer cells and stromal fibroblasts surrounding the tumor has been recently shown. This interaction enhanced tPA proteolytic activity and increased cell migration and invasion (Roda et al., 2009).

The aim of this study was to perform the molecular and functional characterization of the *D. immitis* GAPDH and GAL showing their capabilities as plasminogen-binding proteins, their relationships with the endothelium-dependent components of the fibrinolytic system and confirming their presence on the surface of the parasite.

2. Materials and methods

2.1. Parasite material

Adult worms of *D. immitis* were obtained from hearts of infected dogs from Gran Canary (Canary Islands, Spain) through the jugular vein using Flexible Alligator Forceps.

2.2. RNA isolation, RT-PCR, and cloning of GAPDH and GAL cDNA

Total RNA from adult worms was extracted using the NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. First-strand cDNA was synthesized from *D. immtis* adult worms RNA using the first-strand cDNA synthesis kit (Roche) as recommended by the manufacturer. The cDNA sequence of the *D. immitis* GAPDH and GAL were amplified using the following primers:

GAPDHFwd (5´-ATGAGCAAACCAAAGATTGGAATC)
GAPDHRev (5´-TTATCTGCTGGCGATGTAAGAG)
GALFwd (5´-ATGCACCACAACGAATATGAAACGAATTAC)
GALRev (5´-CTAGTGCATTTGAATACCGCTCACTTC)

The primers from GAPDH were designed on the consensus sequence resulting after the alignment of GAPDH cDNA sequences from *O. volvulus* and *Brugia malayi* (GenBank accession numbers U96177.1 and U18137.1 respectively). The primers from GAL were designed on the sequence of GAL cDNA sequences from *D. immitis* (GenBank accession number AF237485.1). PCR was performed in 1 cycle at 94 °C for 5 m, 35 cycles at 94 °C for 1 m, 46 °C for 46 s and 72 °C for 1 min 30 s, and 1 cycle at 72 °C for 5 m. The PCR products were electrophoresed in an agarose gel and the bands were purified from the gel using the StrataPrep DNA Gel Extraction kit (Stratagene) as recommended by the manufacturers. The GAPDH and GAL PCR products were cloned into the pSC-A vector using the StrataClone PCR Cloning kit (Stratagene) following the manufacturer's instructions. Both clones were purified with the Machery-Nagel NucleoSpin Plasmid kit.

2.3. Expression and purification of the rDiGADPH and rDiGAL

PCR products containing the whole rDiGADPH and rDiGAL coding sequences were cloned into the TOPO vector (Gateway System, Invitrogen) following the manufacturer's instructions. The recombinant plasmids were transformed into the *Escherichia coli* XL1B. Transformed cells were grown in LB-agar plates with ampicillin (100 μg/ml) overnight at 37 °C. Three colonies for each molecule were grown in liquid LB plus ampicillin overnight at 37 °C in agitation, and cells were harvested for plasmid extraction. Extracted plasmids were digested with *Eco*RI to check the insert presence. The TOPO vectors containing the fragments of interest were used for a ligation reaction with the pDEST7 vector (Gateway System, Invitrogen) following the manufacturer's instructions. Ligation reaction was transformed into XL1B cells and grown in LB-agar plates with ampicillin (100 μg/ml) overnight at 37 °C. Three colonies for each molecule were grown in liquid LB plus ampicillin overnight at 37 °C in agitation, and cells were harvested for plasmid extraction. Extracted plasmids were digested with *Eco*RI to check the insert presence. Vectors containing the inserts of interest were sequenced with the T7 primer (Sequencing Service of the Salamanca

University) to check for the correct reading frame. The vectors containing the molecule of interest in reading frame were used to transform BL-21 expression cells. These were grown in liquid LB plus ampicillin (100 µg/ml) overnight at 37 °C in agitation. Cultures were diluted 1:20 in fresh medium and further growth until reaching an optical density (OD) of 0.5 at 600 nm. Then, expression of the recombinant proteins was induced by adding L-arabinose at a final concentration of 0.2% and further growing at 37 °C for 4 h in agitation. The induced cells were harvested and sonicated in a buffer containing 50 mM Na_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 8 for rDiGADPH and 8 M urea, 100 mM NaH_2PO_4 and 10 mM Tris–Cl, pH 7.9 for rDiGAL. After a 20 min centrifugation step at 10,000 × *g*, the supernatant was applied to a HIS-Select® Nickel Affinity Gel (Sigma) for affinity purification of the histidine-tagged rDiGADPH and rDiGAL, according to the manufacturer's instructions. Urea was eliminated for rDiGAL by washing the column with wash buffer (100 mM NaH_2PO_4, and 10 mM Tris–Cl pH 6.3) containing decreasing concentrations of urea (from 6 M to 0 M). Then, the recombinant proteins were eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 7.9). The eluted rDiGAPDH and rDiGAL were dialyzed in PBS for 24 h at 4 °C and stored at –80 °C until use. The purity and yield of each protein obtained after purification were assessed in 12% polyacrylamide gels using Coomasie blue staining. The densitometry was calculated with the PDQUEST program (Bio-Rad).

2.4. Bioinformatic analyses

The deduced amino-acid sequence of rDiGAPDH and rDiGAL were analyzed using the following bioinformatic tools: BLAST searching of the homologous sequences in the NCBI and Swissprot/Uniprot databases (http://www.ncbi.nlm.nih.gov/, http://www.uniprot.org/); analysis of conserved domains with SMART (http://smart.embl-heidelberg.de); theoretical isoelectric point (p*I*) and the molecular weight (MW) calculations (http://www.expasy.org/tools/pi_tool.html); prediction of transmembrane domains with the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0); prediction of signal peptides with SignalP 3.0 (Bendtsen et al., 2004) (http://www.cbs.dtu.dk/services/SignalP); search for glycosyl–phosphatidyl anchors in the sequence with the big-PI Predictor (Eisenhaber et al., 2000) (http://mendel.imp.ac.at/sat/gpi/gpi_server.html); multiple sequence alignment with ClustalW 2.1 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and prediction of the secondary structures and three-dimensional modeling with the Swiss-

Model server (Arnold et al., 2006; http://swissmodel.expasy.org/) using as templates the X-ray crystal structure of a GAPDH from *B. malayi* (code pdb: 4K9D) for DiGAPDH (identity of 96.76%) and the crystal structure of *Toxascaris leonine* GAL (code pdb: 4HL0) for DiGAL (identity of 90.11%). The 3-D models were visualized with the RasMol software v. 2.7.5.2.

2.5. Plasminogen binding assays

To determine whether the rDiGAPDH and rDiGAL would bind plasminogen an ELISA test was carried out as described previously (González-Miguel et al., 2012). In brief, multiwell microplates (Costar) were coated with 0.5 µg/well of each protein, blocked and then incubated with increasing concentrations (from 0 to 30 µg/ml) of human plasminogen (Acris antibodies). After incubation with the corresponding antibodies and with a chromogen, the OD was measured at 492 nm in an easy reader (Bio-Rad). In parallel, competition assays were performed by including 50 mM of the lysine analogue ϵ-aminocaproic acid (ϵACA) during plasminogen incubation.

2.6. Plasminogen activation assays

Plasminogen activation assay was performed in a test volume of 100 µl by measuring the amidolytic activity of generated plasmin (González-Miguel et al., 2012). In each well 2 µg of human plasminogen (Acris antibodies) were incubated in PBS with 3 µg of the chromogenic substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride (Sigma) in the presence of 1 µg of rDiGAPDH or rDiGAL. Activation of plasminogen was initiated by addition of 15 ng of tPA (Sigma). In parallel, plasmin generation was also measured in the absence of tPA. Plates were incubated at 37 °C for 2 h and the hydrolysis of the chromogenic substrate was monitored by measuring absorbance at 405 nm every 30 min. Each sample was analyzed in triplicate.

2.7. Cell culture and stimulation of endothelial cells

Canine aortic endothelial cells (CnAOEC) (Cell Applications, Inc.) were grown in canine endothelial growth mediums (Cell Applications, Inc.). Plates were precoated with an attachment factor solution (Cell Applications, Inc.) and cells were cultured at 37 °C in a humidified atmosphere in the presence of 5% carbon dioxide and 95% air. Medium was changed every 3 days. Endothelial cells (10^6 cells/plate) were plated on 100 mm culture plates and grown for 4 days to obtain confluent cultures and treated

with 1 µg/ml of rDiGAPDH or rDiGAL for 24 h. Non-stimulated cells were used as controls under the same conditions.

2.8. Cell lysates and Western blot analyses

Western blot analysis was performed as previously described (Morchón et al., 2010) with slightly modifications. Treated and control CnAOEC were lysed in ice-cold lysis buffer (20 mM Tris–HCl (pH 7.5), 140 mM NaCl, 10 mM ethylendiaminetetraacetic acid, 10% glycerol, 1% Igepal CA-630, aprotinin, pepstanin, pepstatin, and leupeptin at 1 µg/ml each, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate). Protein samples (10 µg) were separated by SDS–PAGE under reducing conditions and blotted onto polyvinylidine difluoride membranes. Membranes were blocked before incubation with the primary antibodies anti-tPA and anti-uPA (Santa Cruz Biotechnology, Inc.) according to the manufacturer's recommendations. After incubation with HRP-conjugated secondary antibodies, bands were visualized by a luminol-based detection system with p-iodophenol enhancement. Anti-α-tubulin antibody (Oncogene Research Products) was used to confirm loading of comparable amount of protein in each lane. Protein expression was quantified by densitometry using Scion Image Software (Scion).

2.9. Generation of an anti-rDiGAPDH and anti-rDiGAL antisera

Antisera against *D. immitis* rGAPDH and rGAL were generated by subcutaneous immunization of four New Zealand female rabbits with three doses of each protein in 0.2% saponin solution. First dose of 1 mg at the beginning of the experiment, plus two doses of 500 µg 7 and 10 days later. Rabbits were bled 20 days after the last dose. Sera were collected, serially diluted and titred by ELISA. The reactivity and specificity of the sera were also assessed by Western blot on rDiGAPDH, rDiGAL or on ES extract from *D. immitis* adult worms (DiES) containing the corresponding native proteins. In brief, recombinant proteins (2 µg each) and DiES (10 µg) were electrophoresed on 12% SDS–PAGE gels, electrotransferred onto nitrocellulose membranes, blocked with 2% BSA and incubated with the antisera against rDiGAPDH, rDiGAL or with a negative control serum at 1/500 dilution. After washing, immunoblots were incubated with 1/2000 diluted peroxidase-labeled anti-rabbit IgG and revealed with 4-chloro naphthol. Images were digitized with the scanner GS-800 Densitometer (Bio-Rad) using the Quantity One Software v.4.6.5 (Bio-Rad).

2.10. Immunolocalization of proteins in *D. immitis* adult worms

Immunolocalization assays were carried out in microtome-cut 5 µm sections after dehydrate and embed in paraffin *D. immitis* adult worms. The sections were placed on microscope slides, deparaffinized in xylene, rehydrated and blocked with 1% BSA in PBS. Then, sections were firstly incubated with the anti-rDiGAPDH or anti-rDiGAL rabbit antisera or with a negative serum (rabbit preinmune serum), all of them diluted 1/50 in blocking buffer. Secondly, samples were incubated with an anti-rabbit IgG antibody conjugated to Alexa Fluor 594 (Invitrogen) diluted 1/400 in blocking buffer containing phalloidin-Alexa Fluor 488 (Invitrogen) diluted 1/200, which binds to actin microfilaments. All incubations were carried out for 1 h at 37 °C in a humid chamber and between each step, three washes of 5 min with PBS containing 0.05% Tween-20 were performed. Finally, the samples were washed four times, mounted in antifade reagent (Prolong Gold, Invitrogen) and analyzed with a Leica TCS-NT confocal microscope.

2.11. Statistical analysis

The results from the plasminogen-binding assay, plasminogen activation assay and Western blots for the tPA and uPA expression were analyzed with the Student's *t*-test. The results were expressed as the mean ± SD of at least three independent experiments. In all experiments, a significant difference was defined as a *p*-value of <0.05 for a confidence level of 95%.

3. Results

3.1. Amplification, cloning, sequencing, and expression of *D. immitis* GAPDH and GAL

Amplification of *D. immitis* GAPDH and GAL cDNA by RT-PCR resulted in a PCR product of around 1000 and 850 bp respectively. These were cloned into the pSC-A vector and fully sequenced. BLAST analysis of the sequence demonstrated its identity as glyceraldehyde 3-phosphate dehydrogenase and galectin. The GAPDH new sequence was deposited in the Gen-Bank under accession number JQ780095.1. The full *D. immitis* GAPDH and GAL cDNA contained 1020 and 846 nucleotides, encoded proteins of 339 and 280 amino acids, with a theoretical molecular weight of 36,179 and 32,085 Da, and p*I* of 7.11 and 6.08 respectively.

The bioinformatics analyses of the deduced amino acid sequences did not reveal a signal peptide, transmembrane helices or glycosyl-phosphatidyl inositol anchors. The percentage identity between DiGAPDH and homologous sequences from other organisms (*O. volvulus*, *S. bovis*, *Candida albicans*, *Streptococcus pyogenes*, *Bacillus anthracis* and *Trichomonas vaginalis*) whose GAPDH had been previously related with plasminogen-binding activities was analyzed using multiple sequence alignment with the ClustalW program (Fig. 1). The analysis revealed a range of identities between the 94.99% of the filaria *O. volvulus* and the 43.07% of the protozoa *T. vaginalis*. Additionally, seven conserved lysine residues in all the sequences were found and highlighted. Amino-acid conservation of DiGAPDH and DiGAL was analyzed by alignment with homologous sequences from other parasites (Figs. S1 and S2). DiGAPDH and DiGAL revealed a strong identity with the homologous sequences from other filarial nematodes (*B. malayi*, *O. volvulus*, *Loa loa* and *Wuchereria bancrofti*) ranging from 97.46% and 94.99% in the case of DiGAPDH (Fig. S1) and from 96.79% and 94.81% in the case of DiGAL (Fig. S2). These sequences also showed high identities in the alignment with proteins from other non-filarial parasitic helminths. DiGAPDH revealed identities of 87.61%, 76.33% and 73.96% with the GAPDH from *Ascaris suum*, *S. mansoni* and *Fasciola hepatica* (Fig. S1); and DiGAL revealed identities of 88.13 and 82.73 with GAL from *A. suum* and *Haemonchus contortus* (Fig. S2). Conserved lysine residues of DiGAL were also highlighted. In silico three-dimensional modeling of the molecules predicted the 3D structures showing in the case of DiGAPDH a homo-tetramer with 15 α-helices and 4 β-sheets (Fig. 2A). Molecular modeling of DiGAL showing a monomer with the presence of 2 α-helices and 26 β-sheets (Fig. 2B). Conserved lysine residues were highlighted and were visualized on the outside of the proteins.

The *D. immitis* GAPDH and GAL cDNA were cloned into the expression vector TOPO/pDEST. After induction of expression in *E. coli*, the hexahistidine-tagged recombinant proteins were purified under denaturing conditions using nickel affinity chromatography. The purified recombinant proteins rDiGAPDH and rDiGAL had molecular weights of 38.6 kDa and 34.6 kDa in polyacrylamide gel.

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D.immitis        MSKPKIGINGFGRIGRLVLRAAVEK--NTVDVVAVNDPFINIDYMVYMFKYDSTHGRFKG 58
O.volvulus       MSKPKIGINGFGRIGRLVLRAAVEK--DTVEVVAVNDPFINIDYMVYMFKYDSTHGRFKG 58
S.bovis          MSRAKVGINGFGRIGRLVLRAAFQK--NTVDIVSVNDPFINLEYMVYMIKRDSTHGNFQG 58
C.albicans       -MAIKIGINGFGRIGRLVLRVALGR--KDIEVVAVNDPFIAPDYAAYMFKYDSTHGRYKG 57
S.pyogenes       -MVVKVGINGFGRIGRLAFRRIQNI--EGVEVTRIND-LTDPNMLAHLLKYDTTQGRFDG 56
B.anthracis      --MTRVAINGFGRIGRMVFRQAIKE--SAFEIVAINASYPSET-LAHLIKYDTVHGKFDG 55
T.vaginalis      ------------RIGRLVFRACRKLYPKDIQVVAIHD-LGDIKTNVYLLKYDTAHRAFPE 47
                             ****:.:*       . .::. ::        .:::* *:.:  :

D.immitis        NVSAEGGK--LVVTNGQTTHHISVHNSKD-PAEIPWGVDGAEYVVESTGVFT-------- 107
O.volvulus       HVSAEGGK--LIVTNGKTTHQIAVHNSKD-PAEIPWGVEGAEYVVESTGVFT-------- 107
S.bovis          EVSAEDGK--LKVNG----KLISVHFERD-PRNIPWDKDGAEYVVESTGVFT-------- 103
C.albicans       EVTASGDD--LVIDG----HKIKVFQERD-PANIPWGKSGVDYVIESTGVFT-------- 102
S.pyogenes       TVEVKEGG--FEVNG----NFIKVSAERD-PENIDWATDGVEIVLEATGFFA-------- 101
B.anthracis      TVEAFEDH--LLVDG----KMIRLLNNRD-PKELPWTDLGVEVVIEATGKFN-------- 100
T.vaginalis      PVTVDEAKQEFTVGEGADKWVVKSIGGRLGPSQLPWKELGIDVVLESTGIFRTKAEKDAE 107
                  * .      : :         :      :  * :: *    * : *:*:** *

D.immitis        ---TTEKASAHLKGG-AKKVIISAP-SADAPM-FVMGVNNETYDKANNHIISNASCTTNC 161
O.volvulus       ---HTEKASAHLKGG-AKKVIISAP-SADAPM-FVMGVNNDKYDKANNHIISNASCTTNC 161
S.bovis          ---TIDAAKAHTENNRAKKVIISAP-SADAPM-FVVGVNENSYDKS-MSVVSNASCTTNC 157
C.albicans       ---KVEGAQKHIDAG-AKKVIITAP-SADAPM-FVVGVNEDKYTPD-LKIISNASCTTNC 155
S.pyogenes       ---KKEAAEKHLHANGAKKVVITAPGGNDVKT-VVFNTNHDILDGT-ETVISGASCTTNC 156
B.anthracis      ---AKEKAILHVEAG-AKKVILTAPGKNEDVT-IVVGVNEDQLDITKHTVISNASCTTNC 155
T.vaginalis      GKIKKDGYDGHLVSG-AKKVVLSVPSADEIECTLVLGVNDEDLKPE-TKCISNASCTTNC 165
                      :    *    . ****:::.*    :     .*...*.:           :*.*******

D.immitis        LAPLAKVIHDKFGIIEGLMTTVHATTATQKTVDGPS-GKLWRDGRGAGQNIIPASTGAAK 220
O.volvulus       LAPLAKVIHDKFGIIEGLMTTVHATTATQKTVDGPS-GKLWRDGRGAGQNIIPASTGAAK 220
S.bovis          LAPLAKVIHDNFEIVEGLMTTVHSFTATQKVVDGPS-SKLWRDGRGAMQNIIPASTGAAK 216
C.albicans       LAPLAKVVNDTFGIEEGLMTTVHSITATQKTVDGPS-HKDWRGGRTASGNIIPSSTGAAK 214
S.pyogenes       LAPMAKALHDAFGIQKGLMTTIHAYTGDQMILDGPHRGGDLRRARAGAANIVPNSTGAAK 216
B.anthracis      LAPVVKVLDEQFGIENGLMTTVHAYTNDQKNIDNPH--KDLRRARACGQSIIPTTTGAAK 213
T.vaginalis      LGPVAKTLNNAFGIRNGFMTTVHSYTNDQVVADTMH--KDLRRARAAGMNIIPTSTGAAI 223
                 *.*:.*.:.: * * :*:***:*: *  *   *          * .*    .*:* :****

D.immitis        AVGKVIPDLNGK-LTGMAFRVPTPDVSVVDLTCRLQKGATMDEIKAAVKEAAA-GPMKGI 278
O.volvulus       AVGKVIPDLNGK-LTGMASRVPTPDVSVVDLTCRLQKGASMDEIKAAVKEAAA-GPMKGI 278
S.bovis          AVGKVIPALNGK-LTGMAFRVPTPDVSVVDLTCRLGKKATYDQIKAVVKAAAS-GPMKGI 274
C.albicans       AVGKVIPELNGK-LTGMSLRLPTTDVSVVDLTVRLKKAASYEEIAPAIKKASE-GPLKGV 272
S.pyogenes       AIGLVIPELNGK-LDGAAQRVPVPTGSVTELVVTLDKNVSVDEINAAMKAASN-DSFG-- 272
B.anthracis      ALAKVLPHLNGK-LHGMALRVPTPNVSLVDLVVDVKRDVTVEAINDAFKTVAN-GALKGI 271
T.vaginalis      ALPKVCHGLPPKSLDGFALRVPTITGSLVDLTVNVNAKVTKEEVNAALKKATEEGSLKGI 283
                 *:   *    *  * * * : *:*.    *:.:*.   :    .: : :   ..* .:   ..:

D.immitis        LEYTEDQVVSSDFIGDAHSSIFDALACISLNPN-----FVKLIAWYDNEYGYSNRVVDLI 333
O.volvulus       LEYTEDQVVSSDFVGDPHSSIFDALACISLNPN-----FVKLIAWYDNEYGYSNRVVDLI 333
S.bovis          LEYSDDEVVSSDFIGNSSSSIFDAKAGISLNDN-----FVKLISWYDNEFGYSCRVVDLI 329
C.albicans       LGYTEDAVVSTDFLGSSYSSIFDEKAGILLSPT-----FVKLISWYDNEYGYSTKVVDLL 327
S.pyogenes       --YTEDPIVSSDIVGVSYGSLFDATQTKVMEVDGSQ--LVKVVSWYDNEMSYTAQLVRTL 328
B.anthracis      VEFSEEPLVSIDFNTNTHSAIIDGLSTMVMGDR-----KVKVLAWYDNEWGYSRRVVDLV 326
T.vaginalis      MTYVTDPIVSSDIIGCQYSSIVDALSTKVLPNPEGQGTLVKVLSWYDNEWMYSCRCADIF 343
                    :  : :** *:      .::.*       :         **:::*****  *: :  .   .
                                                 Identity (%)
D.immitis        SYIASR---------- 339            -
O.volvulus       SYNASK---------- 339            94.99
S.bovis          THMHKVDHA------- 338            75.74
C.albicans       EHVA------------ 331            67.98
S.pyogenes       EYFAKIAK-------- 336            46.90
B.anthracis      TLVVDELAKQENVQHI 342            44.94
T.vaginalis      HRLEKYL--------- 350            43.07
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Figure 1. Alignment of the *D. immitis* GAPDH sequence (AFL46382) with the GAPDH from *O. volvulus* (CAA70607), *S. bovis* (ACC78613), *C. albicans* (AAC49800), *S. pyogenes* (AAK33348), *B. anthracis* (AIF58743) and *T. vaginalis* (AAA30325), which have previously been associated with plasminogen-binding activities. The percentage of sequence identity between *D. immitis* sequence and the others is indicated. The amino acids conserved in all the sequences are labeled with asterisks, and conservative and semiconservative substitutions are labeled with two and one point, respectively. Conserved lysine residues are shaded in yellow.

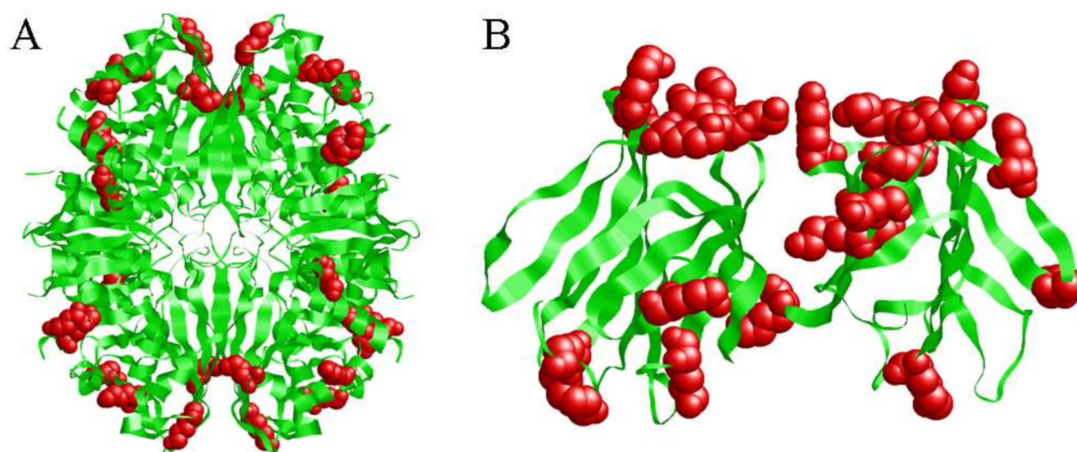

Figure 2. Molecular modeling of *D. immitis* GAPDH (A) and GAL (B). The secondary structure of the proteins were predicted with the Swiss-Model web server (http://swissmodel.expasy.org/) by analogy with the X-ray crystallography available models. The three-dimensional models of the molecules were visualized with the RasMol application v. 2.7.5.2. Conserved lysine residues of proteins were highlighted as red balls.

3.2. rDiGAPDH and rDiGAL bind plasminogen

The binding level of plasminogen to rDiGAPDH and rDiGAL was assessed by ELISA (Fig. 3). Analyses showed that both recombinant proteins bind plasminogen and that this binding is directly proportional to the amount of plasminogen. Comparing the results obtained by both recombinant proteins, rDiGAPDH showed higher plasminogen-binding capacity than rDiGAL (Fig. 3). The negative control consisting of wells coated only with BSA showed some non-specific binding activity, but always with values significantly lowers than those obtained by rDiGAPDH and rDiGAL ($p < 0.05$). To determine whether or not lysine residues are involved in binding, a competition experiment including 50 mM ϵACA was carried out. In this case the binding was inhibited about 90% in the case of rDiGAPDH and approximately 70% in the case of rDiGAL, resulting in slightly higher optical densities than the negative control (Fig. 3).

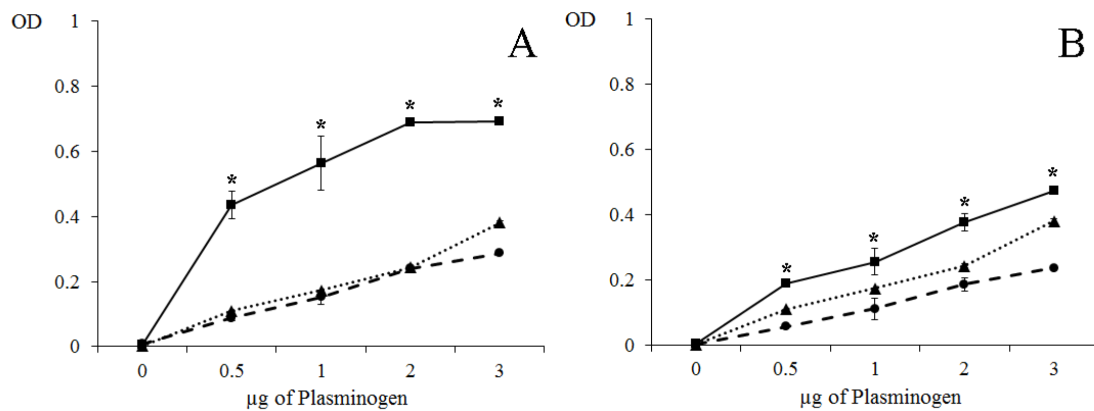

Figure 3. Plasminogen binding to 0.5 µg of rDiGAPDH (A) or rDiGAL (B) measured over a range of plasminogen concentrations using a microtiter plate method. (■) Incubation with increasing concentrations of plasminogen, 0–30 µg/ml. (▲) Competition assay with 50 mM εACA included during plasminogen incubation. (●) Negative control consisted of wells coated only with BSA. Each point is the mean of three replicates ± SD. The asterisk (*) designates significant ($p < 0.05$) differences.

3.3. rDiGAPDH and rDiGAL enhance the activation of plasminogen by tPA

In order to assess the ability of rDiGAPDH and rDiGAL to activate plasminogen and generate plasmin on their own, the amidolytic activity of plasmin generated in the presence or absence of tPA was measured. Negative controls replacing each recombinant protein for BSA or tPA were also used. Fig. 4 shows the capacity of rDiGAPDH and rDiGAL to stimulate plasmin generation by tPA obtaining optical densities significantly higher than the negative controls ($p < 0.05$). Both proteins obtained similar results and plasminogen-activation did not occur in the absence of tPA. Furthermore this effect is inhibited by 50 mM εACA, indicating the involvement of lysine residues in the process.

3.4. rDiGAPDH and rDiGAL enhance the expression of uPA and not of tPA in canine endothelial cells

To study the possible effect of rDiGAPDH and rDiGAL on the expression of the main activators of fibrinolysis (tPA and uPA), the parasitic proteins were employed to stimulate CnAOEC in culture. Proteins from rDiGAPDH/rDiGAL-treated or untreated vascular endothelial cell extracts were separated by SDS–PAGE and analyzed by Western blotting using anti-tPA or anti-uPA antibodies. As shown in Fig. 5, both proteins induced a significant increase in uPA protein expression after 24 h of

stimulation ($p < 0.01$), being this increase higher in the case of rDiGAPDH stimulation. None of the proteins led to significant differences in the protein expression of tPA.

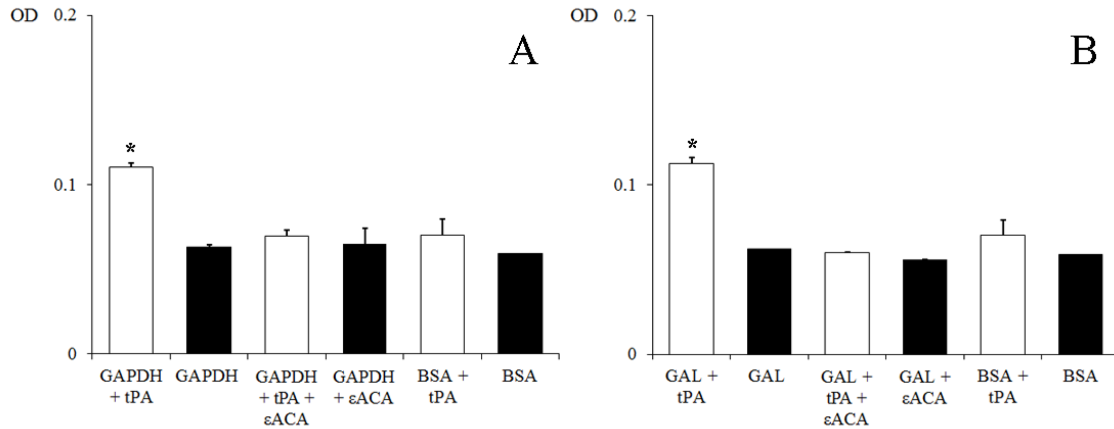

Figure 4. Plasminogen activation and plasmin generation by rDiGAPDH (A) and rDiGAL (B). (□) 15 ng of tPA was added to mixtures containing 2 µg of human plasminogen, 3 µg of D-Val-Leu-Lys 4-nitroanilide dihydrochloride (Sigma) and 1 µg of each recombinant protein (or BSA as negative control) in the presence or absence of 50 mM of εACA in a test volume of 100 µl. (■) No tPA was added to reaction mixtures. Optical densities measured at 405 nm after 120 min of incubation. Each point is the mean of three replicates ± SD. The asterisk (*) designates significant ($p < 0.05$) differences.

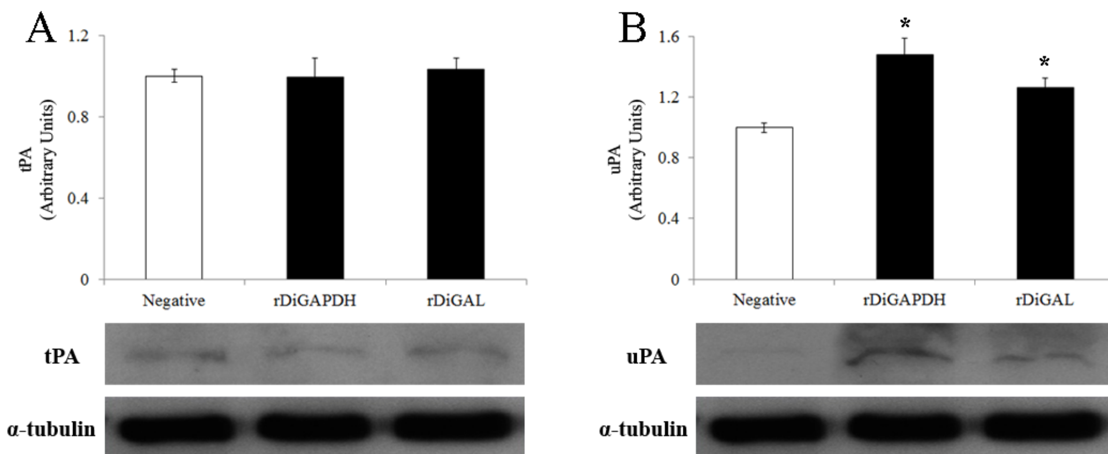

Figure 5. Effect of rDiGAPDH and rDiGAL on the expression of tPA and uPA in canine vascular endothelial cells. Protein extracts from lysed rDiGAPDH or rDiGAL treated or untreated confluent cell cultures were analyzed by Western blot for tPA and uPA. α-tubulin served as a protein control. Results were expressed as the mean ± SD of at least 3 independent experiments. The asterisk (*) designates significant ($p<0.05$) differences from control cells. (■) Stimulated endothelial cells with 1µg/ml of rDiGAPDH or rDiGAL. (□) Non-treated control cells.

3.5. Immunolocalization of DiGAPDH and DiGAL

In a first step, antisera against rDiGAPDH and rDiGAL were generated. The reactivity and specificity of these antisera were tested in ELISA and Western blot prior to their use in the immunolocalization studies. The antibody titers of these antisera were higher than 1/500, with an OD of 1.12 and 1.07, respectively, while a negative serum showed an OD of 0.12 and 0.16 at the same dilution. The anti-rDiGAPDH and anti-rDiGAL antisera reacted strongly and specifically with the recombinant proteins and with the native GAPDH and GAL proteins in the DiES extract in the Western blot analyses. The negative sera showed no reactivity with any of the proteins tested (data not shown).

The anatomical localization of DiGAPDH and DiGAL was carried out in histological sections of *D. immitis* adult worms by immunofluorescence using the rabbit polyclonal antisera previously generated. As shown in Fig. 6, all sections showed green fluorescence throughout the soma of the parasite, as a result of the binding of phalloidin-Alexa Fluor 488, actin ligand which serves as a positive control of the technique. Sections incubated with the anti-rDiGAPDH or anti-rDiGAL antisera showed, in addition, specific reactivity (in red) against the parasitic GAPDH and GAL, respectively. Both proteins are located scattered throughout all the soma, being especially abundant in the cuticle (reflected by an orange color in the overlay of Phalloidin-Alexa Fluor 488 + Alexa Fluor 594 images). Sections incubated with a rabbit negative serum showed no specific red fluorescence from recombinant proteins.

4. Discussion

Two recent in vitro studies demonstrated the participation of the ES and surface antigens of *D. immitis* in the activation of the fibrinolytic system. In addition, some of the antigens responsible for this enhancement were identified (González-Miguel et al., 2012 and González-Miguel et al., 2013). Taking into account these previous data and the importance of the anticlotting mechanisms for *D. immitis*, a parasite that survives for years in the pulmonary arteries of its host, the objective of this work was to investigate the participation of the *D. immitis* GAPDH and GAL in the fibrinolytic system activation using recombinant forms of both proteins. This involves knowing whether or not these proteins are able to bind plasminogen, stimulate plasmin generation, interact

with the expression of the main fibrinolytic activators and express in an antigenic compartment of the parasite in contact with the blood of the host.

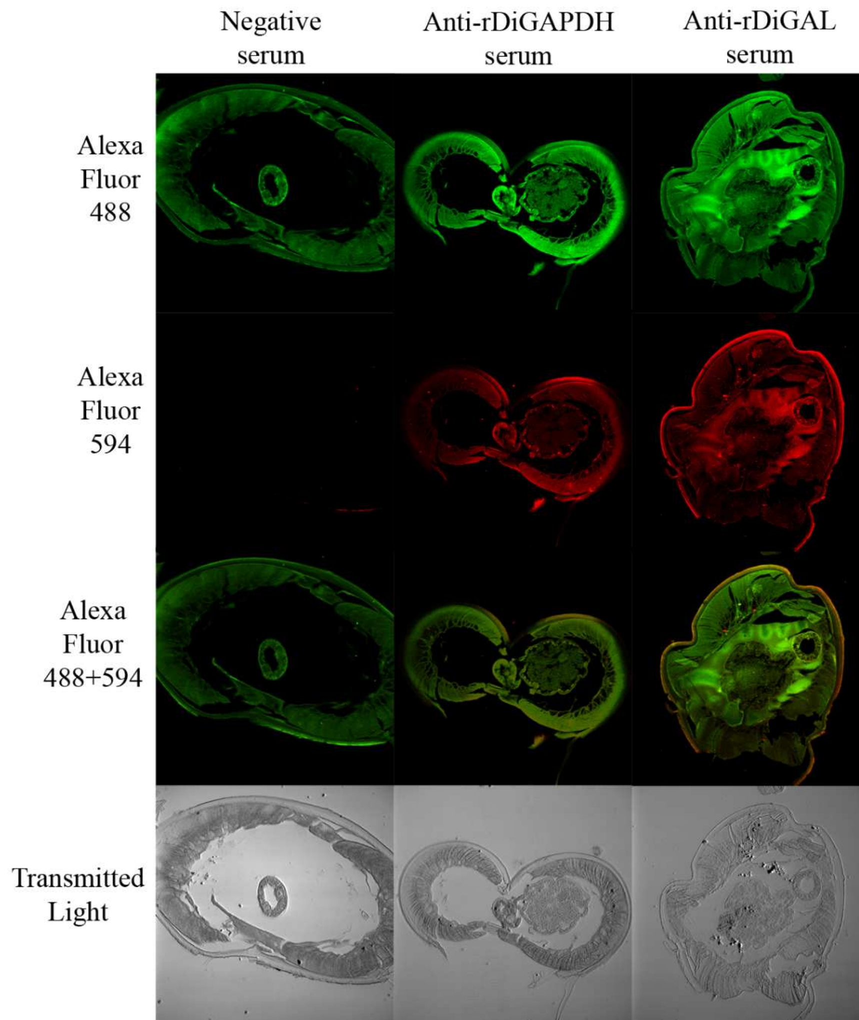

Figure 6. Immunolocalization of DiGAPDH and DiGAL in sections from *D. immitis* adult worms. Images of parasite sections incubated with phalloidin-Alexa Fluor 488 (in green, specific binding to Actin) plus the negative or the anti-rDiGAPDH or anti-rDiGAL rabbit sera and an anti-rabbit IgG-Alexa Fluor 594 (in red). Corresponding transmitted light images are also adressed. Magnification 4X.

Two peptide sequences of 339 and 280 amino acids were respectively obtained by cloning and sequencing of the *D. immitis* GAPDH and GAL cDNAs. The subsequent bioinformatics analysis have highlighted the high degree of evolutionary conservation of these proteins, both in the structural characteristics of their 3D models, and in the multiple sequence alignments carried out with homologous proteins from other helminth parasite species. On the other hand, none of the two proteins showed structural

motifs for their transport or expression on the cell surface (signal peptide, transmembrane motifs or GPI anchors). However, both proteins have been identified by immunoproteomic techniques in the ES and surface extracts of *D. immitis* (González-Miguel et al., 2012 and González-Miguel et al., 2013). This may be related to unconventional mechanisms of protein transport, as for example with the association of these proteins with exosome-like secretion vesicles. This has been postulated as an extracellular transport mechanism both for glycolytic enzymes from several groups of parasites (Gómez-Arreaza et al., 2014) and for galectins (Nickel, 2003).

Both the rDiGAPDH and rDiGAL showed ability to bind plasminogen (higher in the case of rDiGAPDH) and to stimulate plasmin generation in an in vitro system. Plasminogen activation occurred only in the presence of the tPA, as observed previously using the ES and surface antigens from *D. immitis* (González-Miguel et al., 2012 and González-Miguel et al., 2013) or GAPDH from different bacteria, fungi or parasites species (reviewed by Figuera et al., 2013). In addition, to our knowledge, this is the first time that GAL is proposed as a physiological receptor of plasminogen. Competition assays with the ϵ-ACA acid revealed the involvement of lysine residues from both proteins in the binding of plasminogen. The interaction between plasminogen and their receptors has been related to the presence of carboxyl-terminal lysine residues (Plow et al., 1995). DiGAPDH alignment with homologous sequences from other organisms that have been related to plasminogen-binding activities (Figuera et al., 2013) shows that the carboxyl-terminal domain of these proteins are not highly conserved, and that in some cases domains lack lysines (see Fig. 1). However, there are highly conserved internal lysine residues in the amino acid sequences of DiGAPDH and DiGAL. Therefore, it is possible that conserved internal lysine residues are involved in the binding of plasminogen to these proteins as it has been postulated for the enolase of *Streptococcus pneumoniae* (Ehinger et al., 2004). In addition, after viewing the spatial location of the conserved internal lysine residues of the DiGAPDH and DiGAL in their 3D models, these residues seem to be located externally in these molecules, which would facilitate the accessibility of plasminogen.

rDiGAPDH and rDiGAL did not cause a stimulation of basal tPA production in canine endothelial cell cultures. However, it has been shown that whole *D. immitis* ES antigens are able to produce an overexpression of this fibrinolytic activator in human

endothelial cells in culture (González-Miguel et al., 2012). This suggests that other molecules of *D. immitis* are responsible for this process. On the other hand, rDiGAPDH and rDiGAL produced a significant stimulation of the basal uPA production in canine endothelial cells in culture. To our knowledge, this is the first time that the relationship between a parasitic antigen and the overproduction of an activator of fibrinolysis in an in vitro system has been demonstrated. This could have particular relevance since uPA, in addition to its role as activator of fibrinolysis, plays a key role in tissue remodeling inducing proliferation and cell migration (Nicholl et al., 2005a), and high levels in its expression are related to cardiovascular disease (Fuhrman, 2012).

In order to ascertain whether the ability of rDiGAPDH and rDiGAL to bind plasminogen may have relevance in vivo, it is necessary that these proteins are expressed and/or located in tissues of the parasite in close contact with the host blood (Hawley et al., 2000). Immunofluorescence study indicates that DiGAPDH and DiGAL are especially abundant on the surface of *D. immitis*, in addition to have an intracellular localization which is expected for a glycolytic enzyme and a lectin. In the case of the GAPDH, this is consistent with the results of a recent study in which it was identified as one of the five most abundant proteins in the cuticle of *D. immitis* (Morchón et al., 2014).

The ability of these molecules to enhance the activation of fibrinolysis could be an important survival mechanism for *D. immitis* in its intravascular environment. However, overstimulation of the plasminogen/plasmin system has been related to cellular invasion and intra-organic migration in different pathogens (Jong et al., 2003 and Bernal et al., 2004). In addition, the overproduction of plasmin has been linked with the proliferation and migration of vascular cells and with the degradation of extracellular matrices in humans (Nicholl et al., 2005b; Yang et al., 2005; Roth et al., 2006 and Hayashi et al., 2009). This suggests a possible involvement of the over-activation of the fibrinolytic system by *D. immitis* antigens in the long-term development of the pathogenic mechanisms that occur during cardiopulmonary dirofilariosis.

In conclusion, we have shown that *D. immitis* GAPDH and GAL are able to bind plasminogen and enhance plasmin generation by tPA with the involvement of lysine

residues. In addition, these proteins stimulate the expression of the fibrinolytic activator uPA on canine endothelial cells in culture and they are expressed on the surface of the worms. Therefore, DiGAPDH and DiGAL could be used by *D. immitis* to stimulate the activation of the fibrinolytic system through the plasminogen binding to its surface or excreted molecules, as a mechanism to avoid blood clot formation in its close environment.

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References

Arnold, K., Bordoli, L., Kopp, J., Schwede, T., 2006. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22, 195–201.

Astorgues-Xerri, L., Riveiro, M.E., Tijeras-Raballand, A., Serova, M., Neuzillet, C., Albert, S., Raymond, E., Faivre, S., 2014. Unraveling galectin-1 as a novel therapeutic target for cancer. Cancer Treat. Rev. 40, 307–319.

Bendtsen, J.D., Nielsen, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340, 783–795.

Bernal, D., de la Rubia, J.E., Carrasco-Abad, A.M., Toledo, R., Mas-Coma, S., Marcilla, A., 2004. Identification of enolase as a plasminogen-binding protein in excretory–secretory products of *Fasciola hepatica*. FEBS Lett. 563, 203–206.

Bhattacharya, S., Ploplis, V.A., Castellino, F.J., 2012. Bacterial plasminogen receptors utilize host plasminogen system for effective invasion and dissemination. J. Biomed. Biotechnol. 2012, 482096.

Cesarman-Maus, G., Hajjar, K.A., 2005. Molecular mechanisms of fibrinolysis. Br. J. Haematol. 129, 307–321.

Crowe, J.D., Sievwright, I.K., Auld, G.C., Moore, N.R., Gow, N.A., Booth, N.A., 2003. *Candida albicans* binds human plasminogen: identification of eight plasminogen-binding proteins. Mol. Microbiol. 47, 1637–1651.

de la Torre-Escudero, E., Manzano-Román, R., Pérez-Sánchez, R., Siles-Lucas, M., Oleaga, A., 2010. Cloning and characterization of a plasminogen-binding surface-associated enolase from *Schistosoma bovis*. Vet. Parasitol. 173, 76–84.

Ehinger, S., Schubert, W.D., Bergmann, S., Hammerschmidt, S., Heinz, D.W., 2004. Plasmin(ogen)-binding alpha-enolase from *Streptococcus pneumoniae*: crystal structure and evaluation of plasmin(ogen)-binding sites. J. Mol. Biol. 343(4), 997–1005.

Eisenhaber, B., Bork, P., Yuan, Y., Loeffler, G., Eisenhaber, F., 2000. Automated annotation of GPI anchor sites: case study *C. elegans*. TIBS 25, 340–341.

Erttmann, K.D., Kleensang, A., Schneider, E., Hammerschmidt, S., Büttner, D.W., Gallin, M., 2005. Cloning, characterization and DNA immunization of an *Onchocerca volvulus* glyceraldehyde-3-phosphate dehydrogenase (Ov-GAPDH). Biochim. Biophys. Acta 1741, 85–94.

Figuera, L., Gómez-Arreaza, A., Avilán, L., 2013. Parasitism in optima forma: Exploiting the host fibrinolytic system for invasion. Acta Trop. 128, 116–123.

Fuhrman, B., 2012. The urokinase system in the pathogenesis of atherosclerosis. Atherosclerosis 222, 8–14.

Genchi, C., Kramer, L.H., Prieto, G., 2001. Epidemiology of canine and feline dirofilariasis: a global view. In: Simón, F., Genchi, C. (Eds.), Heartworm infection in humans and animals. Ediciones Universidad de Salamanca, Salamanca, Spain, pp. 121–134.

Gómez-Arreaza, A., Acosta, H., Quiñones, W., Concepción, J.L., Michels, P. a M., Avilán, L., 2014. Extracellular functions of glycolytic enzymes of parasites: Unpredicted use of ancient proteins. Mol. Biochem. Parasitol. 193, 75–81.

González-Miguel, J., Morchón, R., Mellado, I., Carretón, E., Montoya-Alonso, J.A., Simón, F., 2012. Excretory/secretory antigens from *Dirofilaria immitis* adult worms interact with the host fibrinolytic system involving the vascular endothelium. Mol. Biochem. Parasitol. 181, 134–140.

González-Miguel, J., Morchón, R., Carretón, E., Montoya-Alonso, J.A., Simón, F., 2013. Surface associated antigens of *Dirofilaria immitis* adult worms activate the host fibrinolytic system. Vet. Parasitol. 196, 235–240.

Hayashi, M., Matsuzaki, Y., Shimonaka, M., 2009. Impact of plasminogen on an in vitro wound healing model based on a perfusion cell culture system. Mol. Cell. Biochem. 322, 1–13.

Hawley, S.B., Green, M.A., Miles, L.A., 2000. Discriminating between cell surface and intracellular plasminogen-binding proteins: heterogeneity in profibrinolytic plasminogen-binding proteins on monocytoid cells. Thromb. Haemost. 84, 882–890.

Jolodar, A., Fischer, P., Bergmann, S., Büttner, D.W., Hammerschmidt, S., Brattig, N.W., 2003. Molecular cloning of an alpha-enolase from the human filarial parasite *Onchocerca volvulus* that binds human plasminogen. Biochim. Biophys. Acta. 1627, 111–120.

Jong, A.Y., Chen, S.H., Stins, M.F., Kim, K.S., Tuan, T.L., Huang, S.H., 2003. Binding of *Candida albicans* enolase to plasmin(ogen) results in enhanced invasion of human brain microvascular endothelial cells. J. Med. Microbiol. 52, 615–622.

Joseph, G.T., Huima, T., Klion, A., Lustigman, S., 2000. A novel developmentally regulated galectin of *Onchocerca volvulus*. Mol Biochem Parasitol. 106, 187–195.

Lama, A., Kucknoor, A., Mundodi, V., Alderete, J.F., 2009. Glyceraldehyde-3-phosphate dehydrogenase is a surface-associated, fibronectin-binding protein of *Trichomonas vaginalis*. Infect. Immun. 77, 2703–2711.

Mebius, M.M., van Genderen, P.J., Urbanus, R.T., Tielens, A.G., de Groot, P.G., van Hellemond, J.J., 2013. Interference with the host haemostatic system by schistosomes. PLoS Pathog. 9, e1003781.

Morchón, R., González-Miguel, J., Mellado, I., Velasco, S., Rodríguez-Barbero, A., Simón, F., 2010. Adult *Dirofilaria immitis* excretory/secretory antigens upregulate the production of prostaglandin E2 and downregulate monocyte transmigration in an “in vitro” model of vascular endothelial cell cultures. Vet. Parasitol. 170, 331–335.

Morchón, R., González-Miguel, J., Carretón, E., Kramer, L.H., Valero, L., Montoya-Alonso, J.A., Simón, F., Siles-Lucas, M., 2014. Proteomic analysis of the somatic and surface compartments from *Dirofilaria immitis* adult worms. Vet. Parasitol. 203, 144–152.

Nicholl, S.M., Roztocil, E., Davies, M.G., 2005a. Urokinase-induced smooth muscle cell responses require distinct signaling pathways: a role for the epidermal growth factor receptor. J. Vasc. Surg. 41, 672–681.

Nicholl, S.M., Roztocil, E., Galaria, I.I., Davies, M.G., 2005b. Plasmin induces smooth muscle cell proliferation. J. Surg. Res. 127, 39–45.

Nickel, W., 2003. The mystery of nonclassical protein secretion. A current view on cargoproteins and potential export routes. Eur. J. Biochem. 270, 2109–2119.

Plow, E.F., Herren, T., Redlitz, A., Miles, L.A., Hoover-Plow, J.L., 1995. The cell biology of the plasminogen system. FASEB J. 9(10), 939–945.

Ramajo-Hernández, A., Pérez-Sánchez, R., Ramajo-Martín, V., Oleaga, A., 2007. *Schistosoma bovis*: plasminogen binding in adults and the identification of plasminogen-binding proteins from the worm tegument. Exp. Parasitol. 115, 83–91.

Roda, O., Ortiz-Zapater, E., Martínez-Bosch, N., Gutiérrez-Gallego, R., Vila-Perelló, M., Ampurdanés, C., Gabius, H.J., André, S., Andreu, D., Real, F.X., Navarro, P., 2009. Galectin-1 is a novel functional receptor for tissue plasminogen activator in pancreatic cancer. Gastroenterology 136, 1379–1375.

Roth, D., Piekarek, M., Paulsson, M., Christ, H., Bloch, W., Krieg, T., Davidson, J.M., Eming, S.A., 2006. Plasmin modulates vascular endothelial growth factor-A-mediated angiogenesis during wound repair. Am. J. Pathol. 168, 670–684.

Tristan, C., Shahani, N., Sedlak, T.W., Sawa, A., 2011. The diverse functions of GAPDH: views from different subcellular compartments. Cell. Signal. 23, 317–323.

Venco, L., 2007. Heartworm (*Dirofilaria immitis*) disease in dogs. In: Genchi, C., Ronaldi, L., Cringoli, G. (Eds.), *Dirofilaria immitis* and *D. repens* in Dog and Cat and Human Infections. Rolando Editore, Italia, pp. 117–125.

Yang, Z., Eton, D., Zheng, F., Livingstone, A.S., Yu, H., 2005. Effect of tissue plasminogen activator on vascular smooth muscle cells. J. Vasc. Surg. 42, 532–538.

Supplementary material

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D.immitis        MSKPKIGINGFGRIGRLVLRAAVEKNTVDVVAVNDPFINIDYMVYMFKYDSTHGRFKGNV 60
L.loa            MSKPKIGINGFGRIGRLVLRAAVEKDTVDVVAVNDPFINIDYMVYMFKYDSTHGRFKGNV 60
W.bancrofti      ----------FGRIGRLVLRAAVEKDTVDVVAVNDPFINIDYMVYMFKYDSTHGRFKGSV 50
B.malayi         MSKPKVGINGFGRIGRLVLRAAVEKDTVDVVAVNDPFINIDYMVYMFKYDSTHGRFKGSV 60
O.volvulus       MSKPKIGINGFGRIGRLVLRAAVEKDTVEVVAVNDPFINIDYMVYMFKYDSTHGRFKGHV 60
A.suum           MSKPKVGINGFGRIGRLVLRAAVEKDTVEVVAVNDPFINIDYMVYMFKYDSTHGRFKGDV 60
S.mansoni        MSRAKVGINGFGRIGRLVLRAAFLKNTVDVVSVNDPFIDLEYMVYMIKRDSTHGTFPGEV 60
F.hepatica       MSKPKVGINGFGRIGRLVLRAAVERGTVEVVAVNDPFIDLNYMVYMFKYDSTHGTFKGDV 60
                           ************. :.**:**:******:::*****:* ***** * * *

D.immitis        SAEGGKLVVTN-GQTTHHISVHNSKDPAEIPWGVDGAEYVVESTGVFTTTEKASAHLKGG 119
L.loa            SAEGGKLVVTN-GKRTHHISVHNSKDPAEIPWGVDGAEYVVESTGVFTTTEKASAHLKGG 119
W.bancrofti      SAEGGKLVVTN-GKTTHHISVHNSKDPAEIPWGVDGAEYVVESTGVFTTTDKASAHLKGG 109
B.malayi         SAEGGKLIVTN-GKTTHHISVHNSKDPAEIPWGVDGAEYVVESTGVFTTTDKASAHLKGG 119
O.volvulus       SAEGGKLIVTN-GKTTHQIAVHNSKDPAEIPWGVEGAEYVVESTGVFTHTEKASAHLKGG 119
A.suum           HAEGGKLVVSTPGKATHHITVHNSKDPSEIAWSADGAEYVVESTGVFTTVEKASAHLKGG 120
S.mansoni        STENGKLKVNG-----KLISVHCERDPANIPWDKDGAEYVVESTGVFTTIDKAQAHIKNN 115
F.hepatica       SVHDKKLCING-----MRISVYNERSPETIPWDKDSAEYVVESTGVFTTMEKAQAHLKNN 115
                  ... ** :.        *:*: .:.*  *.*. :.************  :**.**:*..

D.immitis        -AKKVIISAPSADAPMFVMGVNNETYDKANNHIISNASCTTNCLAPLAKVIHDKFGIIEG 178
L.loa            -AKKVVISAPSADAPMFVMGVNNDKYDKANNHIISNASCTTNCLAPLAKVIHDKFGIIEG 178
W.bancrofti      -AKKVIISAPSADAPMFVMGVNNDKYDKANNHIISNASCTTNCLAPLAKVIHDKFGIIEG 168
B.malayi         -AKKVIISAPSADAPMFVMGVNNDMYDKANNHIISNASCTTNCLAPLAKVIHDKFGIIEG 178
O.volvulus       -AKKVIISAPSADAPMFVMGVNNDKYDKANNHIISNASCTTNCLAPLAKVIHDKFGIIEG 178
A.suum           -AKKVIISAPSADAPMFVMGVNEDKYDGANNHVISNASCTTNCLAPLAKVINDKFGIVEG 179
S.mansoni        RAKKVIISAPSADAPMFVVGVNENSYEKSMS-VVSNASCTTNCLAPLAKVIHDKFEIVEG 174
F.hepatica       RAKKVIISAPSPDAPMFVVGVNHDKYDKCMN-VVSNASCTTNCLSPLAKVLHENFGIEEG 174
                  ****:*****.******:***.: *:  .  . ::*********:*****::::* * **

D.immitis        LMTTVHATTATQKTVDGPSGKLWRDGRGAGQNIIPASTGAAKAVGKVIPDLNGKLTGMAF 238
L.loa            LMTTVHATTATQKTVDGPSGKLWRDGRGAAQNIIPASTGAAKAVGKVIPDLNGKLTGMAF 238
W.bancrofti      LMTTVHATTATQKTVDGPSGKLWRDGRGAGQNIIPASTGAAKAVGKVIPDLNGKLTGMAF 228
B.malayi         LMTTVHATTATQKTVDGPSGKLWRDGRGAGQNIIPASTGAAKAVGKVIPGLNGKLTGMAN 238
O.volvulus       LMTTVHATTATQKTVDGPSGKLWRDGRGAGQNIIPASTGAAKAVGKVIPDLNGKLTGMAS 238
A.suum           LMTTVHATTATQKTVDGPSGKQWRDGRGAGQNIIPASTGAAKAVGKVIPQLNGKLTGMAF 239
S.mansoni        LMTTVHSFTATQKVVDGPSSKLWRDGRGAMQNIIPASTGAAKAVGKVIPALNGKLTGMAF 234
F.hepatica       LMTTIHSYTATQRVVDGPSVKLWRDGRGAAQNIIPASTGAAKAVGKVIPDLNGKLTGMAF 234
                 ****:*: ****:.***** * ******* ****************** ********

D.immitis        RVPTPDVSVVDLTCRLQKGATMDEIKAAVKEAAAGPMKGILEYTEDQVVSSDFIGDAHSS 298
L.loa            RVPTPDVSVVDLTCRLQKGATMDEIKAAVKEAANGPMKGILDYTEDQVVSTDFVGDTHSS 298
W.bancrofti      RVPTPDVS---------------------------------------------------- 236
B.malayi         RVPTPDVSVVDLTCRLQKGATMDEIKAAVKEAANGPMKGILEYTEDQVVSTDFTGDTHSS 298
O.volvulus       RVPTPDVSVVDLTCRLQKGASMDEIKAAVKEAAAGPMKGILEYTEDQVVSSDFVGDPHSS 298
A.suum           RVPTPDVSVVDLTARLEKAATMDEIKAAIKEAAAGPMKGILGYTEDSVVSTDFVGDSHSS 299
S.mansoni        RVPTPDVSVVDLTCRLGKGASYEEIKAAVKAAASGPLKGILEYTEDEVVSSDFVGSTSSS 294
F.hepatica       RVPTPDVSVVDLTCKLSRPTTYDQIKAALKAAAEGPMKGILEYTEDQVVSMDFRGCSASS 294
                 ********
                                                                    Identity (%)
D.immitis        IFDALACISLNPNFVKLIAWYDNEYGYSNRVVDLISYIASR--- 339    -
L.loa            IFDALACISLNPNFVKLIAWYDNEYGYSNRVVDLISYIASR--- 339    97.46
W.bancrofti      --------------------------------------------        96.46
B.malayi         IFDSLACISLNPNFVKLIAWYDNEYGYSNRVVDLISYIASR--- 339    95.58
O.volvulus       IFDALACISLNPNFVKLIAWYDNEYGYSNRVVDLISYNASK--- 339    94.99
A.suum           IFDANACISLNPHFVKLISWYDNEFGYSCRVVDLIAYISKH--- 340    87.61
S.mansoni        IFDAKAGISLNNNFVKLVSWYDNEFGYSCRVVDLITHMHKVDHA 338    76.33
F.hepatica       IFDAGAGLALNDTFVKIVAWYDNEYGYSCRVIDLINHMHTIDHL 338    73.96
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Figure S1. Alignment of the *D. immitis* GAPDH sequence (AFL46382) with the GAPDH from *L. loa* (EJD75047), *W. bancrofti* (EJW73626), *B. malayi* (P48812), *O. volvulus* (O01360), *A. suum* (BAB68543), *S. mansoni* (P20287) and *F. hepatica* (AIE44418). The percentage of sequence identity between *D. immitis* sequence and the others is indicated. The amino acids conserved in all the sequences are labelled with asterisks, and conservative and semiconservative substitutions are labelled with two and one point, respectively.

```
D.immitis         MHHNEYETNYPVPYRSKLTESFEPGQTLLVKGKTAEDSVRFTINLHNTSADFSGNDVPLH 60
B.malayi          -MPNEYETIYPVPYRSKLTESFEPGQTLLVKGKTAEDSVRFTINLHNTSADFSGNDVPLH 59
O.volvulus        -MTNEYETNYPVPYRSKLTESFEPGQTLLVKGKTAEDSVRFTINLHNTSADFSGNDVPLH 59
L.loa             -MANEYETNYPVPYRSKLTETFDPGQTLLVKGKTAEDSVRFTVNLHNTSADFSGNDVPLH 59
W.bancrofti       ------------------------------------------------------------
A.suum            ---MATETSYPVPYRSKLTEPFEPGQTLIIKGKTAEDSVRFTINLHNTSADFSGNDVPLH 57
H.contortus       ---MAFETNYPIPYRSKLTEPFEPGQTLTVKGKTGEDSVRFTINLHNSSADFSGNDVPLH 57


D.immitis         ISVRFDEGKIVFNTFSKGEWGKEERKSNPYKKGDDIDIRIRAHDSKYTIYVDQKEVKEYE 120
B.malayi          ISVRFDEGKIVFNTFSKGEWGKEERKSNPYKKGDDIDIRIRAHDSKYTIYVEQKEVKEYE 119
O.volvulus        ISVRFDEGKIVFNTFSKGEWGKEERKSNPYKKGDDIDIRIRAHDSKYTIYVDQKEVKEYE 119
L.loa             ISVRFDEGKIVFNTFSKGEWGKEERKSNPYKKGDDIDIRIRAHDSKYTIYVDQKEVKEYE 119
W.bancrofti       ---------IVFNTFSKGEWGKEERKSNPYKKGDDIDIRIRAHDSKYTIYVDQKEVKEYE 51
A.suum            ISVRFDEGKIVFNTFSKGEWGKEERKSNPYKKGDDIDIRIRAHDSKFSISVDQKEVKEYE 117
H.contortus       VSVRFDEGKIVCNSFAKGEWGKEERKSNPYKKGDDIDIRIRAHDSKFQIFVDQKELKEYE 117
                           ** *:*:******************************: * *:***:****

D.immitis         HRVPLSS-TRFSIDGDILVTYIHWGGKYYPVPYESGLSGEGLVPGKSLLIFATPEKKGKR 179
B.malayi          HRVPLSSVTHFSIDGDVLVTYIHWGGKYYPVPYESGLSGESLMPGKSLLIFATPEKKGKR 179
O.volvulus        HRVPLSAVTHFSIDGDVLVTYIHWGGKYYPVPYESGLSGEGLVPGKSLLIFATPEKKGKR 179
L.loa             HRVPLSSVTHFSIDGDVLVTYIHWGGKYYPVPYESGLSGEGLVPGKSLLIFATPEKKGKR 179
W.bancrofti       HRVPLSSVTHFSIDGDVLITYIHWGGKYYPVPYESGLSGDGLVPGKSLLIFATPEKKGKR 111
A.suum            HRVPLSSVTHFSVDGDILITYIHWGGKYYPVPYESGLAGDGLAPGKSLLIFATPEKKGKR 177
H.contortus       HRLPLSSVTHFSIDGDVLITYIHWGGKYYPVPYESGLAGEGLAPGKSIFLYGMPEKKGKR 177
                  **:***: *:**:***:*:******************:*:.* ****:::.  *******

D.immitis         FHINLLKKNGDIALHFSPRFDEKAIVRNSLIAGEWGNEEREGKMILEKGIGFDLEIKNEE 239
B.malayi          FHINLLKKNGDIALHFNPRFDEKAIVRNSLIAGEWGNEEREGKMILEKGIGFDLEIKNEE 239
O.volvulus        FHINLLKKNGDIALHFNPRFDEKAIVRNSLIAGEWGNEEREGKMILEKGIGFDLEIKNEE 239
L.loa             FHINLLKKNGDIALHFNPRFDEKAIVRNSLIAGEWGNEEREGKMILEKGIGFDLEIKNEE 239
W.bancrofti       FHINLLKKNGDIALHFNPRFDEKV------------------------------------ 135
A.suum            FHINLLKKNGDIALHFNPRFDEKAIVRNSLISGEWGNEEREGKNPLEKGIGCDLEFRNEE 237
H.contortus       FHINLLKKNGDIALHFNPRFDEKAVVRNSLISNEWGNEEREGKMPFEKAVGFDLEIKNEE 237
                  ****************.******.
                                                                 Identity (%)
D.immitis         YAFQIFINSERYATYAHRLDPHEINGLQIGGDLEVSGIQMH 280      -
B.malayi          YAFQIFINSERYATYAHRLDPHEINGLQIGGDLKVSGIQMH 280      96.79
O.volvulus        YAFQIFINGERYATYAHRLDPREINGLQIGGDLEVSGIQMH 280      96.07
L.loa             YAFQIFINSERYATYAHRVDPHEINGLQIGGDLEVSGIQMR 280      96.07
W.bancrofti       -----------------------------------------          94.81
A.suum            YAFQIYVDGERFATYAHRLDPHDINGLQIGGDVEVTGIQMV 278      88.13
H.contortus       YAFQIMVNGERFASYAHRLEPHELNGLQIGGDVEITGIQLH 278      82.73
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Figure S2. Alignment of the *D. immitis* galectin sequence (AAF37720) with the galectins from *B. malayi* (Q9NGY0), *O. volvulus* (Q25597), *L. loa* (EFO26688), *W. bancrofti* (EJW74751), *A. suum* (F1KZZ8) and *H. contortus* (AAD11972). The percentage of sequence identity between *D. immitis* sequence and the others is indicated. The amino acids conserved in all the sequences are labelled with asterisks, and conservative and semiconservative substitutions are labelled with two and one point, respectively. Conserved lysine residues are shaded in yellow.

CVARTO CAPÍTVLO

"Can the activation of plasminogen/plasmin system of the host by metabolic products of *Dirofilaria immitis* participate in heartworm disease endarteritis?"

¿Puede la activación del sistema plasminógeno/plasmina del hospedador por los productos metabólicos de *Dirofilaria immitis* participar en la endarteritis de la dirofilariosis cardiopulmonar?

Javier González-Miguel, Rodrigo Morchón, Elena Carretón, José Alberto Montoya-Alonso, Fernando Simón

Parasites & Vectors (2015) 8:194.
Factor de impacto (2014): 3,25.

Resumen

Antecedentes: La endarteritis proliferativa es uno de los mecanismos patológicos clave en la dirofilariosis cardiopulmonar, una parasitosis cosmopolita causada por *Dirofilaria immitis* y que afecta a perros y gatos de todo el mundo. Se ha demostrado que los antígenos excretores/secretores de los vermes adultos de *D. immitis* (DiES) fijan plasminógeno (PLG) y activan la fibrinolisis, lo que puede suponer un mecanismo de supervivencia para el parásito en su entorno intravascular. Sin embargo, la sobreproducción de plasmina (producto final de la ruta) ha sido relacionada con procesos patológicos similares a los descritos en la endarteritis proliferativa. El objetivo de este trabajo es relacionar la aparición de esta condición patológica con la activación del sistema PLG/plasmina del hospedador por DiES.

Métodos: La proliferación celular a través de la técnica del cristal violeta, la migración celular mediante un ensayo de cicatrización de heridas y la destrucción de la matriz extracelular mediante la medición de la degradación del colágeno y los niveles de metaloproteasas de matriz, fueron estudiados en un modelo *in vitro* utilizando células endoteliales y musculares lisas vasculares de perro. Estas células fueron tratadas con una mezcla de DiES + PLG. Células sin tratar, células estimuladas solamente con DiES o con PLG, o con una mezcla de DiES + PLG + εACA (un inhibidor de la conversión PLG-plasmina) fueron empleadas como controles. Además, el efecto de DiES en la expresión de los activadores fibrinolíticos tPA y uPA, el inhibidor PAI-1 y el receptor de PLG Anexina A2 fue analizado en ambos tipos de cultivos por *western blot*.

Resultados: La plasmina generada por la unión de DiES + PLG produjo un aumento significativo en la proliferación y migración celular de las células endoteliales y del músculo liso, así como un aumento en la destrucción de la matriz extracelular basada en una degradación mayor del colágeno de tipo I y en un nivel incrementado de la metaloproteasa de matriz-2. DiES también induce un aumento en la expresión de tPA y uPA en los cultivos de células endoteliales, así como una disminución en la expresión de PAI-1 en ambos tipos de células.

Conclusiones: Nuestro estudio muestra una interrelación entre la plasmina causada por la activación de la fibrinolisis por los productos metabólicos de *D. immitis* y la aparición de procesos patológicos similares a los descritos en la aparición de la endarteritis proliferativa en la dirofilariosis cardiopulmonar.

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Parasites & Vectors

RESEARCH Open Access

Can the activation of plasminogen/plasmin system of the host by metabolic products of *Dirofilaria immitis* participate in heartworm disease endarteritis?

Javier González-Miguel[1*], Rodrigo Morchón[1], Elena Carretón[2], José Alberto Montoya-Alonso[2] and Fernando Simón[1]

Abstract

Background

Proliferative endarteritis is one of the key pathological mechanisms of cardiopulmonary dirofilariosis, a cosmopolitan parasitosis caused by *Dirofilaria immitis* affecting dogs and cats around the world. It has been shown that the excretory/secretory antigens from *D. immitis* adult worms (DiES) bind plasminogen (PLG) and activate fibrinolysis, which can lead to a survival mechanism for the parasite in its intravascular environment. However, overproduction of plasmin (final product of the route) has been related to pathological processes similar to those described in proliferative endarteritis. The aim of this study is to relate the appearance of this pathological condition with the activation of the PLG/plasmin system of the host by DiES.

Methods

Cell proliferation through the crystal violet technique, cell migration by wound healing assay and degradation of the extracellular matrix by measuring collagen degradation and levels of matrix metalloproteinases were studied in an "*in vitro*" model using canine vascular endothelial and smooth muscle cells. These cells were treated with a mixture of DiES + PLG. Untreated cells, cells only stimulated with DiES or with PLG, or with a mixture of DiES + PLG + εACA (an inhibitor of the PLG-plasmin conversion) were employed as controls. In addition, the effect of DiES on the expression of the fibrinolytic activators tPA and uPA, the inhibitor PAI-1 and the PLG receptor Annexin A2 was analyzed in both types of cultures by western blot.

Results

Plasmin generated by DiES + PLG binding produced a significant increase in the cell proliferation and migration of the endothelial and smooth muscle cells, as well as an increase in the destruction of the extracellular matrix based on a further degradation of Type I Collagen and an increased level of matrix metalloproteinase-2. DiES also induce an increase in the expression of tPA and uPA in endothelial cells in culture, as well as a decrease in the expression of PAI-1 in both types of cells.

Conclusions

Our study reports an interrelationship between plasmin caused by fibrinolysis activation by metabolic products of *D. immitis* and the appearance of pathological events similar to those described in the emergence of proliferative endarteritis in the cardiopulmonary dirofilariosis.

Key words: *Dirofilaria immitis*; excretory/secretory antigens; fibrinolysis; plasmin; endothelial cells; smooth muscle cells; proliferative endarteritis.

1. Background

Dirofilaria immitis is the filaroid nematode that causes the canine and feline cardiopulmonary dirofilariosis, a vector-borne parasitosis with cosmopolitan distribution. *D. immitis* is also responsible for the human pulmonary dirofilariosis, a clinical entity characterized by the formation of benign lung nodules that may be confused with carcinomas in radiology [1].

The dog acts as definitive host and reservoir of the parasite. The adult worms lodge in its pulmonary artery and right ventricle of the heart causing a chronic inflammatory pathology at vascular level [2]. One of the key factors of the disease is the appearance of proliferative endarteritis, which has resulted in the formation of intravascular microvilli. This process has previously been described as being accompanied by an increase and migration of the arterial wall cells [3-6], as well as the destruction of the extracellular matrix (ECM) [7]. These changes cause disorganization of the endothelium and reduction of the vascular lumen in the pulmonary arteries, with the consequent extension of pathology to the pulmonary parenchyma [8]. On the other hand, the simultaneous death of groups of adult worms can trigger an acute disease characterized by the exacerbation of the inflammatory reactions and the emergence of serious thromboembolic events threatening the life of the affected hosts [9]. However, *D. immitis* possesses the ability to regulate these pathological mechanisms and survive for long periods (over 7 years) in their intravascular environment. Recently, it has been shown that both excretory/secretory and surface-associated antigens of *D. immitis* interact with the fibrinolytic system of the host binding PLG and generating plasmin [10,11].

PLG is a glycoprotein predominantly released by the liver into the blood circulation. After its activation PLG becomes a serine protease (plasmin), whose main target are the fibrin clots. Under physiological conditions, this process is strictly regulated at the vascular level for the complex formed by receptors that bind PLG through carboxyterminal lysine residues (Annexin A2, among others) and activators [tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA)], whose activity is inhibited primarily by the plasminogen activator inhibitor-1 (PAI-1) [12,13].

The activation of the fibrinolytic system by *D. immitis* antigens and the consequent maintenance of haemostasis, a priori beneficial for both the parasite and host,

could have pathological consequences. An over-activation of the PLG/plasmin system has been related to cell invasion and intra-organic migration of different pathogens [14,15]. In addition, in human cardiovascular research the overproduction of plasmin has also been linked with the proliferation and migration of human vascular cells and with the degradation of extracitoplasmatic matrices [16-19]. These mechanisms have similarities with those that cause the formation of microvilli in the cardiopulmonary dirofilariosis, although their molecular aspects have not been conveniently studied to date in this parasitosis.

The objective of this work is to demonstrate that the overproduction of plasmin by the antigens of *D. immitis* may relate to the mechanisms described in the formation of microvilli at the vascular level in cardiopulmonary dirofilariosis. For this purpose, cell proliferation and migration, degradation of the ECM and expression of some components of the fibrinolytic system were studied in canine vascular endothelial and smooth muscle cells in culture stimulated with the parasitic antigens and PLG.

2. Methods

2.1. Cell culture

Canine endothelial cells (CnAOEC) and canine smooth muscle cells (CnAOSMC) from Cell Applications, INC were respectively grown in canine endothelial and canine smooth muscle cell growth mediums (Cell Applications, INC). CnAOEC plates were precoated with an attachment factor solution (Cell Applications, INC). Cells were cultured at 37°C in a humidified atmosphere in the presence of 5% carbon dioxide and 95% air. Medium was changed every 3 days. Expansion was carried out by trypsinizing the cells, (Trypsin/EDTA, Cell Applications, INC), and re-plating them when the proliferating cells had reached a sufficient density. Passaging was performed at ratios of 1:6 (CnAOEC) or 1:3 (CnAOSMC). Cell counts were performed using the equipment Countess® Automated Cell Counter (Invitrogen) following the manufacturer’s instructions.

2.2. Reagents and stimulation of CnAOEC and CnAOSMC

DiES were prepared as previously described [12] with minor modifications and stored at −80°C. In brief, live worms (25) obtained from a naturally infected dog were washed in sterile phosphate-buffered saline solution (PBS) pH 7.2 and incubated for 24 h

in 50 ml of Eagle's minimum essential medium (EMEM) supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin at 37°C. A cocktail of protease inhibitors was added to the medium following the methodology described by Maizels *et al*. [20]. The medium was dialyzed against water for 24 h and filtered through an Amicon YC05 membrane (Millipore). The protein concentration of DiES was measured by DC protein assay commercial kit (Bio-Rad). DiES extract was tested for the presence of endotoxin contamination using a quantitative *Limulus* amebocyte lysate test (BioWhittaker). The endotoxin quantity was under the sensitivity level of cell stimulation (<0.4 U/mg protein).

For stimulations, CnAOEC and CnAOSMC were grown for 4 days to obtain confluent cultures and were treated with 1 µg/ml of DiES [21], 10 µg/ml of PLG (Acris Antibodies) [17] or with a mixture of both treatments. Untreated cells and cells treated with DiES + PLG + 50 mM of the lysine analogue ε-aminocaproic acid (εACA) as an inhibitor of PLG activation were used as control cells under the same conditions.

2.3. Cell proliferation assay

Cells were plated on 24-well plates to a density of 10^4 CnAOEC/well or 1.5×10^4 CnAOSMC/well and allowed to attach overnight. After stimulation cell proliferation was analyzed by crystal violet nuclei staining over 10 days to determine the number of viable cells, as previously described [22]. Briefly, every two days, cells were rinsed with PBS, fixed with 4% formaldehyde for 10 min and stained with 0.2% crystal violet for 30 min at room temperature. After several rinses with PBS, the cells were allowed to dry overnight and crystal violet bound to cells was extracted by incubation with 2 ml/well of 10% acetic acid. The absorbance of the samples was then measured at 595 nm and transformed to "number of viable cells" using a curve that correlated absorbance and number of endothelial or smooth muscle cells previously determined.

2.4. Cell migration assay

Cell migration was assessed by quantifying the percentage of wound closure in the wound-healing assay [23]. In brief, CnAOEC and CnAOSMC were cultured in 60 mm plates (3×10^5 cells/plate) and allowed to attach overnight before wound creation. Confluent monolayers were wounded using a sterile pipette tip and the medium was exchanged for fresh medium before cell stimulation to remove cellular debris. The extent of wound closure of the treated and control cells was monitored over a time course by

microscopy and determined along 8 hours by calculating the migrated distance/total wound distance.

2.5. Collagen degradation assay

The concentration of collagen in the supernatants was analyzed by ELISA. Treated and control cells were cultured with medium for 48 hours. Then the culture supernatants were collected, filtered and added to multi-well plates (Costar). After incubation overnight at 4°C, wells were blocked with 1% BSA in PBS and incubated with a rabbit anti-Type I Collagen antibody (1:2500) (Acris Antibodies), followed by a peroxidase-conjugated goat anti-rabbit IgG (Sigma) at 1:500 dilution. All incubations were performed for 1 h at 37°C and between each step washed three times with PBS wash buffer (PBS containing 0.05% $Tween_{20}$). Ortho-phenylene-diamine was used as a chromogen. Optical densities (OD) were measured at 492 nm in an Easy Reader (Bio-Rad).

2.6. Matrix metalloproteinases (MMPs) levels assays

The levels of the MMP-2 and MMP-9 metalloproteinases in the culture media of the different experimental groups were analyzed by gelatin zymography according to the methodology described by Marangoni *et al*. [24]. Media samples employed in the collagen degradation assays were electrophoresed on a 10% polyacrylamide gel copolymerized with 1% gelatin (Sigma) together with a MMP marker (Cosmobio) as a positive control. The gels were washed for 1 hour in 2.5% Triton X-100 and incubated for 20 hours at 37°C in agitation in an enzymatic activation buffer ph 7.5 (50 mM Tris; 200 mM NaCl; 5 mM CaCl2; 0,2% Brij-35). The gels were finally stained with Coomassie blue. The positivity was assessed as appearance of clear bands on a dark background with molecular weights of 72 kDa (MMP-2) and 92 kDa (MMP-9). The levels of the MMPs were calculated after measuring the density of the existing bands, which is directly proportional to the amount of gelatin degraded into the gel.

2.7. Cell lysates and Western blot analyses

Western blot analyses were performed as previously described [21] with slight modifications. CnAOEC and CnAOSMC previously treated with 1 µg/ml of DiES for 24 h were lysed in ice-cold lysis buffer (20 mM Tris–HCl (pH 7.5), 140 mM NaCl, 10 mM ethylendiaminetetraacetic acid, 10% glycerol, 1% Igepal CA-630, aprotinin, pepstatin,

and leupeptin at 1 µg/ml each, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate). Non-stimulated cells were used as controls under the same conditions. Protein samples (10 µg) were separated by SDS-PAGE under reducing conditions and blotted onto polyvinylidine difluoride membranes. Membranes were blocked before incubation with primary antibodies: anti-tPA, anti-uPA, anti-Annexin A2 and anti-PAI-1 (Santa Cruz Biotechnology Inc) according to the manufacturer's recommendations. After incubation with HRP-conjugated secondary antibodies, bands were visualized by a luminol-based detection system with p-iodophenol enhancement. Anti-α-tubulin antibody (Oncogene Research Products) was used to confirm loading of comparable amount of protein in each lane. Protein expression was quantified by densitometry using Scion Image Software (Scion).

2.8. Statistical analysis

Cell proliferation and migration, collagen degradation and MMP levels significance measurements (comparisons between groups) were performed by ANOVA and corrected for repeated measurements when appropriate. If ANOVA revealed overall significant differences, individual means were evaluated post hoc using Bonferroni's procedure. The results from the Western blots for the tPA, uPA, annexin A2 and PAI-1 expression were analyzed with the Student's *t*-test. All the results were expressed as the mean ± SD of three experiments performed with duplicates. In all experiments, a significant difference was defined as a p-value of <0.05 for a confidence level of 95%.

3. Results

3.1. DiES produce proliferation of CnAOEC and CnAOSMC via PLG/plasmin system

The effect of DiES and PLG on the proliferation of endothelial and smooth muscle cells was quantified using the crystal violet technique in a period of 10 days (Figure 1). Both cultures showed typical curves of cell growth in all experimental groups with a progressive growth between days 0 and 8 post-treatment, experiencing cell death and an evident decrease of viable cells between days 8 and 10 post-treatment. Crystal violet staining showed an increase significantly greater in the number of viable cells in CnAOEC and CnAOSMC in culture stimulated with DiES + PLG than that showed by other experimental groups on day 8 post-treatment ($p < 0.05$), indicating that this treatment stimulates the proliferation of CnAOEC and CnAOSMC.

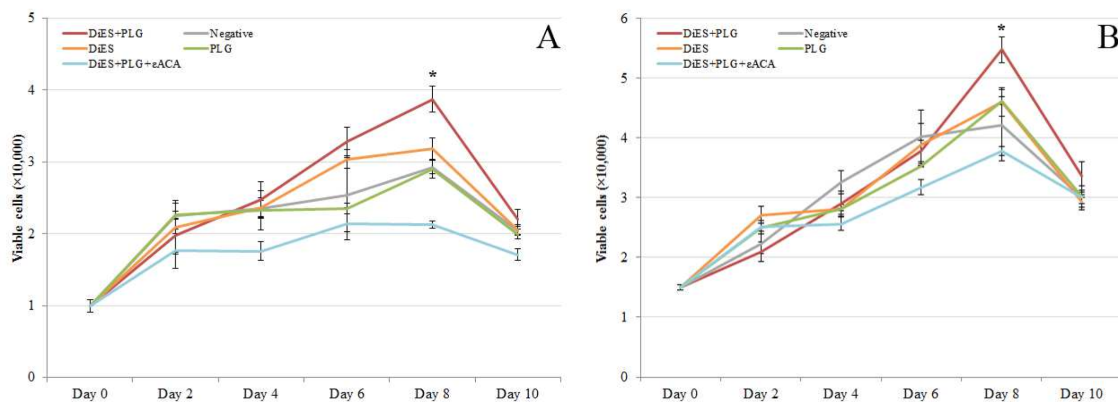

Figure 1. Cell proliferation assay performed by the crystal violet technique measuring cell viability over a 10 days period. The experiment was carried out in canine endothelial (A) and smooth muscle cells (B) untreated (▬) or treated with 1 μg/ml of DiES + 10 μg/ml of PLG (▬), 1 μg/ml of DiES (▬), 10 μg/ml of PLG (▬), or with 1 μg/ml of DiES + 10 μg/ml of PLG + 50 mM of the εACA (▬).Results are expressed as number of viable cells (x 10,000). Each point is the mean ± SD from three independent experiments. The asterisk (*) designates significant ($p < 0.05$) differences between DiES + PLG treatment and control groups.

3.2. DiES produce migration of CnAOEC and CnAOSMC via PLG/plasmin system

Wound Healing assay was performed to assess migration of endothelial (Figure 2A) and smooth muscle cells (Figure 2B). The quantification was carried out by measuring the distance of migration in comparison with negative control (untreated cells) up to 8 hours post-treatment. In both CnAOEC and CnAOSMC cultures a significant increase of cell migration after stimulation with DiES + PLG with respect to the other experimental groups ($p < 0.05$) occurred, this increase was most pronounced in cultured endothelial cells.

3.3. DiES produce ECM degradation of CnAOEC and CnAOSMC via PLG/plasmin system

To examine ECM degradation, Type I Collagen in the culture supernatant of treated and untreated CnAOEC and CnAOSMC were measured by ELISA (Figure 3). A lower concentration of Type I Collagen and therefore a further degradation of the secreted collagen by the cells was observed in the CnAOEC and CnAOSMC stimulated with DiES + PLG than that obtained by the control cells ($p < 0.05$). In addition, the same culture media from treated and untreated cells was analyzed with gelatin zymography for MMP-2 and MMP-9 levels (Figure 4). Density of the bands was measured by the Quantity One Software (Bio-Rad). The results show a significantly higher level of MMP-2 in the

CnAOEC and CnAOSMC treated with DiES + PLG than that obtained in the control cells ($p < 0.05$). No significant differences in the MMP-9 levels were observed (Figure 4).

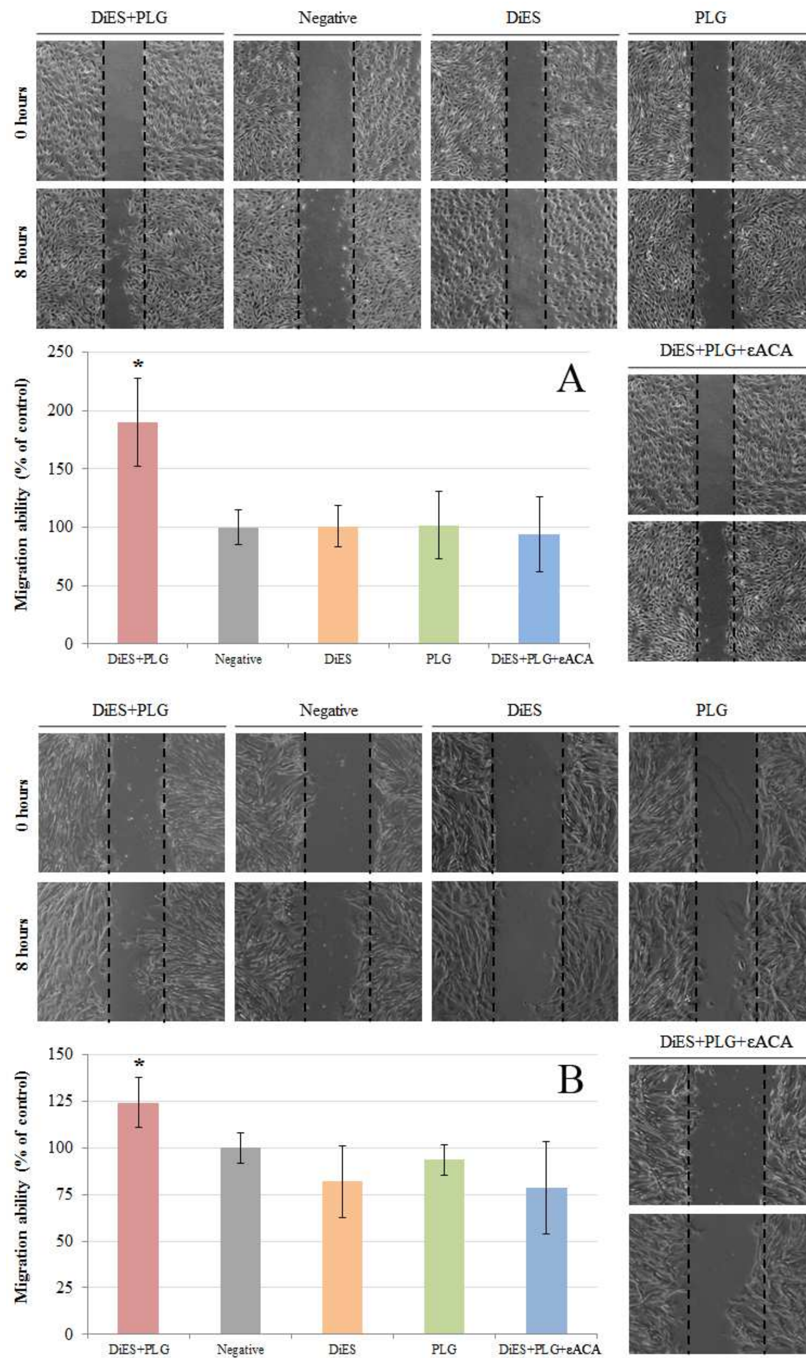

Figure 2. Cell migration by Wound-Healing assay. Confluent cell cultures were wounded post-treatment and migration distances were measured at 8 hours. The experiment was carried out in canine endothelial (A) and smooth muscle cells (B) untreated (▬) or treated with 1 µg/ml of DiES + 10 µg/ml of PLG (▬), 1 µg/ml of DiES (▬), 10 µg/ml of PLG (▬), or with 1 µg/ml of DiES + 10 µg/ml of PLG + 50 mM of the εACA (▬).The results were expressed as percentage of the migration ability of the negative control cells (100%). Each point is the mean ± SD from three independent experiments. The asterisk (*) designates significant ($p < 0.05$) differences between DiES + PLG treatment and control groups.

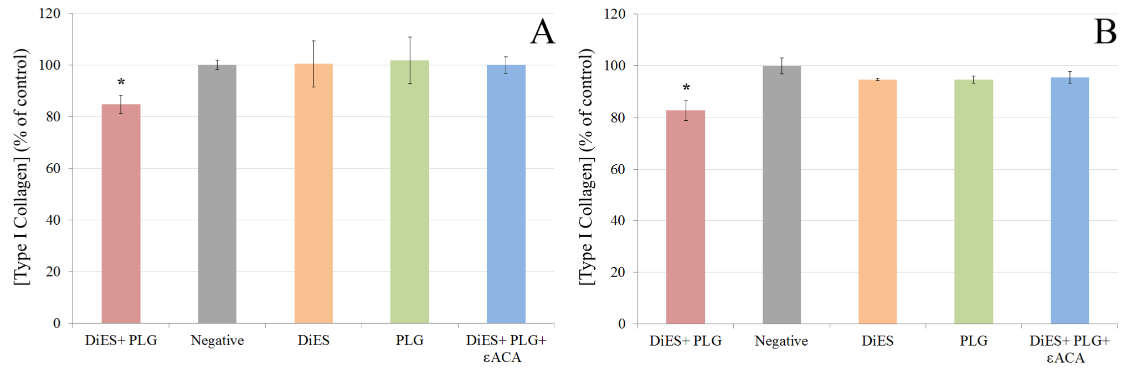

Figure 3. Type I Collagen degradation assay measured in culture supernatants from canine endothelial (A) and smooth muscle cells (B) untreated ( ) or treated with 1 µg/ml of DiES + 10 µg/ml of PLG ( ), 1 µg/ml of DiES ( ), 10 µg/ml of PLG ( ), or with 1 µg/ml of DiES + 10 µg/ml of PLG + 50 mM of the εACA ( ).The results were expressed as percentage of the Type I Collagen concentration in the culture supernatant from negative control cells (100%). Each point is the mean ± SD from three independent experiments. The asterisk (*) designates significant ($p < 0.05$) differences between DiES + PLG treatment and control groups.

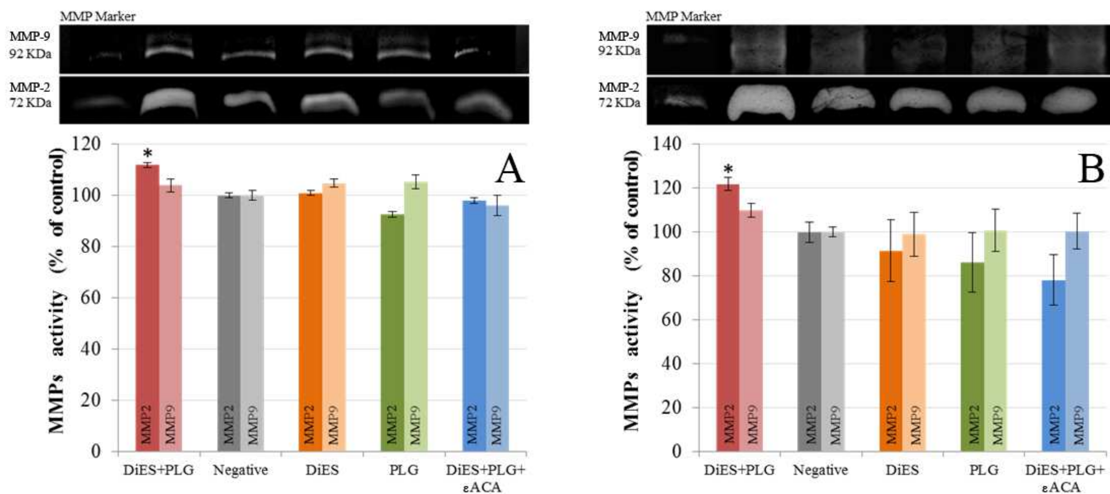

Figure 4. Representative zymography of culture supernatants from canine endothelial (A) and smooth muscle cells (B) untreated ( ) or treated with 1 µg/ml of DiES + 10 µg/ml of PLG ( ), 1 µg/ml of DiES ( ), 10 µg/ml of PLG ( ), or with 1 µg/ml of DiES + 10 µg/ml of PLG + 50 mM of the εACA ( ). Note the gelatinolytic bands associated with MMP-2 (72 KDa) and MMP-9 (92 KDa) levels. The results are expressed as percentage of the MMPs levels in the culture supernatant from negative control cells (100%). Each point is the mean ± SD from three independent experiments. The asterisk (*) designates significant ($p < 0.05$) differences between DiES + PLG treatment and control groups.

3.4. Effect of DiES on the fibrinolytic system components (tPA, uPA, Annexin II and PAI-1) expression in CnAOEC and CnAOSMC

To determine the effect of DiES on some of the main components of the fibrinolytic system, proteins from DiES-treated or untreated CnAOEC and CnAOSMC extracts were separated by SDS–PAGE and analyzed by Western blotting using anti-tPA, anti-uPA, anti-Annexin A2 and anti-PAI-1 antibodies. DiES induced a marked increase in the expression of the main fibrinolytic activators tPA and uPA in cultured endothelial cells ($p<0.05$) (Figure 5A and B), as well as a slight decrease in the expression of the main fibrinolytic inhibitor PAI-1 in both types of cultures ($p<0.05$) (Figure 5D). Significant differences in the expression of tPA and uPA in CnAOSMC (Figure 5A and B) and in the expression of Annexin A2 in both cell types between DiES-treated or untreated cultures were not found (Figure 5C).

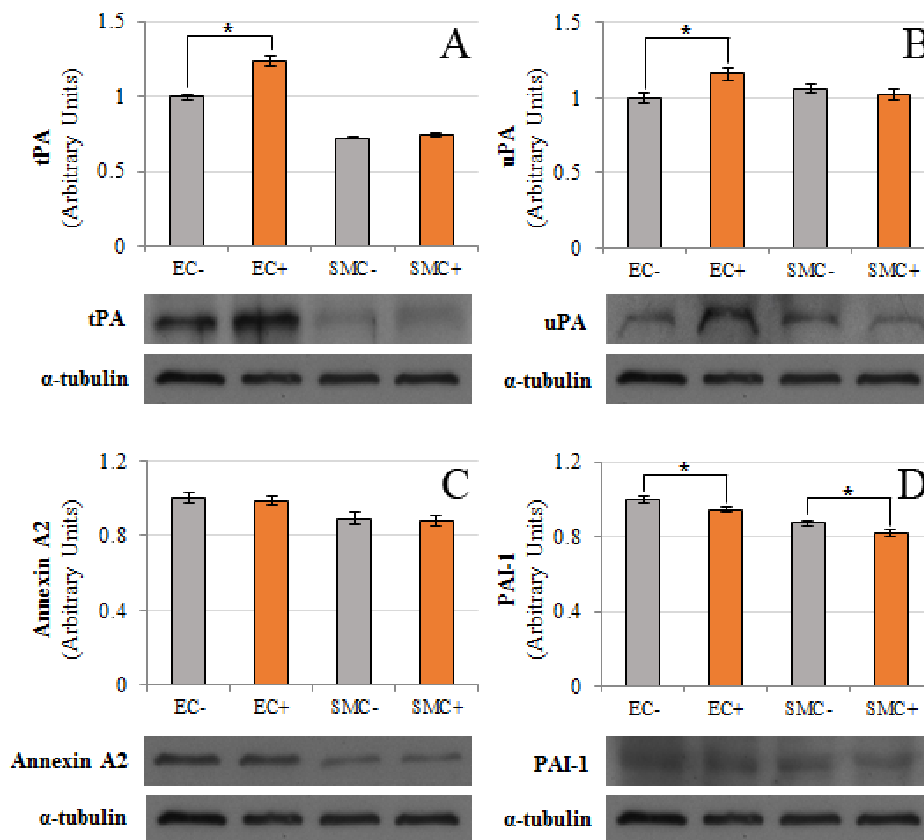

Figure 5. Effect of DiES on the expression of tPA (A), uPA (B), annexin A2 (C) and PAI-1 (D) in canine vascular endothelial (EC) and smooth muscle cells (SMC). Protein extracts from lysed DiES untreated or treated confluent cell cultures were analyzed by Western blot for tPA, uPA annexin A2 and PAI-1. α-tubulin served as a protein control. Results were expressed as the mean ± SD of at least 3 independent experiments. The asterisk (*) designates significant ($p < 0.05$) differences from control cells. (■) Non-treated control cells. (■) Stimulated endothelial or smooth muscle cells with 1μg/ml of DiES.

4. Discussion

Plasmin is the serine protease responsible for initiating the fibrinolytic process through the lysis of the fibrin clots. Apart from its pivotal role in the maintenance of haemostasis, plasmin possesses exceptionally broad specificity for target substrates playing important physiological and pathological roles in tissue remodeling, cell migration, wound healing, angiogenesis, inflammation and degradation of ECM [25]. In addition, plasmin is also upregulated in chronic inflammatory diseases, including atherosclerosis and arthritis [26]. The cardiopulmonary dirofilariosis is a chronic disease with an important inflammatory component at the vascular level in which the interaction between the excretory/secretory and surface antigens of its aetiologic agent (*D. immitis*) and the overproduction of plasmin has been recently shown [10,11]. The objective of this work was to establish a relationship between this interaction and the mechanisms responsible for the formation of intravascular microvilli in the cardiopulmonary dirofilariosis. Similar lesions have been previously explained through mechanical damage caused by the sole presence of the adult worms in the pulmonary arteries [27]. However, the pathogenic mechanisms causing the proliferative endarteritis at the molecular level have not been described in cardiopulmonary dirofilariosis.

Firstly, we have developed an “*in vitro*” model of canine endothelial and smooth muscle cells to study the relationship between the excretory/secretory antigens of the parasite and the different cell types of the canine arterial wall. Our data show that stimulation of both types of cultures with DiES + PLG cause the proliferation and migration of CnAOEC and CnAOSMC. Cells treated separately with DiES or PLG did not generate growth curves or migration distances significantly different from non-treated cells. In this sense, Morchón *et al*. [22] observed that the stimulation of human endothelial cells in culture with somatic antigens of *D. immitis* (DiSA) did not alter cell proliferation. Finally, the inhibition of both effects by including the 50 mM εACA in the stimulations demonstrates the participation of plasmin generated by DiES + PLG binding on the proliferation and migration of CnAOEC and CnAOSMC.

Two facts reveal the involvement of plasmin generated by DiES + PLG binding in the degradation of the ECM. First, this treatment for 48 h causes a significant increase in the degradation of the Type I Collagen in the culture media of CnAOEC and CnAOSMC. This molecule represents the main component of the ECM of elastic arteries.

Its alteration has been associated with vascular disease and its degradation products with the proliferation and migration of smooth muscle cells in remodelling arteries [28]. These results are consistent with that observed *in vivo* by Wang *et al*. [7] who reported a significantly lower amount of collagen in heartworm-infected dogs than in clinically normal dogs. The degradation of the ECM is also related to the increase in the levels of MMPs. Our results show a significantly higher level of MMP-2 or gelatinase A in the culture media of CnAOEC and CnAOSMC treated with DiES + PLG for 48 h, than that obtained from the other control groups. The MMP-2 can digest a large number of the ECM molecules including Type I, II, III, IV, V and XI collagens, laminin, aggrecan core protein, etc. [29]. In addition, the pathophysiological study of the action of gelatinases shows that an increase in its activity can be responsible for tissue remodeling, hypertrophy, angiogenesis and chronic inflammation [30].

All these data are consistent with previous studies, which relate the over-activation of the PLG/plasmin system with different processes of cell proliferation and migration, as well as the degradation of the ECM [16-19]. However, none of them linked these pathogenic processes with the plasmin generated through the interaction of a blood-borne pathogen with its host fibrinolytic system. To complete the knowledge of this interaction we analyze the participation of DiES in the basal production of the main components of the fibrinolytic system secreted by the cells of the arterial wall. Our results show that DiES increases the expression of the fibrinolytic activators tPA and uPA in CnAOEC during the first 24 h of stimulation, an effect that is not observed in CnAOSMC cultures. These data are consistent with those obtained previously where an increase in the basal production of tPA in human endothelial cells in culture was demonstrated [10]. In addition, the presence of tPA is required for the activation of PLG in various parasites, including *D. immitis* [31]. The increase in the expression of uPA could have special significance since, in addition to its function as an activator of fibrinolysis, it plays a key role in tissue remodeling inducing cell proliferation and migration [32], and high levels in its expression are related to cardiovascular disease [33]. DiES causes a significant reduction in the production of PAI-1 in CnAOEC and CnAOSMC in culture. PAI-1 is a member of the serine protease inhibitor (SERPIN) superfamily and is the primary physiological inhibitor of the tPA and uPA activity [34]. Finally, DiES does not cause significant changes in the expression of Annexin A2 PLG receptor. However, it has

recently been shown that *D. immitis* is able to excrete numerous antigens which can carry out similar functions as PLG-binding proteins [10].

Conclusions

In conclusion, the data obtained in this study suggest that metabolic products of *D. immitis* may tip the fibrinolytic balance towards the generation of plasmin, and that this fact is related to the appearance of pathological phenomena similar to those described in the formation of intravascular microvilli in cardiopulmonary dirofilariosis.

List of abbreviations

DiES, excretory/secretory antigens from *D. immitis* adult worms; PLG, plasminogen; ECM, extracellular matrix; tPA, tissue plasminogen activator; uPA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; CnAOEC, canine aortic endothelial cells; CnAOSMC, canine aortic smooth muscle cells; εACA, lysine analogue ε-aminocaproic acid; OP, optical density; MMPs, matrix metalloproteinases.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JGM and FS conceived and designed the experiments; JGM and RM performed the experiments; JGM, RM, EC, JAMA and FS analyzed the data; JGM and FS wrote the manuscript. All the authors have read and approved the final version of the manuscript.

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References

1. Simón F, Siles-Lucas M, Morchón R, González-Miguel J, Mellado I, Carretón E, Montoya-Alonso JA: Human and animal dirofilariasis: the emergence of a zoonotic mosaic. *Clin Microbiol Rev* 2012, 25:507-544.
2. Venco L, Genchi C, Simón F: La filariosis cardiopulmonar (*Dirofilaria immitis*) en el perro. In *La filariosis en las especies domésticas y en el hombre*. Edited by Simón F,

Genchi C, Venco L, Montoya MN. Barcelona, Spain: Merial Laboratorios; 2011:19-60.

3. Adcock JL: Pulmonary arterial lesions in canine dirofilariasis. *Am J Vet Res* 1961, 22:655-662.
4. Atwell RB, Sutton RH, Buoro IB: Early pulmonary lesions caused by dead *Dirofilaria immitis* in dogs exposed to homologous antigens. *Br J Exp Pathol* 1986, 67:395-405.
5. Hidaka Y, Hagio M, Horii Y, Murakami T, Naganobu K, Miyamoto T: Histopathological and Enzyme Histochemical Observations on Mast Cells in Pulmonary Arterial Lesion of Dogs with *Dirofilaria immitis* Infestation. *J Vet Med Sci* 2004, 66:1457-1462.
6. Kawabata A, Nakagaki K, Yoshida M, Shirota K: Histopathological comparison of pulmonary artery lesions between raccoon dogs (*Nyctereutes procyonoides*) and domestic dogs experimentally infected with *Dirofilaria immitis*. *J Vet Med Sci* 2008, 70:301-303.
7. Wang JS, Tung KC, Huang CC, Lai CH: Alteration of extracellular collagen matrix in the myocardium of canines infected with *Dirofilaria immitis*. *Vet Parasitol* 2005, 131:261-265.
8. McCall JW, Genchi C, Kramer LH, Guerrero J, Venco L: Heartworm disease in animals and humans. *Adv Parasitol* 2008, 66:193-285.
9. Venco L: Heartworm (*Dirofilaria immitis*) disease in dogs. In *Dirofilaria immitis and D. repens in Dog and Cat and Human Infections*. Edited by Genchi C, Ronaldi L, Cringoli G. Italy: Rolando Editore; 2007:117-125.
10. González-Miguel J, Morchón R, Mellado I, Carretón E, Montoya-Alonso JA, Simón F: Excretory/secretory antigens from *Dirofilaria immitis* adult worms interact with the host fibrinolytic system involving the vascular endothelium. *Mol Biochem Parasitol* 2012, 181:134-140.
11. González-Miguel J, Morchón R, Carretón E, Montoya-Alonso JA, Simón F: Surface associated antigens of *Dirofilaria immitis* adult worms activate the host fibrinolytic system. *Vet Parasitol* 2013, 196:235-240.
12. Miyashita C, Wenzel E, Heiden M. Plasminogen: a brief introduction into its biochemistry and function. *Haemostasis* 1988, 18(Suppl 1):7-13.
13. Cesarman-Maus G, Hajjar KA: Molecular mechanisms of fibrinolysis. *Br J Haematol* 2005, 129:307-321.

14. Jong AY, Chen SH, Stins MF, Kim KS, Tuan TL, Huang SH: Binding of *Candida albicans* enolase to plasmin(ogen) results in enhanced invasion of human brain microvascular endothelial cells. *J Med Microbiol* 2003, 52:615-622.
15. Bernal D, de la Rubia JE, Carrasco-Abad AM, Toledo R, Mas-Coma S, Marcilla A: Identification of enolase as a plasminogen-binding protein in excretory-secretory products of Fasciola hepatica. *FEBS Lett* 2004, 563:203-206.
16. Nicholl SM, Roztocil E, Galaria II, Davies MG: Plasmin induces smooth muscle cell proliferation. *J Surg Res* 2005, 127:39-45.
17. Yang Z, Eton D, Zheng F, Livingstone AS, Yu H: Effect of tissue plasminogen activator on vascular smooth muscle cells. *J Vasc Surg* 2005, 42:532-538.
18. Roth D, Piekarek M, Paulsson M, Christ H, Bloch W, Krieg T, Davidson JM, Eming SA: Plasmin modulates vascular endothelial growth factor-A-mediated angiogenesis during wound repair. *Am J Pathol* 2006, 168:670-684.
19. Hayashi M, Matsuzaki Y, Shimonaka M: Impact of plasminogen on an in vitro wound healing model based on a perfusion cell culture system. *Mol Cell Biochem* 2009, 322:1-13.
20. Maizels RM, Blaxter ML, Robertson BD, Selkirk ME: *Parasite antigen parasite genes: a laboratory manual for molecular parasitology*. Cambridge: Cambridge University Press; 1991.
21. Morchón R, González-Miguel J, Mellado I, Velasco S, Rodríguez-Barbero A, Simón F: Adult *Dirofilaria immitis* excretory/secretory antigens upregulate the production of prostaglandin E2 and downregulate monocyte transmigration in an “in vitro” model of vascular endothelial cell cultures. *Vet Parasitol* 2010, 170:331-335.
22. Morchón R, Rodríguez-Barbero A, Velasco S, López-Belmonte J, Simón F: Vascular endothelial cell activation by adult *Dirofilaria immitis* antigens. *Parasitol Int* 2008, 57:441-446.
23. Yamaguchi M, Ebihara N, Shima N, Kimoto M, Funaki T, Yokoo S, Murakami A, Yamagami S: Adhesion, migration, and proliferation of cultured human corneal endothelial cells by laminin-5. *Invest Ophthalmol Vis Sci* 2011, 52:679-684.
24. Marangoni NR, Melo GD, Moraes OC, Souza MS, Perri SH, Machado GF: Levels of matrix metalloproteinase-2 and metalloproteinase-9 in the cerebrospinal fluid of dogs with visceral leishmaniasis. *Parasite Immunol* 2011, 33:330-334.

25. Law RH, Abu-Ssaydeh D, Whisstock JC: New insights into the structure and function of the plasminogen/plasmin system. *Curr Opin Struct Biol* 2013, 23:836-841.
26. Syrovets T, Simmet T: Novel aspects and new roles for the serine protease plasmin. *Cell Mol Life Sci* 2004, 61:873-885.
27. Atwell RB, Buoro IB, Sutton RH: Experimental production of lesions in canine pulmonary arteries similar to those produced by *Dirofilaria immitis* infection. *Vet Rec* 1985, 116:539-541.
28. Bou-Gharios G, Ponticos M, Rajkumar V, Abraham D: Extra-cellular matrix in vascular networks. *Cell Prolif* 2004, 37:207-220.
29. Nagase H, Visse R, Murphy G: Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc Res* 2006, 69:562-573.
30. Klein T, Bischoff R: Physiology and pathophysiology of matrix metalloproteases. *Amino Acids* 2011, 41:271-290.
31. Figuera L, Gómez-Arreaza A, Avilán L: Parasitism in optima forma: Exploiting the host fibrinolytic system for invasion. *Acta Trop* 2013, 128:116-123.
32. Nicholl, S.M., Roztocil, E., Davies, M.G., 2005a. Urokinase-induced smooth muscle cell responses require distinct signaling pathways: a role for the epidermal growth factor receptor. J. Vasc. Surg. 41, 672–681.
33. Fuhrman B: The urokinase system in the pathogenesis of atherosclerosis. *Atherosclerosis* 2012, 222:8-14.
34. Iwaki T, Urano T, Umemura K: PAI-1, progress in understanding the clinical problem and its aetiology. *Br J Haematol* 2012, 157:291-298.

QVINTO CAPÍTVLO

“Fibrinolysis and proliferative endarteritis: two related processes in chronic infections? The model of the blood-borne pathogen *Dirofilaria immitis*”

Fibrinolisis y endarteritis proliferativa: ¿Dos procesos relacionados en las infecciones crónicas? El modelo del patógeno sanguíneo *Dirofilaria immitis*

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Resumen

La interacción entre los patógenos sanguíneos y la fibrinolisis es uno de los mecanismos más importantes que intervienen en la invasión y en el establecimiento de los agentes infecciosos en sus hospedadores. Sin embargo, la sobreproducción de plasmina (producto final de la ruta) ha sido relacionada en otros contextos con la proliferación y migración de las células de la pared arterial y con la degradación de la matriz extracelular. Recientemente hemos identificado antígenos con capacidad para activar la fibrinolisis pertenecientes a *Dirofilaria immitis*, un parásito sanguíneo cuyo proceso patológico clave (la endarteritis proliferativa) se produce por mecanismos similares a los indicados anteriormente. El objetivo de este trabajo es estudiar cómo dos de estos antígenos [la actina (ACT) y la fructosa-bifosfato aldolasa (FBAL)] altamente conservados en los patógenos, activan la fibrinolisis y establecer una relación entre esta activación y el desarrollo de la endarteritis proliferativa durante la dirofilariosis cardiopulmonar. Se demostró que ambas proteínas fijan plasminógeno, potencian la generación de plasmina, estimulan la expresión de los activadores fibrinolíticos tPA y uPA en cultivos de células endoteliales y que se encuentran en la superficie del verme en contacto con la sangre del hospedador. Técnicas de enzimoinmunoensayo, *western blot* e inmunofluorescencia fueron utilizadas para este propósito. Adicionalmente, la implicación de residuos de lisina en esta interacción se analizó mediante bioinformática. La participación de la plasmina generada por la unión de la ACT/FBAL y el plasminógeno en la proliferación y migración celular, así como en la degradación de la matriz extracelular se demostró en un modelo *in vitro* de cultivos de células endoteliales y del músculo liso. Los resultados obtenidos indican que la ACT y la FBAL de *D. immitis* activan la fibrinolisis, lo que podría ser utilizado por el parásito como un mecanismo de

supervivencia para evitar la formación de coágulos. Sin embargo, la sobreproducción a largo plazo de plasmina puede desencadenar procesos patológicos similares a los descritos en la aparición de la endarteritis proliferativa. Procesos similares podrían ocurrir en otros patógenos sanguíneos debido al alto grado de conservación evolutiva de estos antígenos.

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RESEARCH ARTICLE

Fibrinolysis and Proliferative Endarteritis: Two Related Processes in Chronic Infections? The Model of the Blood-Borne Pathogen *Dirofilaria immitis*

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Abstract

The interaction between blood-borne pathogens and fibrinolysis is one of the most important mechanisms that mediate invasion and the establishment of infectious agents in their hosts. However, overproduction of plasmin (final product of the route) has been related in other contexts to proliferation and migration of the arterial wall cells and degradation of the extracellular matrix. We have recently identified fibrinolysis-activating antigens from *Dirofilaria immitis*, a blood-borne parasite whose key pathological event (proliferative endarteritis) is produced by similar mechanisms to those indicated above. The objective of this work is to study how two of this antigens [actin (ACT) and fructose-bisphosphate aldolase (FBAL)] highly conserved in pathogens, activate fibrinolysis and to establish a relationship between this activation and the development of proliferative endarteritis during cardiopulmonary dirofilariasis. We demonstrate that both proteins bind plasminogen, enhance plasmin generation, stimulate the expression of the fibrinolytic activators tPA and uPA in endothelial cell cultures and are located on the surface of the worm in contact with the host's blood. ELISA, western blot and immunofluorescence techniques were employed for this purpose. Additionally, the implication of lysine residues in this interaction was analyzed by bioinformatics. The involvement of plasmin generated by the ACT/FBAL and plasminogen binding in cell proliferation and migration, and degradation of the extracellular matrix were shown in an "in vitro" model of endothelial and smooth muscle cells in culture. The obtained results indicate that ACT and FBAL from *D. immitis* activate fibrinolysis, which could be used by the parasite like a survival mechanism to avoid the clot formation. However, long-term overproduction of plasmin can trigger pathological events similar to those described in the emergence of proliferative endarteritis. Due to the high degree of evolutionary

conservation of these antigens, similar processes may occur in other blood-borne pathogens.

1. Introduction

The interaction between pathogens and their hosts at molecular level is the key point that mediates invasion and establishment of the infection. One of these events is the prevention of blood clotting through interaction with the hemostatic system, which is used by many blood-borne pathogens as a survival mechanism [1]. The fibrinolytic system, one of the main anticlotting mechanisms of the hemostatic system, is composed by plasminogen (PLG), an abundant component of blood and zymogen of serine protease plasmin, enzyme responsible for degrading fibrin clots. The conversion of PLG into plasmin is regulated by binding to receptors via its five kringle domains, which have affinity for lysine residues and PLG activators [tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA)] [2]. On the other hand, plasmin is believed to play an important role in a number of physiological and pathophysiological processes such as tissue remodeling, wound healing, angiogenesis or inflammation [3]. Overproduction of plasmin has been also linked with the proliferation and migration of human vascular cells and with the degradation of the extracellular matrix (ECM) [4–6]. In addition, plasmin is also upregulated in chronic inflammatory diseases, including atherosclerosis and arthritis [7].

Activation of fibrinolysis by pathogen antigens has been widely studied in bacteria where the expression of fibrinolytic receptors is considered an effective system for invasion and dissemination [8]. In addition, the ability to interact with the fibrinolytic system has been recently found in many eukaryotic pathogens causing parasitic infections, such as *Leishmania mexicana*, *Plasmodium falciparum*, *Fasciola hepatica*, *Schistosoma bovis* or *Onchocerca volvulus* [9]. *Dirofilaria immitis* is a zoonotic filaria responsible for canine and feline cardiopulmonary dirofilariasis and human pulmonary dirofilariasis [10]. These are chronic pathological processes that occur in the pulmonary arteries, where *D. immitis* adult worms survive for long periods (over 7 years) causing an inflammatory and thrombotic disease. Its key factor is the appearance of proliferative endarteritis, which results in the formation of intravascular microvilli. It has been described that this process is accompanied by an increase and migration of the cells of the arterial wall [11–14], as well as the destruction of the ECM [15]. These changes cause

disorganization of the endothelium and reduction of the vascular lumen in the pulmonary arteries, with the consequent extension of pathology to the pulmonary parenchyma [16].

In previous research, we have studied the interaction between the excretory/secretory (ES) and surface antigens of *D. immitis* and the fibrinolytic system of the host. The ability of these antigenic complexes to bind PLG, generate plasmin in a tPA-dependent manner and to induce an overexpression of the fibrinolytic activator tPA in vascular endothelial cells in culture was demonstrated. Additionally, we have respectively identified a total of 10 and 11 PLG-binding proteins in the ES and surface extracts of the parasite, which included different isoforms of highly conserved proteins like ACT and FBAL [17,18].

Despite the fact that the pathogenic mechanisms described in the formation of intravascular microvilli during cardiopulmonary dirofilariasis are similar to those associated with the pathophysiology of plasmin, a relationship between plasmin over-production during *Dirofilaria* infection and their pathological implications has not yet been established. The aim of the present work is to study the participation of two highly conserved proteins of *D. immitis* (ACT and FBAL) in the activation of the fibrinolytic system of the host and to establish its relationship with the proliferative endarteritis pathogenic mechanisms in the cardiopulmonary dirofilariasis.

2. Materials and methods

2.1. Parasite material

Adult worms of *D. immitis* were obtained from hearts of naturally infected dogs from Gran Canary (Canary Island, Spain) through the jugular vein using Flexible Alligator Forceps. The surgeries were carried out by veterinarians at the Veterinary Medicine Service of Las Palmas de Gran Canaria University (Canary Island, Spain) as part of a routine practice for treating animals. The pet owners were adequately informed and gave their verbal consent to the use of the samples in the study.

2.2. RNA isolation, RT-PCR, and cloning of DiACT and DiFBAL cDNA

RNA isolation, RT-PCR and cloning of the cDNA from the selected proteins were carried out as described in detail previously [19]. Total RNA from adult worms was extracted using the NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. Then, first-strand cDNA was synthesized from *D. immtis*

adults worms RNA using the first-strand cDNA synthesis kit (Roche) as recommended by the manufacturer. The cDNA sequence of the *D. immitis* actin (DiACT) and fructose-bisphosphate aldolase (DiFBAL) were amplified using the following primers:

ACTFwd (5´-ATGTGTGACGACGACGTTGCGG)
ACTRev (5´-CTAGAAACATTTGCGATGAACAATTG)
FBALFwd (5´-ATGACCTCTTACTCACAGTTTCTG)
FBALRev (5´-TTAGTATGCATGATTAGCAATGTAG)

The primers from DiACT were designed on the consensus sequence resulting after the alignment of ACT cDNA sequences from *Loa loa* and *Brugia malayi* (GenBank accession numbers XM_003146804.1 and XM_001894784.1 respectively). The primers from DiFBAL were designed on the consensus sequence resulting after the alignment of FBAL cDNA sequences from *O. volvulus*, *B. malayi* and *L. loa* (GenBank accession numbers AF155220.1, XM_001894495.1 and XM_003138767.1 respectively). PCR amplifications were performed in 1 cycle at 94°C for 5 min, 35 cycles at 94°C for 1 min, 46°C for 46 sec, and 72°C for 1 min 30 sec and 1 cycle at 72°C for 5 min. The PCR products were finally electrophoresed in an agarose gel and the bands were purified using the StrataPrep DNA Gel Extraction kit (Stratagene). The DiACT and DiFBAL PCR products were cloned into the pSC-A vector using the StrataClone PCR Cloning kit (Stratagene) following the manufacturer's instructions. Both clones were purified with the Machery-Nagel NucleoSpin Plasmid kit.

2.3. Expression and purification of the rDiACT and rDiFBAL proteins

PCR products containing the whole DiACT and DiFBAL coding sequences were cloned into the TOPO vector (Invitrogen) following the manufacturer's instructions. The recombinant plasmids were transformed into the *Escherichia coli* XL1B. Transformed cells were grown in LB-agar plates with ampicillin (100 µg/ml) overnight at 37°C. Three colonies for each molecule were grown in liquid LB plus ampicillin overnight at 37°C in agitation, and cells were harvested for plasmid extraction. Extracted plasmids were digested with EcoRI to check the insert presence. Transformed vectors were used for a ligation reaction with the pDEST vector (Invitrogen). Ligation reaction was used for transformant selection as above-mentioned. Vectors containing the inserts of interest were used to transform BL-21 cells. These were grown in liquid LB plus ampicillin (100

μg/ml) overnight at 37°C in agitation. Cultures were diluted 1:10 in fresh medium and further grown until reaching an optical density (OD) of 0.5. Then, expression of the recombinant protein was induced by 0.2% L-arabinose 20% at 37°C for 4 h in agitation. The induced cells were harvested and sonicated in a buffer containing 8M urea, 100mM NaH_2PO_4 and 10mM Tris-Cl, pH 7.9. After a 20 min centrifugation step at 10.000 x g, the supernatant was applied to a HIS-Select Nickel Affinity Gel (Sigma) for affinity purification of the histidine-tagged rDiACT and rDiFBAL, according to the manufacturer's instructions. Urea was eliminated by washing the column with wash buffer (100mM NaH2PO4, and 10mM Tris-Cl pH 6.3) containing decreasing concentrations of urea (from 6M to 0M). Then, the recombinant proteins were eluted with elution buffer (50mM NaH_2PO_4, 300mM NaCl and 250mM imidazole, pH 7.9). The eluted rDiACT and rDiFBAL were dialysed in PBS for 24 h at 4°C and stored at –80°C until use. The purity and yield of each protein obtained after purification were assessed in 12% polyacrylamide gels using Coomasie blue staining.

2.4. Bioinformatic analyses

The deduced amino-acid sequence of rDiACT and rDiFBAL were analysed using the following bioinformatic tools: BLAST searching of the homologous sequences in the NCBI and Swissprot/Uniprot databases (http://www.ncbi.nlm.nih.gov/, http://www.uniprot.org/); analysis of conserved domains with SMART (http://smart.embl-heidelberg.de); theoretical isoelectric point (pI) and the molecular weight (MW) calculations (http://www.expasy.org/tools/pi_tool.html); prediction of transmembrane domains with the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0); prediction of signal peptides with SignalP 3.0 [20] (http://www.cbs.dtu.dk/services/SignalP); search for glycosyl-phosphatidyl anchors in the sequence with the big-PI Predictor [21] (http://mendel.imp.ac.at/sat/gpi/gpi_server.html); multiple sequence alignment with ClustalW 2.1 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and prediction of the secondary structures and three-dimensional modelling with the Swiss-Model server [22] (http://swissmodel.expasy.org/). The 3-D models were visualized with the RasMol software v.2.7.5.2.

2.5. PLG binding assays

The ability of rDiACT and rDiFBAL to act as plasminogen-binding proteins was assessed by ELISA. Following the methodology described by González-Miguel et al. (2012) multiwell microplates (Costar) were coated with 0.5 µg/well of each recombinant protein. Then, plates were blocked and incubated with increasing amounts (from 0 µg to 3 µg) of human PLG (Acris antibodies). After incubation with the corresponding antibodies and with a chromogen, binding was revealed by measuring OD at 492 nm in an Easy Reader (Bio-Rad). Between each incubation, plates were washed three times with PBS containing 0.05% $Tween_{20}$ (PBST). In order to study the involvement of lysine residues in this binding, competition assays were performed by including 50 mM of the lysine analogue ε-aminocaproic acid (εACA) during PLG incubation [17].

2.6. PLG activation assays

The participation of rDiACT and rDiFBAL in plasmin generation was studied following the methodology previously described [17]. In brief, 2 µg of human PLG (Acris antibodies) were incubated in PBS with 3 µg of the chromogenic substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride (Sigma) in the presence of 1 µg of each recombinant protein in a final volume of 100 µl. Activation of PLG was initiated by addition of 15 ng of t-PA (Sigma), however, plasmin generation in the absence of tPA was also analyzed. After incubation of the plates (1 h at 37°C) the hydrolysis of the chromogenic substrate, which is directly proportional to the amidolytic activity of generated plasmin was revealed by measuring absorbance at 405 nm. Each sample was analyzed in triplicate.

2.7. Generation of an anti-rDiFBAL antiserum

Antiserum against rDiFBAL was generated by subcutaneous immunization of two New Zealand female rabbits with 3 doses of protein in 0.2% saponin solution. First dose of 1 mg at the beginning of the experiment, plus 2 doses of 500 µg 7 and 10 days later. Rabbits were sacrificed by an intravenous overdose of pentobarbital and bled 20 days after the last dose. Serum was collected, serially diluted and titred by ELISA. The reactivity and specificity of the serum was also assessed by Western blot. Animal procedures for this purpose complied with the Spanish (Real Decreto RD53/2013) and the European Union (European Directive 2010/63/EU) guidelines on animal experimentation for the protection and humane use of laboratory animals, and were conducted at the accredited Animal Experimentation Facility (Servicio de

Experimentación Animal) of the University of Salamanca (Register number: PAE/SA/001). Procedures were approved by the Ethics Committee of the University of Salamanca and all efforts were made to minimize suffering. These included good practice for housing and care minimizing discomfort, distress and pain in animals.

2.8. Immunolocalization of DiACT and DiFBAL in *D. immitis* adult worms

Confocal microscopy studies were carried out on adult worm sections. *D. immitis* adult worms were fixed in 10% buffered formalin and embedded in paraffin. For immunofluorescence, 6 µm-thick sections were placed on polylisinated slides, deparaffinised in xylene (2×8 min each), and rehydrated. Sections were then blocked with 1% BSA in PBST for 1 h at 37°C, after which they were incubated with the anti-rDiFBAL rabbit serum diluted 1:50 in blocking buffer for 1 h at 37°C. Samples were washed three times with PBST and incubated at 4°C overnight with an anti-rabbit IgG antibody conjugated to Alexa Fluor 594 (Molecular Probes) diluted 1:400 in blocking buffer containing phalloidin-Alexa Fluor 488 (Molecular Probes) diluted 1:200, which specifically binds to ACT microfilaments. The samples were then washed four times and mounted in an antifade reagent (Prolong Gold, Molecular Probes). Negative controls were carried out by serum from a non-immunized rabbit.

2.9. Cell culture

Canine endothelial cells (CnAOEC) and canine smooth muscle cells (CnAOSMC) from Cell Applications, INC were respectively grown in canine endothelial and canine smooth muscle cell growth mediums (Cell Applications, INC). CnAOEC plates were precoated with an attachment factor solution (Cell Applications, INC). Cells were cultured at 37°C in a humidified atmosphere in the presence of 5% carbon dioxide and 95% air. Medium was changed every 3 days. Expansion was done by trypsinizing the cells (Trypsin/EDTA, Cell Applications, INC) and replating them when the proliferating cells had reached a sufficient density. Passaging was done at ratios of 1:6 (CnAOEC) or 1:3 (CnAOSMC). Cell counts were performed using the equipment Countess Automated Cell Counter (Invitrogen) following the manufacturer's instructions.

2.10. Reagents and stimulation of CnAOEC and CnAOSMC

For stimulations, CnAOEC and CnAOSMC were grown for 4 days to obtain confluent cultures and were treated with 1 µg/ml of rDiACT or rDiFBAL, 10 µg/ml of

PLG (Acris Antibodies) [4] or with a mixture of both treatments (rDiACT + PLG or rDiFBAL + PLG). Untreated cells and cells treated with rDiACT/rDiFBAL + PLG + 50 mM of εACA as an inhibitor of PLG activation were used as control cells under the same conditions.

2.11. Cell lysates and Western blot analyses

Western blot analyses were performed as described in detailed previously [23] with slightly modifications. CnAOEC and CnAOSMC previously treated with 1µg/ml of rDiACT or rDiFBAL for 24 h were lysed in ice-cold lysis buffer (20mM Tris—HCl (pH 7.5), 140mM NaCl, 10mM ethylendiaminetetraacetic acid, 10% glycerol, 1% Igepal CA-630, aprotinin, pepstatin, and leupeptin at 1µg/ml each, 1mM phenylmethylsulfonyl fluoride, and 1mM sodium orthovanadate). Non-stimulated cells were used as controls under the same conditions. Protein samples (10 µg) were separated by SDS-PAGE under reducing conditions and electrotransferred onto polyvinylidine difluoride membranes. Then, membranes were blocked before incubation with the following primary rabbit polyclonal antibodies: anti-tPA and anti-uPA (Santa Cruz Biotechnology Inc) according to the manufacturer's recommendations. After incubation with HRP-conjugated anti-rabbit secondary antibodies, bands were visualized by a luminol-based detection system with p-iodophenol enhancement. Anti-α-tubulin antibody (Oncogene Research Products) was used as control to confirm loading of comparable amount of protein in each lane. Protein expression was quantified by densitometry using the PDQuest Software v.8.0.1 (Bio-Rad).

2.12. Cell proliferation assay

Cells were plated on 24-well plates to a density of 10^4 CnAOEC/well or 1.5 x 10^4 CnAOSMC/well and allowed to attach overnight. After stimulations cell proliferation was analyzed by crystal violet nuclei staining over 10 days determining the number of viable cells as previously described [24]. Briefly, every two days, cells were rinsed with PBS, fixed with 4% formaldehyde for 10 min and stained with 0.2% crystal violet for 30 min at room temperature. After several rinses with PBS, the cells were allowed to dry overnight and crystal violet bound to cells was extracted by incubation with 2 ml/well of 10% acetic acid. The absorbance of the samples was then measured at 595 nm and transformed to “number of viable cells” using a curve that correlated absorbance and number of endothelial or smooth muscle cells previously determined.

2.13. Cell migration assay

Cell migration was assessed by quantifying the percentage of wound closure in the wound-healing assay [25]. In brief, CnAOEC or CnAOSMC were cultured in 60 mm plates (3 x 10^5 cells/plate) and allowed to attach overnight before wound creation. Confluent monolayers were wounded using a sterile pipette tip and the medium was exchanged for fresh medium before cell stimulation to remove cellular debris. The extent of wound closure of the treated and control cells was monitored over a time course by microscopy and determined along 8 hours by calculating the migrated distance/total wound distance.

2.14. Collagen degradation assay

The concentration of collagen in the supernatants was analyzed by ELISA. Treated and control cells were cultured with medium for 48 hours. Then the culture supernatants were collected, filtered and added to multi-well plates (Costar). After incubation overnight at 4°C, wells were blocked with 1% BSA in PBS and incubated with a rabbit anti-Type I Collagen antibody (1:2500) (Acris Antibodies), and then with a peroxidase-conjugated goat anti-rabbit IgG (Sigma) at 1:500 dilution. All incubations were performed for 1 h at 37°C and between each step washed three times with PBST. Ortho-phenylene-diamine was used as a chromogen. ODs were measured at 492 nm in an Easy Reader (Bio-Rad).

2.15. Matrix Metalloproteinase (MMP) levels assays

The levels of MMP-2 and MMP-9 metalloproteinases in the culture media of the different experimental groups was analyzed by gelatin zymography [26] according to the methodology described by Marangoni et al. (2011) [27]. Media samples employed in the collagen degradation assays were electrophoresed on a 10% polyacrylamide gel copolymerized with 1% gelatin (Sigma) together with a MMP marker (Cosmobio) as a positive control. The gels were washed for 1 hour in 2.5% Triton X-100 and incubated for 20 hours at 37°C in agitation in an enzymatic activation buffer ph 7.5 (50 mM Tris; 200 mM NaCl; 5 mM $CaCl_2$; 0,2% Brij-35). The gels were finally stained with Coomassie blue. The positivity was assessed as appearance of clear bands on a dark background with molecular weights of 72 kDa (MMP-2) and 92 kDa (MMP-9). The levels of MMPs were calculated after measuring the intensity of the existing bands, which is directly proportional to the amount of gelatin degraded into the gel [28].

2.16. Statistical analysis

The results from the PLG binding assay, PLG activation assay and Western blots for the tPA and uPA expression were analyzed with the Student's t-test. Cell proliferation and migration, collagen degradation and MMP levels significance measurements (comparisons between groups) were performed by ANOVA and corrected for repeated measurements when appropriate. If ANOVA revealed overall significant differences, individual means were evaluated post hoc using Bonferroni's procedure. All the results were expressed as the mean ± SD of three experiments performed with duplicates. In all experiments, a significant difference was defined as a p-value of <0.05 for a confidence level of 95%.

3. Results

3.1. Amplification, cloning, sequencing, and expression of *D. immitis* ACT and FBAL

Amplification of *D. immitis* ACT and FBAL cDNA by RT-PCR respectively resulted in PCR products of around 1100 and 1000 bp. After their cloning into the pSC-A vector, fragments were fully sequenced and their identities demonstrated as actin and fructose bisphosphate aldolase by BLAST analysis. The new sequences were respectively deposited in the Gen-Bank under accession numbers JQ780093.1 and JQ780094.1. The full cDNA respectively contained 1131 and 1092 nucleotides, encoded a protein of 376 and 363 amino acids, with a theoretical molecular weight of 41.820 and 39.423 Da, and pI of 5.29 and 7.65.

The bioinformatics analyses of the deduced amino acid sequences did not reveal a signal peptide, transmembrane helices or glycosyl-phosphatidyl inositol anchors. The percentage identity between DiACT/DiFBAL and homologous sequences from other organisms was analyzed using multiple sequence alignment with the ClustalW program (Figs 1 and 2). The analysis revealed that DiACT and DiFBAL are highly conserved proteins. Thus, in the alignment of these sequences with homologous proteins from other filarial nematodes (*Wuchereria bancrofti*, *O. volvulus*, *B. malayi* and *L. loa*) DiACT and DiFBAL respectively obtained identities of 100% and range of identities between 94.21 and 96.69%. These identities also obtained high values when DiACT and DiFBAL were aligned with homologous proteins from other parasitic helminthes (Figs 1 and 2). Aditionally, a PLG-binding domain to actin within amino acids 56 to 70 (GDEAQSKRGILTLKY) and 19 and 12 conserved lysine residues in the DiACT and

DiFBAL alignment were respectively found and highlighted. In silico three-dimensional modelling of the molecules predicted the 3D structures showing in the case of DiACT a monomer with 20 α-helices and 19 β-sheets (Fig 3A). Molecular modelling of DiFBAL showing a homo-tetramer with the presence of 14 α-helices and 13 β-sheets (Fig 3B). The PLG-binding domain (GDEAQSKRGILTLKY) and the conserved lysine residues were highlighted and were visualized on the outside of the proteins.

The *D. immitis* ACT and FBAL cDNA were cloned into the expression vector TOPO/pDEST. After induction of expression in *E. coli*, the recombinant proteins were purified under denaturing conditions using nickel affinity chromatography. The purified rDiACT and rDiFBAL respectively had molecular weights of 43.6 kDa and 41.6 kDa in polyacrylamide gel.

3.2. rDiACT and rDiFBAL bind PLG

An ELISA was carried out to determine the binding level of PLG to rDiACT and rDiFBAL (Fig 4). Analyses showed that both recombinant proteins bind PLG in a similar way and that this binding is directly proportional to the amount of PLG (Fig 4). Wells coated only with BSA, used as negative control, showed some non-specific binding activity, but always with values significantly lowers than those obtained by rDiACT and rDiFBAL ($p<0.05$). The addition of 50 mM εACA respectively resulted in the inhibition of about 70% and 90% of the binding level of rDiACT and rDiFBAL, demonstrating the involvement of lysine residues in the process (Fig 4).

```
D.immitis      MCDDDVAALVIDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQ 60
W.bancrofti    MCDDDVAALVIDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQ 60
O.volvulus     MCDDDVAALVIDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQ 60
B.malayi       MCDDDVAALVIDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQ 60
L.loa          MCDDDVAALVIDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQ 60
A.suum         MCDDDVAALVIDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQ 60
H.sapiens      -MDDDIAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQ 59
S.bovis        MADEEVQALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQ 60
                 *::: ***:*************************************************

D.immitis      SKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM 120
W.bancrofti    SKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM 120
O.volvulus     SKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM 120
B.malayi       SKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM 120
L.loa          SKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM 120
A.suum         SKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM 120
H.sapiens      SKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM 119
S.bovis        SKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM 120
               ************************************************************

D.immitis      TQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVLDTGDGVTHTVPIYEGYALPHAILRLD 180
W.bancrofti    TQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVLDTGDGVTHTVPIYEGYALPHAILRLD 180
O.volvulus     TQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVLDTGDGVTHTVPIYEGYALPHAILRLD 180
B.malayi       TQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVLDTGDGVTHTVPIYEGYALPHAILRLD 180
L.loa          TQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVLDTGDGVTHTVPIYEGYALPHAILRLD 180
A.suum         TQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVLDTGDGVTHTVPIYEGYALPHAILRLD 180
H.sapiens      TQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVMDSGDGVTHTVPIYEGYALPHAILRLD 179
S.bovis        TQIMFETFNTPAMYVGIQAVLSLYASGRTTGIVLDSGDGVTHTVPIYEGYALPHAILRLD 180
               ***************.*****************:*:************************

D.immitis      LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSLEKS 240
W.bancrofti    LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSLEKS 240
O.volvulus     LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSLEKS 240
B.malayi       LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSLEKS 240
L.loa          LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSLEKS 240
A.suum         LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSLEKS 240
H.sapiens      LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSLEKS 239
S.bovis        LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSLEKS 240
               ************************************************************

D.immitis      YELPDGQVITIGNERFRCPEALFQPSFLGMESTGIHETTYNSIMKCDVDIRKDLYANTVL 300
W.bancrofti    YELPDGQVITIGNERFRCPEALFQPSFLGMESTGIHETTYNSIMKCDVDIRKDLYANTVL 300
O.volvulus     YELPDGQVITIGNERFRCPEALFQPSFLGMESTGIHETTYNSIMKCDVDIRKDLYANTVL 300
B.malayi       YELPDGQVITIGNERFRCPEALFQPSFLGMESTGIHETTYNSIMKCDVDIRKDLYANTVL 300
L.loa          YELPDGQVITIGNERFRCPEALFQPSFLGMESTGIHETTYNSIMKCDVDIRKDLYANTVL 300
A.suum         YELPDGQVITIGNERFRCPEALFQPSFLGMESCGIHETTYNSIMKCDVDIRKDLYANTVL 300
H.sapiens      YELPDGQVITIGNERFRCPEALFQPSFLGMESCGIHETTFNSIMKCDVDIRKDLYANTVL 299
S.bovis        YELPDGQVITIGNERFRCPEALFQPSFLGMESAGVHETTFNSIMKCDVDIRKDLYANTVL 300
               ******************************** *:****:********************

D.immitis      SGGTSMFPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISK 360
W.bancrofti    SGGTSMFPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISK 360
O.volvulus     SGGTSMFPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISK 360
B.malayi       SGGTSMFPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISK 360
L.loa          SGGTSMFPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISK 360
A.suum         SGGTSMFPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISK 360
H.sapiens      SGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISK 359
S.bovis        SGGTTMFPGIADRMQKEITALAPSTMKIKIVAPPERKYSVWIGGSILASLSTFQQMWISK 360
               ****:*:***********************:*****************************
                                           Identity (%)
D.immitis      QEYDESGPSIVHRKCF 376     -
W.bancrofti    QEYDESGPSIVHRKCF 376     100
O.volvulus     QEYDESGPSIVHRKCF 376     100
B.malayi       QEYDESGPSIVHRKCF 376     100
L.loa          QEYDESGPSIVHRKCF 376     100
A.suum         QEYDESGPSIVHRKCF 376     99.73
H.sapiens      QEYDESGPSIVHRKCF 375     97.60
S.bovis        QEYDESGPGIVHRKCF 376     96.54
               ********.*******
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Fig. 1. Multiple sequence alignment of DiACT with homologous proteins. Alignment of the *D. immitis* ACT sequence (I3WTW3) with the ACT from *W. bancrofti* (EJD75047), *O. volvulus* (EJW73626), *B. malayi* (P48812), *L. loa* (O01360), *Ascaris suum* (BAB68543), *Homo sapiens* (P20287) and *S. bovis* (AIE44418). The percentage of sequence identity between *D. immitis* sequence and the others is indicated. The amino acids conserved in all the sequences are labelled with asterisks, and conservative and semiconservative substitutions are respectively labelled with two and one point. Conserved lysine residues are shaded in yellow. The PLG-binding domain (GDEAQSKRGILTLKY) are shaded in grey.

```
D.immitis       ----------------------------------------------------MTSYSQFL 8
O.volvulus      ----------------------------------------------------MTSYSQFL 8
L.loa           MQPNFFTKYYVISGDTSSTAKHLVDEGNSSAVNATSDTTAVDAEATAAVVEKMTSYSPFL 60
B.malayi        ----------------------------------------------------MTSYSQFL 8
W.bancrofti     ----------------------------------------------------MTSYSQFL 8
A.suum          ----------------------------------------------------MASYSQYL 8
H.contortus     ----------------------------------------------------MASHSQYL 8
S.japonicum     ----------------------------------------------------MPRFPPYL 8
                                                                    *. .. :*

D.immitis       TEAQKDELRQIANQIVAPGKGILAADESVGSMDKKLKPIGLENVEENRRLYRQLLFTAGD 68
O.volvulus      TDAQKDELRKIANQIVTPGKGILAADESTGSMDKKLKPIGLENVEENRRLYRQLLFTAGD 68
L.loa           TQAQKDELRQIANQIVAPGKGILAADESTGSMDKKLKPIGLENVEENRRLYRQLLFTAGD 120
B.malayi        TDAQKDELRQIANQIVTPGKGILAADESTGSMDKKLKPIGLENVEENRRLYRQLLFTAGD 68
W.bancrofti     TEAQKDELRQIANQIVTPGKGILAADESTGSMDKKLKPIGLENVEENRRLYRQLLFTAGD 68
A.suum          TKAQEDELRQIAQAIVAPGKGILAADESTGSMDKKLKPIGLENVEENRRLYRQILFTSSD 68
H.contortus     TKEQEDELRGIANAIVAPGKGILAADESTGSMDKKMQNIGTENTEEQRRKYRQLLFTASP 68
S.japonicum     TEAQEDDLRQIAQAICAPGKGILAADESTATMGKRLQQIGVENNEENRRLYRQLLFSADH 68
                *. *:*:** **: * :***********..:*.*::: ** ** **:** ***:**::.

D.immitis       EMSKYISGVIMFHESFYHKADDGTPFVQILQKKGILPGIKVDKGVVPMAGTVGEGTTQGL 128
O.volvulus      EMSKYISGVIMFHETFYQKGDDGTPFVQILQKKGILPGIKVDKGVIPMAGTVGEGTTQGL 128
L.loa           EMSKYISGVIMFHETFYQKGDDGTPFVQILQKKGILPGIKVDKGVVPMAGTVGEGTTQGL 180
B.malayi        EMSKYISGVIMFHETFYQKADDGTPFVQVLQKKGILPGIKVDKGVIPMAGTVGEGTTQGL 128
W.bancrofti     EMSKYISGVIMFHETFYQKADDGTPFVQVLQKKGILPGIKVDKGVIPMAGTVGEGTTQGL 128
A.suum          EISKHISGVIMFHETFYQKTDDGTPFVEVLKKKGIIPGIKVDKGVVPMAGTIGEGTTQGL 128
H.contortus     EMSKHISGVIMFHETFYQKTDDGTRFVDVLKKQGVIPGIKVDKGVVPMAGTVGEGTTQGM 128
S.japonicum     KLAQNISGVILFEETLHQKSDDGKTLPTLLAERHIIPGIKVDKGVVPLAGTDNETTTQGL 128
                :::: *****:*.*::::* ***. :  :* :: ::*********:*:*** .* ****:

D.immitis       DDLNSRCAQYKKGGAQFAKWRCVHKIGATTPSHMALVEIAEVLARYASICQQHGLVPIVE 188
O.volvulus      DDLNSRCAQYKKDGAQFAKWRCVHKIGATTPSHMALVEIAEVLARYASICQQHGLVPIVE 188
L.loa           DDLNSRCAQYKKDGAQFAKWRCVHKIGATTPSHMALVEIAEVLARYASICQQHGLVPIVE 240
B.malayi        DDLNNRCAQYKKDGAQFAKWRCVHKIGATTPSHMALVEIAEVLARYASICQQHGLVPIVE 188
W.bancrofti     DDLNNRCAQYKKDGAQFAKWRCVHKIGATTPSHMALVEIAEVLARYASICQQHGLVPIVE 188
A.suum          DDLNARCAQYKKDGAQFAKWRCVHKIGATTPSHMALVEIAEVLARYASICQQHGLVPIVE 188
H.contortus     DDLNARCAQYKKDGAQFAKWRCVHKISATTPSHMALVEIAEVLARYASICQQNGLVPIVE 188
S.japonicum     DDLASRCAEYRKLGCRFAKWRCVLKISSHTPSYQAMLENANVLARYASICQQNGLVPIVE 188
                ***  ***:*:* *.:******* **.: ***: *::* *:***********:*******

D.immitis       PETLPDGEHDVHRCQKVTELVLSYTYKALIDHHVYLEGTLLKPNMCMPGMQFKG-QCSHE 247
O.volvulus      PETLPDGEHDVHRCQKVTELVLSYTYKALIDHHVYLEGTLLKPNMCMPGMQFKG-QCSHE 247
L.loa           PETLPDGEHDLHRCQKVTELVLSYTYKALIDHHVYLEGTLLKPNMCMPGMQFKG-QCSHE 299
B.malayi        PETLPDGEHDLHRCQKVTELVLSYTYKALIDHHVYLEGTLLKPNMCMPGMQYKG-QCSHE 247
W.bancrofti     PETLPDGEHDLHRCQKVTELVLSYTYKALIDHHVYLEGTLLKPNMCMPGMQYKG-KCSHE 247
A.suum          PETLPDGDHDLDRCQKITEAVLSYTYKALIDHHVFLEGTLLKPNMVTPGMQGP--KVSHE 246
H.contortus     PEILPDGEHDIHRCQKITETVLSYCYRALNDHHVYLEGTLLKPNMVTAGQAFKGTKPSHD 248
S.japonicum     PEVLPDGDHDLLTAQRVTEEVLSFVYKALADHHVYLEGTLLKPNMVTAGQACKK-AYTPQ 247
                ** ****:**:  .*::** ***: *:** ****:**********  .*         : :

D.immitis       EIARATVTALRRTVPVAVPGIVFLSGGQSEEDATLNLNAINQFSGKKPWALTFSYGRALQ 307
O.volvulus      EIARATVTALQRTVPVAVPGIVFLSGGQSEEDATLNLNAINQFPGKKPWALTFSYGRALQ 307
L.loa           DIARATVTALQRTVPVAVPGVVFLSGGQSEEDATLNLNAINQFPGKKPWALTFSYGRALQ 359
B.malayi        DIARATVTALQRTVPVAVPGIVFLSGGQSEEDATLNLNAINQFPGKKPWALTFSYGRALQ 307
W.bancrofti     DIARATVTALQRTVPVAVPGIVFLSGGQSEEDATLNLNAINQFPGKKPWALTFSYGRALQ 307
A.suum          EIARATVTCLQRTVPVAVPGIVFLSGGQSEEDATLNLNAINQYQGKKPWALTFSYGRALQ 306
H.contortus     EIALATVTALQRAVPAAVPGVVFLSGGQSEEDATLNLNAMNKLQTKKPWALTFSYGRALQ 308
S.japonicum     ENALATVRALQRTVPPAVPGITFLSGGQSELDATKNLNEINKIPGPKPWALTFSFGRALQ 307
                : * *** .*:*:** ****:.******** *** *** :*:    ********:*****

                                                                     Identity (%)
D.immitis       ASALAAWGGKPENIQAAKEAFLKRAEANSLAQLGKYTGG-AGSGAAGENLYIANHAY 363     -
O.volvulus      ASALAAWGGKPENIQAAKEAFLKRAQANSLAQLGKYTGG-AGSGAAGENLYIANHAY 363     96.69
L.loa           ASALAAWGGKPENIQAAKEAFLKRAQANSLAQLGKYTGG-AGSGAAAQNLYVANHAY 415     95.59
B.malayi        ASALAAWGGKLENIQAAKEAFLKRAQANSLAQLGKYAGG-AGSGAAAQNLYVANHAY 363     94.49
W.bancrofti     ASALAAWGGKLENIQAAKEAFLKRAQANSLAQLGKYAGG-ADSGAAAQNLYVANHAY 363     94.21
A.suum          ASVLAAWGGKKENEEAAKAAFLKRAQANGLAALGKYTGG-GAGSAAGQSLYIANHAY 362     85.64
H.contortus     ASAMAKWSGKDENVPAAKAVFMQRAQANSLAALGKYSGDPNADKAASQSLFVANHAY 365     77.41
S.japonicum     ASVLATWQGKKENVHSAQEEFLKLAKANGAAAVGKFEGD-MGTALGDKSLFVANHAY 363     63.64
                **.:* * ** **   :*:  *:: *:**. * :**: *.       . :.*::*****
```

Fig. 2. Multiple sequence alignment of DiFBAL with homologous proteins. Alignment of the *D. immitis* FBAL sequence (I3WTW4) with the FBAL from *O. volvulus* (Q9U9R9), *L. loa* (J0DRR2), *B. malayi* (A8P3ES), *W. bancrofti* (J9EVQ4), *A. suum* (U1M5S0), *Haemonchus contortus* (R4H2V1) and *S. japonicum* (C1LB95). The percentage of sequence identity between *D. immitis* sequence and the others is indicated. The amino acids conserved in all the sequences are labelled with asterisks, and conservative and semiconservative substitutions are respectively labelled with two and one point. Conserved lysine residues are shaded in yellow.

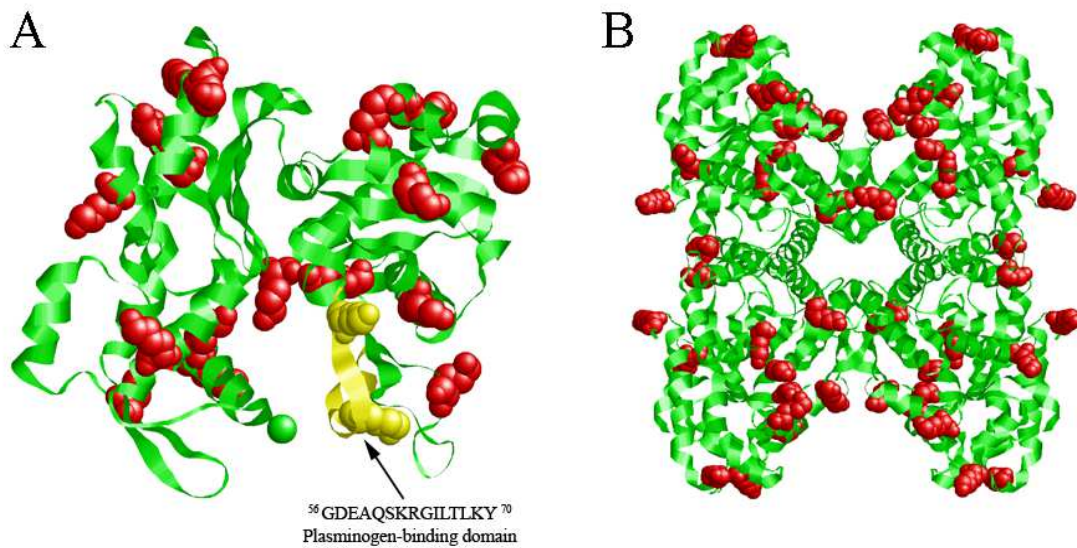

Fig. 3. Molecular modelling of *D. immitis* ACT and FBAL. The secondary structure of the proteins was predicted with the Swiss-Model web server (http://swissmodel.expasy.org/) by analogy with the X-ray crystallography available models. The three-dimensional models of DiACT (A) and DiFBAL (B) were visualized with the RasMol application v. 2.7.5.2. Conserved lysine residues of proteins were highlighted as red balls. The PLG-binding domain (GDEAQSKRGILTLKY) is highlighted in yellow.

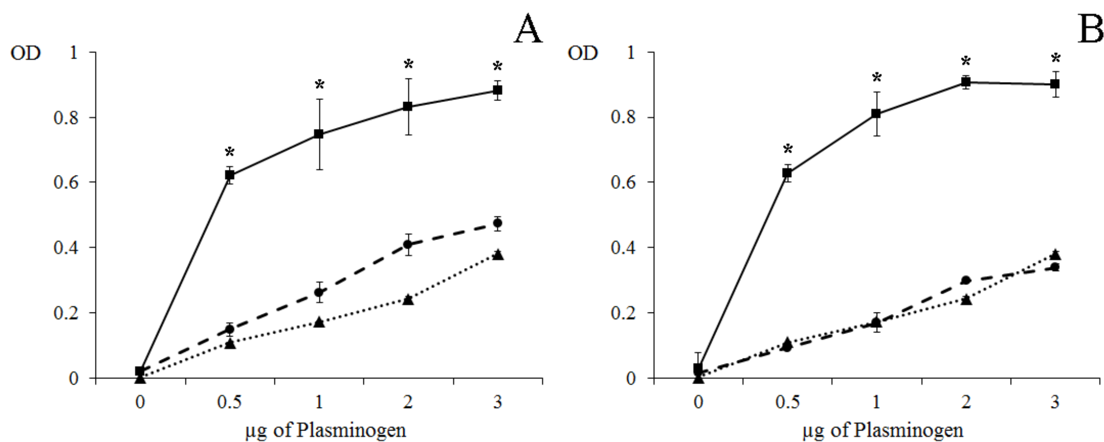

Fig. 4. PLG binding assay of rDiACT and rDiFBAL. PLG binding to 0.5 μg of rDiACT (A) or rDiFBAL (B) analyzed over a range of PLG amounts using a microtiter plate method. (■) Incubation with increasing amounts of PLG, 0–3 μg. (▲) Competition assay with 50 mM εACA included during PLG incubation. (●) Wells coated with BSA used as negative control. Each point is the mean ± SD from three independent experiments. The asterisk (*) designates significant ($p<0.05$) differences.

3.3. rDiACT and rDiFBAL enhance the activation of PLG by tPA

In order to determine the ability of rDiACT and rDiFBAL to activate PLG and generate plasmin on their own, the amidolytic activity of plasmin generated in the

presence or absence of tPA was measured. Negative controls replacing each recombinant protein for BSA or tPA were also used. Fig 5 shows the capacity of rDiGAPDH and rDiGAL to stimulate plasmin generation by tPA obtaining ODs significantly higher than the negative controls ($p<0.05$). Both proteins obtained similar results and PLG activation did not occur in the absence of tPA. In addition, this effect is inhibited by 50 mM εACA, indicating the involvement of lysine residues in the process (Fig 5).

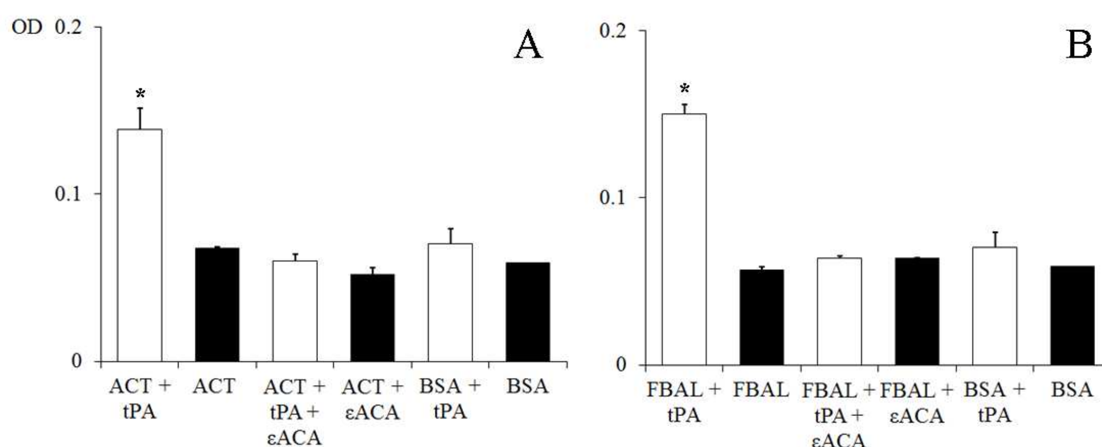

Fig. 5. PLG activation assay of rDiACT and rDiFBAL. PLG activation and plasmin generation by rDiACT (A) or rDiFBAL (B). (□) 15 ng of tPA was added to mixtures which contained 2 µg of human PLG, 3 µg of D-Val-Leu-Lys 4-nitroanilide dihydrochloride (Sigma) and 1 µg of each recombinant protein (or BSA as negative control) in the presence or absence of 50 mM of εACA in a test volume of 100 µl. (■) Reaction mixtures in absence of tPA. Each point is the mean ± SD from three independent experiments. The asterisk (*) designates significant ($p<0.05$) differences.

3.4. Effect of rDiACT and rDiFBAL on the fibrinolytic system activators (tPA and uPA) expression in CnAOEC and CnAOSMC

To complete the study of the effect of rDiACT and rDiFBAL on the fibrinolytic system activation, proteins from rDiACT or rDiFBAL-treated or untreated CnAOEC and CnAOSMC extracts were separated by SDS-PAGE and analyzed by Western blotting using anti-tPA and anti-uPA antibodies. rDiACT and rDiFBAL induced a marked increase in the expression of the main fibrinolytic activators tPA and uPA in cultured endothelial cells ($p<0.05$) (Fig 6A and 6C). This increase was greater in the case of the uPA expression and significantly higher in cells stimulated with rDiFBAL (Fig 6C). Significant differences in the expression of tPA and uPA in CnAOSMC between rDiACT/rDiFBAL-treated or untreated cultures were not found (Fig 6B and 6D).

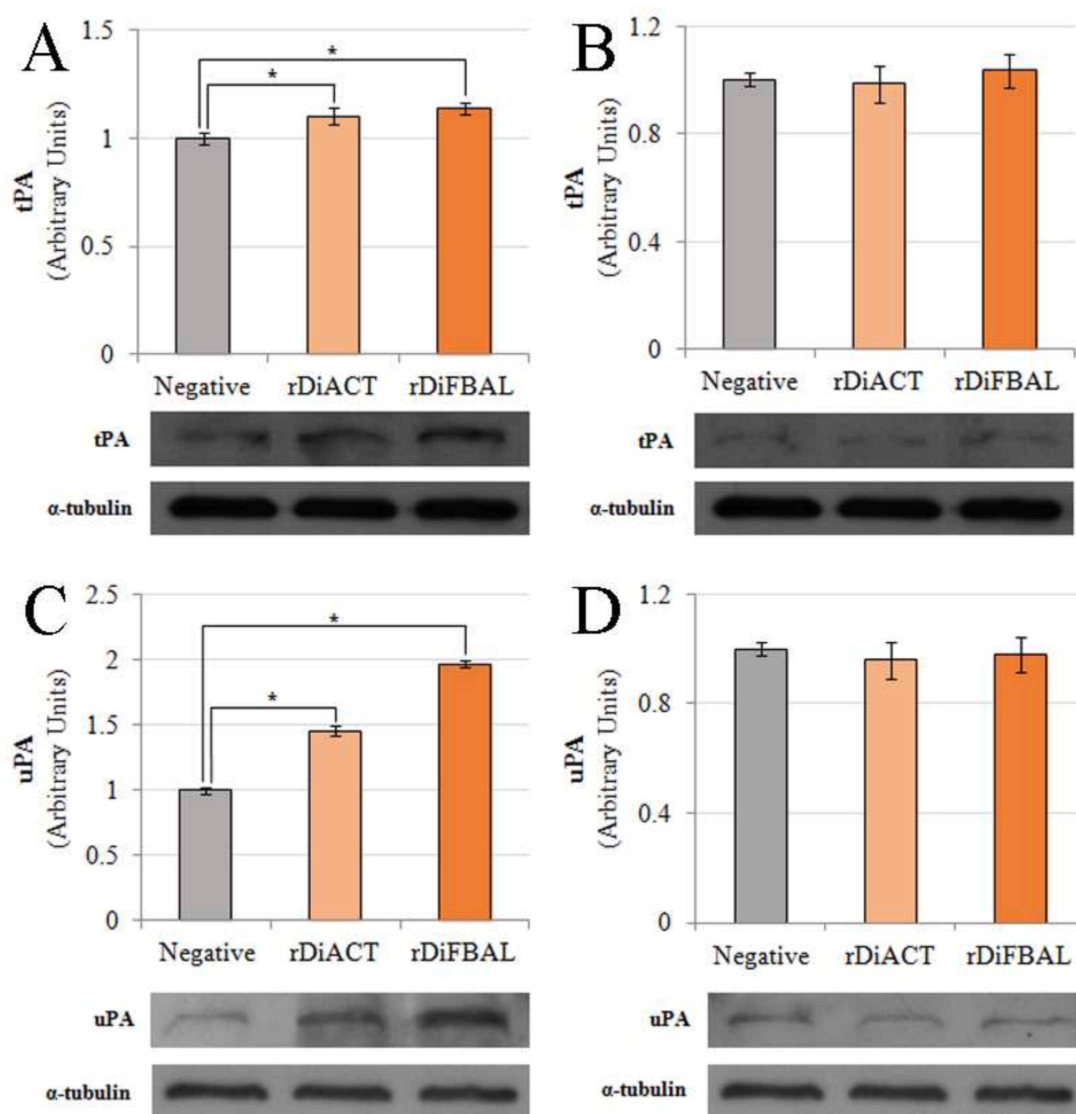

Fig. 6. Effect of rDiACT and rDiFBAL on the fibrinolytic system activators expression. Effect of rDiACT and rDiFBAL on the expression of tPA (A and B) and uPA (C and D) in canine vascular endothelial (A and C) and smooth muscle cells (B and D). Protein extracts from lysed rDiACT or rDiFBAL treated or untreated confluent cell cultures were analyzed by Western blot for tPA and uPA. α-tubulin served as a protein control. Data are shown as representative images or means ± SD from three independent experiments. The asterisk (*) designates significant ($p<0.05$) differences from control cells. (■) Stimulated cells with 1µg/ml of rDiACT. (■) Stimulated cells with 1µg/ml of rDiFBAL. (■) Non-treated control cells.

3.5. Immunolocalization of DiACT and DiFBAL in sections from *D. immitis* adult worms

Immunolocalization of proteins was carried out by the use of a commercially available high-affinity ligand (in the case of ACT) and a rabbit polyclonal antisera (in the case of FBAL). The reactivity and specificity of this antiserum was tested in ELISA and

Western blot prior to their use in the immunolocalization studies. The antibody titers of this antiserum were higher than 1/500, with an OD of 1.15 while the negative serum showed an OD of 0.13 at the same dilution. The anti-rDiFBAL antiserum reacted strongly and specifically against the recombinant protein, while the negative serum showed no reactivity in the Western blot analysis (not shown).

The anatomical localization of DiACT and DiFBAL was carried out in histological sections of *D. immitis* adult worms by immunofluorescence. As shown in Fig 7, all sections showed green fluorescence throughout the soma of the parasite, as a result of the binding of phalloidin-Alexa Fluor 488, ACT high-affinity ligand which serves also as a positive control of the technique. Sections incubated with the anti-rDiFBAL antiserum showed, in addition, specific reactivity (in red) against the parasitic FBAL due to the binding of the anti-rabbit IgG antibody conjugated to Alexa Fluor 594. Both proteins are located scattered throughout all the soma, being especially abundant in the cuticle (reflected by an orange color in the overlay of Phalloidin-Alexa Fluor 488 + Alexa Fluor 594 images). Sections incubated with a rabbit negative serum showed no specific red fluorescence from recombinant proteins.

3.6. rDiACT, but not rDiFBAL, produces proliferation of CnAOEC and CnAOSMC via PLG/plasmin system

The effect of rDiACT or rDiFBAL and PLG on the proliferation of endothelial and smooth muscle cells was quantified using the crystal violet technique in a period of 10 days (Fig 8). Both cultures showed typical curves of cell growth in all experimental groups with a progressive growth between days 0 and 6 or 8 post-treatment in the CnAOEC or CnAOSMC cultures, experiencing cell death and an evident decrease of viable cells from there until the end of the experiment (day 10 post-treatment). Crystal violet staining showed an increase significantly greater in the number of viable cells in cultures stimulated with rDiACT + PLG than that showed by other experimental groups on days 4 and 6 post-treatment (CnAOEC) or day 8 post-treatment (CnAOSMC) ($p<0.05$), indicating that this treatment stimulates the proliferation of CnAOEC and CnAOSMC in culture. Significant differences in cell proliferation between cells stimulated with rDiFBAL + PLG and other experimental groups were not found in both types of cultures.

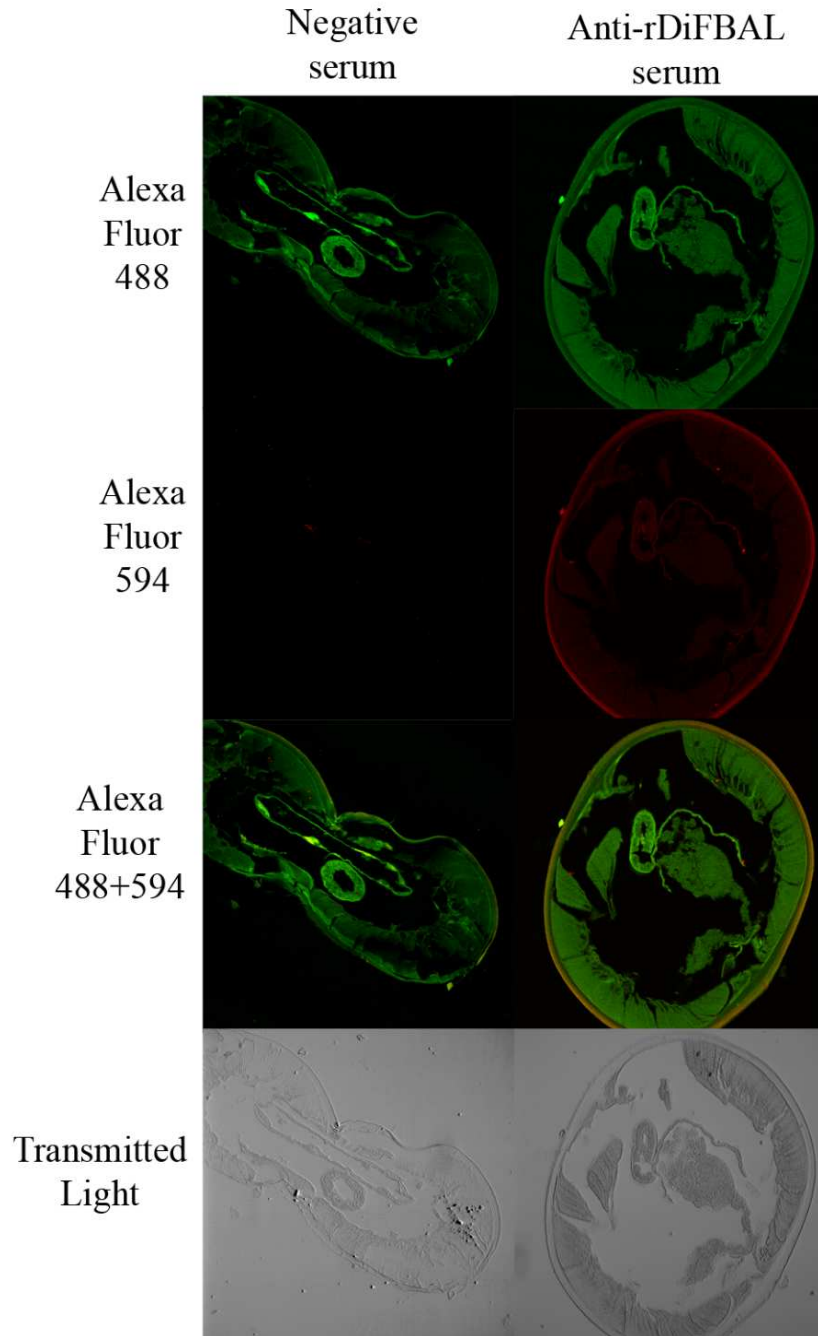

Fig. 7. Immunolocalization of DiACT and DiFBAL in sections from *D. immitis* adult worms. Representative images from three independent experiments of parasite sections incubated with phalloidin-Alexa Fluor 488 (in green, specific binding to ACT) plus the negative or the anti-rDiFBAL rabbit sera and an anti-rabbit IgG-Alexa Fluor 594 (in red). Corresponding transmitted light images are also addressed. Magnification 4X.

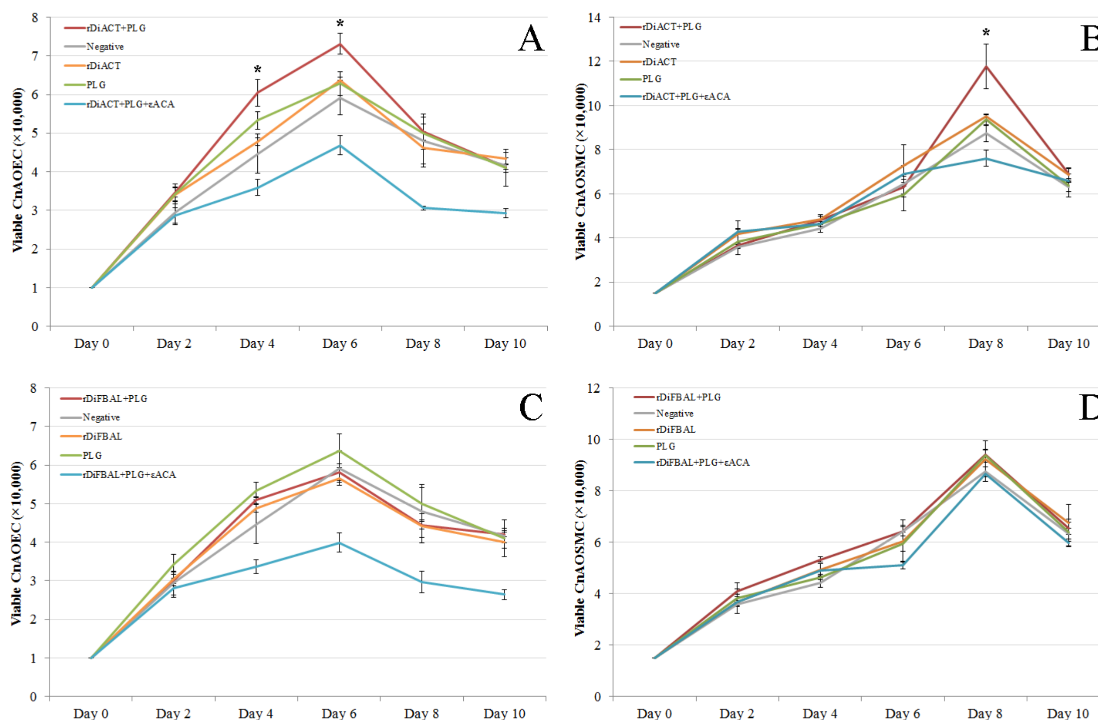

Fig. 8. Cell proliferation assay performed by the crystal violet technique measuring cell viability over a 10 days period. The experiment was carried out in canine endothelial (A and C) and smooth muscle cells (B and D) untreated (▬) or treated with 1 µg/ml of rDiACT or rDiFBAL + 10 µg/ml of PLG (▬), 1 µg/ml of rDiACT or rDiFBAL (▬), 10 µg/ml of PLG (▬), or with 1 µg/ml of rDiACT or rDiFBAL + 10 µg/ml of PLG + 50 mM of the εACA (▬). Results were expressed as number of viable cells (x 10,000). Each point is the mean ± SD from three independent experiments. The asterisk (*) designates significant ($p<0.05$) differences between rDiACT + PLG treatment and control groups.

3.7. rDiACT and rDiFBAL produce migration of CnAOEC and CnAOSMC via PLG/plasmin system

A Wound Healing assay was performed to assess migration of endothelial (Fig 9) and smooth muscle cells (Fig 10). The quantification was carried out by measuring the distance of migration in comparison with negative control (untreated cells) to 8 hours post-treatment. In both CnAOEC and CnAOSMC cultures a significant increase of cell migration after stimulation with rDiACT or rDiFBAL + PLG with respect to the other experimental groups ($p<0.05$) occurred, being this increase most pronounced in cultured smooth muscle cells. After comparing the effect of both parasitic proteins, rDiACT showed higher values of migration ability in both types of cell cultures with respect to those obtained by rDiFBAL.

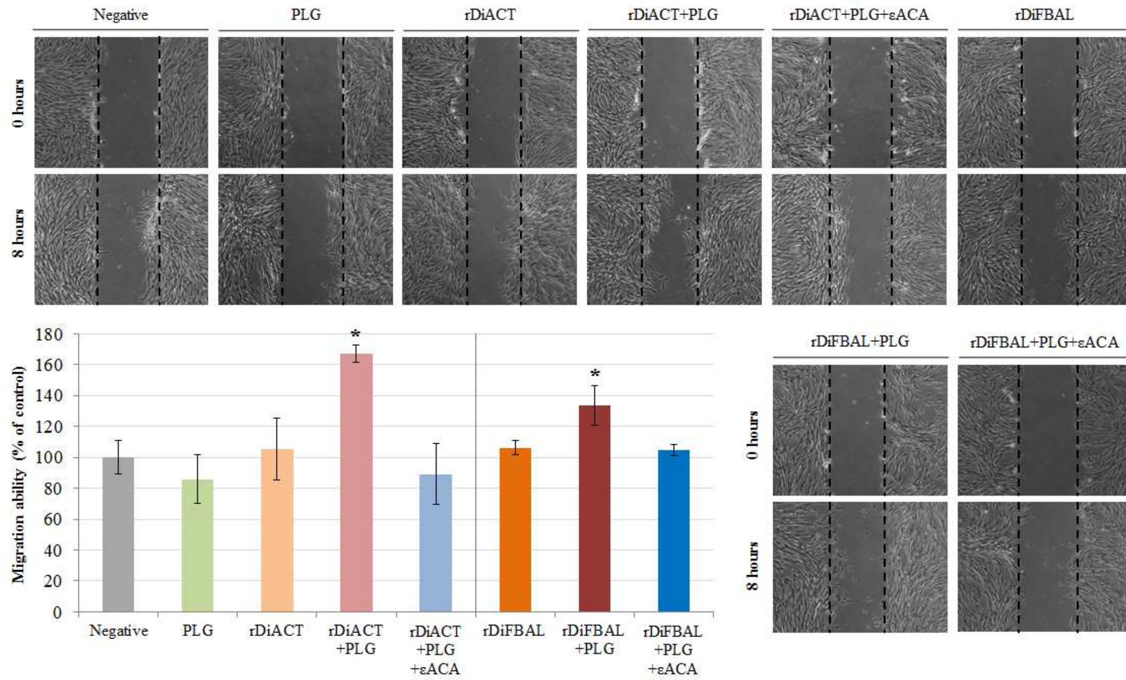

Fig. 9. CnAOEC migration by Wound-Healing assay. Confluent cell cultures were wounded post-treatment and migration distances were measured at 8 hours. The experiment was carried out in canine endothelial cells untreated (▬) or treated with 10 µg/ml of PLG (▬), 1 µg/ml of rDiACT/rDiFBAL (▬/▬), 1 µg/ml of rDiACT/rDiFBAL + 10 µg/ml of PLG (▬/▬) or with 1 µg/ml of rDiACT/rDiFBAL + 10 µg/ml of PLG + 50 mM of the εACA (▬/▬). The results were expressed as percentage of the migration ability of the negative control cells (100%). Data are shown as representative images or means ± SD from three independent experiments. The asterisk (*) designates significant ($p<0.05$) differences between rDiACT or rDiFBAL + PLG treatment and control groups.

3.8. rDiACT and rDiFBAL produce ECM degradation of CnAOEC and CnAOSMC via PLG/plasmin system

To examine ECM degradation, Type I Collagen in the culture supernatant of treated and untreated CnAOEC and CnAOSMC were measured by ELISA (Fig 11). A lower concentration of Type I Collagen and therefore a further degradation of the secreted collagen by the cells was observed in the culture media of CnAOEC and CnAOSMC stimulated with rDiACT/rDiFBAL + PLG than that obtained by the control cells ($p<0.05$). In addition, the same culture media from treated and untreated cells were analyzed with gelatin zymography for MMP-2 and MMP-9 levels (Fig 12). Density of the bands was measured by the Quantity One Software (Bio-Rad). Treatment with rDiACT or rDiFBAL + PLG shows a significantly higher MMP-2 level in the CnAOEC and CnAOSMC culture media and MMP-9 level in the CnAOEC culture media than that obtained by the other treatments ($p<0.05$). In addition, treatment with rDiFBAL + PLG shows an activation of

the latent form of the MMP-9 in the CnAOSMC culture media (show by a clear band of 82 kDa), which does not appear with other treatments. No significant differences in the MMP-9 levels in the culture media of CnAOSMC were observed (Fig 12).

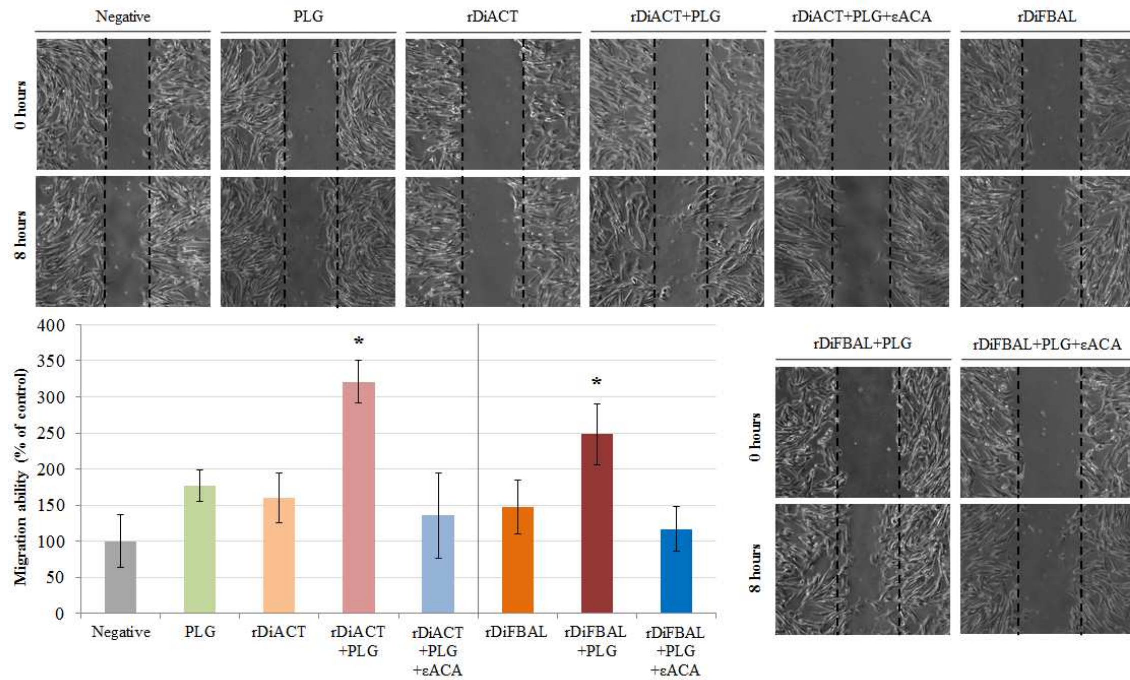

Fig. 10. CnAOSMC migration by Wound-Healing assay. Confluent cell cultures were wounded post-treatment and migration distances were measured at 8 hours. The experiment was carried out in canine smooth muscle cells untreated (▬) or treated with 10 µg/ml of PLG (▬), 1 µg/ml of rDiACT/rDiFBAL (▬/▬), 1 µg/ml of rDiACT/rDiFBAL + 10 µg/ml of PLG (▬/▬) or with 1 µg/ml of rDiACT/rDiFBAL + 10 µg/ml of PLG + 50 mM of the εACA (▬/▬). The results were expressed as percentage of the migration ability of the negative control cells (100%). Data are shown as representative images or means ± SD from three independent experiments. The asterisk (*) designates significant ($p<0.05$) differences between rDiACT or rDiFBAL + PLG treatment and control groups.

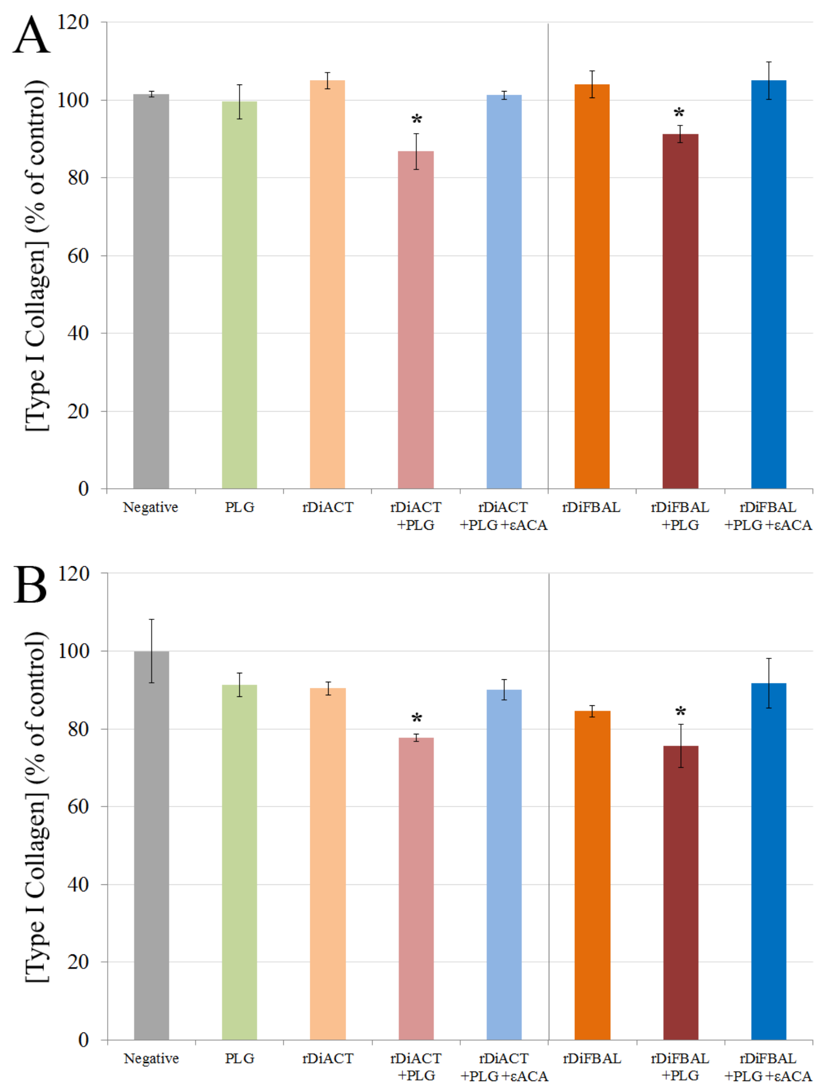

Fig. 11. Type I Collagen degradation assay. Collagen degradation measured in culture supernatants from canine endothelial (A) and smooth muscle cells (B) untreated (▬) or treated with 10 µg/ml of PLG (▬), 1 µg/ml of rDiACT/rDiFBAL (▬/▬), 1 µg/ml of rDiACT/rDiFBAL + 10 µg/ml of PLG (▬/▬) or with 1 µg/ml of rDiACT/rDiFBAL + 10 µg/ml of PLG + 50 mM of the εACA (▬/▬). The results were expressed as percentage of the Type I Collagen concentration in the culture supernatant from negative control cells (100%). Each point is the mean ± SD from three independent experiments. The asterisk (*) designates significant ($p<0.05$) differences between rDiACT or rDiFBAL + PLG treatment and control groups.

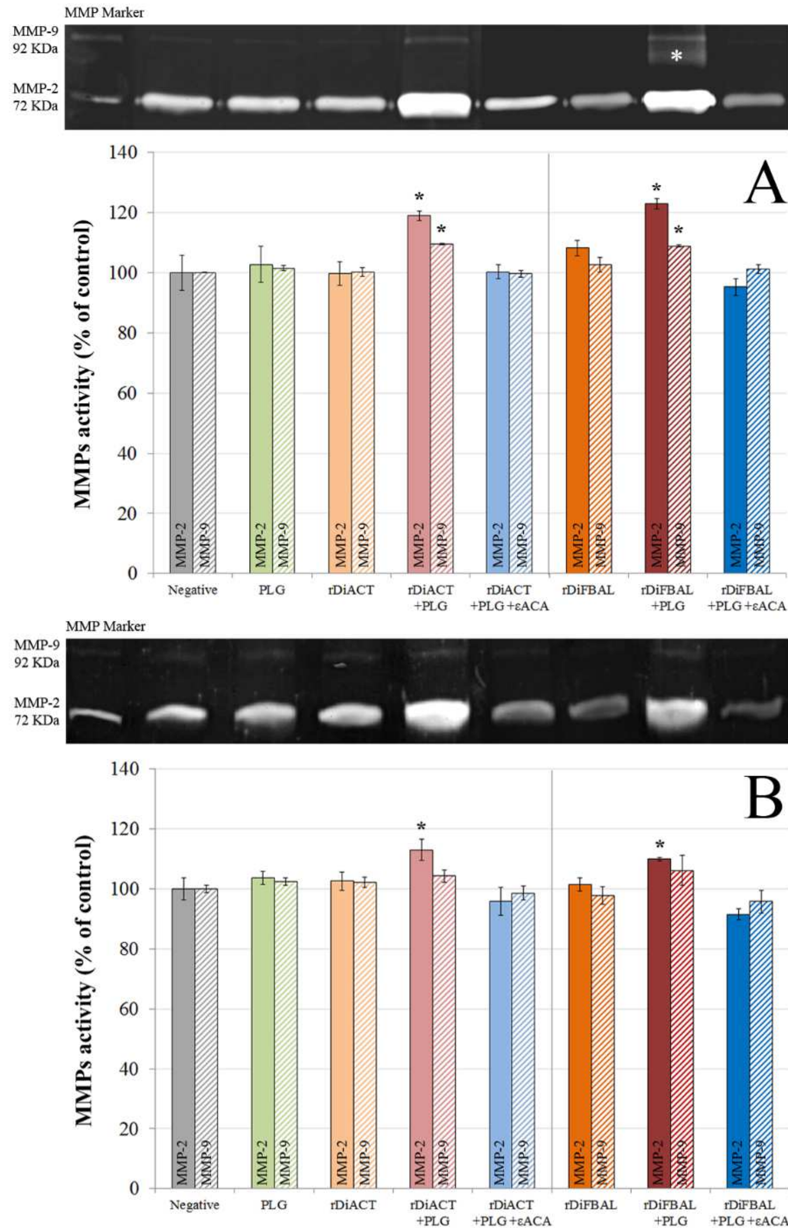

Fig. 12. MMP-2 and 9 levels assay. Representative zymography of MMP-2 (solid bars) and MMP-9 (hatched bars) levels in the culture supernatants from canine endothelial (A) and smooth muscle cells (B) untreated (▬) or treated with 10 µg/ml of PLG (▬), 1 µg/ml of rDiACT/rDiFBAL (▬/▬), 1 µg/ml of rDiACT/rDiFBAL + 10 µg/ml of PLG (▬/▬) or with 1 µg/ml of rDiACT/rDiFBAL + 10 µg/ml of PLG + 50 mM of the εACA (▬/▬). Note the gelatinolytic bands associated with MMP-2 (72 KDa) and MMP-9 (92 KDa) levels, as well as with the MMP-9 activated form (marked with a white asterisk at 82KDa. The results were expressed as percentage of the MMPs levels in the culture supernatant from negative control cells (100%). Data are shown as representative images or means ± SD from three independent experiments. The asterisk (*) designates significant ($p<0.05$) differences between rDiACT or rDiFBAL treatment and control groups.

4. Discussion

The main finding of this study lies in relating how the activation of the fibrinolytic system by two proteins of the blood-borne parasite *D. immitis* (a priori beneficial for both the parasite and host), may cause long-term pathological effects based on the participation of generated plasmin in the emergence of a process of proliferative endarteritis. This study was conducted with the ACT and FBAL of *D. immitis*, highly conserved proteins that were selected among parasite antigens that had been identified as PLG-binding proteins in previous works [17,18]. Both proteins have previously been linked to pro-fibrinolytic activities. The interaction between actin and PLG is well known, as well as the fact that specific binding occurs through lysine residues, which stimulate the tPA-dependent plasmin generation [29]. In addition, its function as PLG receptor has been demonstrated on the surface of endothelial cells [30] and in the tegument of *S. bovis* [31]. Meanwhile, FBAL has been identified as PLG-binding protein in the bacterium *Mycobacterium tuberculosis* [32], the fungal pathogens *Candida albicans* [33] and *Cryptococcus neoformans* [34] and in the helminth parasite *S. bovis* [31].

In this paper, two peptide sequences of 376 and 363 amino acids were respectively obtained by cloning and sequencing of the *D. immitis* ACT and FBAL cDNAs. The subsequent bioinformatic analyses based on the multiple sequence alignments carried out with homologous proteins from other helminth parasites and the homology modelling of their 3D structures have highlighted their high degree of conservation. None of the proteins showed structural motifs for their transport or expression on the cell surface (signal peptide, transmembrane motifs or GPI anchors), despite the fact that both proteins have been previously identified in the secretome and on the surface of *D. immitis* [18,35]. This may be related to unconventional mechanisms of protein transport, as for example with the association of these proteins with exosome-like secretion vesicles. This fact has been recently postulated as an extracellular transport mechanism for glycolytic enzymes from several groups of parasites [36].

In order to assess the interaction of these proteins with the host fibrinolytic system we study their abilities to bind PLG, enhance plasmin generation, stimulate the production of the fibrinolytic activators tPA and uPA, as well as their locations in the adult parasite. Both proteins rDiACT and rDiFBAL showed ability to bind PLG and stimulate plasmin generation by tPA, which are capabilities mediated by the participation of lysine residues,

as it has been demonstrated by competition assays carried out with εACA. Interaction with PLG has been historically associated with the presence of carboxyl-terminal lysine residues in their receptors [37]. However, conserved internal lysine residues have been more recently described as PLG-binding domains as in the case of enolase of *Streptococcus pneumoniae* [38] or human beta-actin, in which a PLG-binding domain within amino acids 55 to 69 (GDEAQSKRGILTLKY) has been identified indicating that Lys^{61} and Lys^{68} are essential for this action [39]. This domain has been conserved in the ACT of *D. immitis* (see Fig 1). In addition, after viewing the spatial location of the conserved lysine residues of the DiACT and DiFBAL in their 3D models, these residues seem to be located externally in these molecules, which would facilitate the accessibility of PLG.

Despite the fact that the generation of plasmin by rDiACT and rDiFBAL is dependent on tPA availability, we demonstrate that both proteins produce a significant stimulation of the basal production not only of this fibrinolytic activator, but also of uPA in canine endothelial cells in culture. This result reinforces the pro-fibrinolytic condition of these proteins, since the participation of tPA and uPA in fibrinolysis is essential in the effective activation of PLG [40]. On the other hand, high levels in the expression of both tPA and uPA have been related to several physiological and pathological processes like tissue remodeling and chronic inflammatory diseases, such as atherosclerosis and arthritis [41,42]. Finally, immunolocalization studies showed that DiACT and DiFBAL, as well as having an intracellular location, they are particularly abundant in the cuticle of *D. immitis*. This fact is essential, so that the interaction of these proteins with the fibrinolytic system may have relevance in vivo, it is necessary that DiACT and DiFBAL are expressed in tissues in direct contact with the blood of the host [43].

Secondly, in order to study the effect of plasmin resulting from the fibrinolytic activation by DiACT and DiFBAL on the proliferative endarteritis in the canine arterial wall, we have developed an “in vitro” model of canine endothelial and smooth muscle cells. Our data demonstrate that stimulation with rDiACT + PLG causes the proliferation of CnAOEC and CnAOSMC, and treatments with rDiACT or rDiFBAL + PLG enhance migration of both types of cells. This would be consistent with the formation of intravascular microvilli occurring during dirofilariosis, which is result of the multiplication and migration of the arterial wall cells [44]. In addition, the binding of both

proteins to PLG causes a significant increase in the degradation of collagen type I and in the levels of MMP-2 in the culture media of CnAOEC and CnAOSMC, as well as in the levels of MMP-9 in the culture media of CnAOEC. Moreover, the binding of rDiFBAL and PLG seems to induce an activation of the latent form of the MMP-9 in the culture media of CnAOSMC. These facts highlight the participation of the rDiACT/rDiFBAL + PLG interaction in the degradation of the ECM needed for the formation of intravascular microvilli. Type I Collagen represents the main component of the ECM of elastic arteries. Its alteration has been associated with vascular disease and its degradation products with the proliferation and migration of smooth muscle cells in remodeling arteries [45]. These results are consistent with those observed in vivo by Wang et al. (2005) who reported a significantly lower amount of collagen in heartworm-infected dogs than in clinically normal dogs [15]. On the other hand, MMPs function in the extracellular environment of cells and degrade ECM molecules from the tissue. Among them, Gelatinases (MMP-2 and MMP-9) can digest a large number of the ECM molecules including type IV, V and XI collagens, laminin, aggrecan core protein, etc. MMP-2, but not MMP-9, also digests collagens I, II and III [46]. In addition, the pathophysiological study of the action of gelatinases shows that an increase in its activity can be responsible for tissue remodeling, hypertrophy, angiogenesis and chronic inflammation [47]. Finally, inhibition of all positive results by including the 50 mM εACA in the stimulations demonstrates the final participation of plasmin generated by binding between DiACT or DiFBAL and PLG on the proliferation and migration of CnAOEC and CnAOSMC, as well as the degradation of the ECM.

These results seem to indicate that *D. immitis* could use DiACT and DiFBAL to shift the fibrinolytic balance towards the generation of plasmin, which might constitute a survival mechanism to avoid the clot formation in its intravascular habitat. On the other hand, in long-term infections as cardiopulmonary dirofilariasis, this overproduction of plasmin could be related to pathological phenomena described in the emergence of proliferative endarteritis. These findings contribute to understand a very complex part of the host-pathogen relationships of dirofilariasis, showing how a process related to the survival of the parasite and the host can lead to a pathogenic mechanism of great importance. Since the ability to bind PLG and enhance plasmin generation of proteins of many pathogens has been shown, and that ACT and FBAL are highly conserved pathogenic antigens, similar events could occur in other infections caused by vascular

pathogens developing chronic processes. The knowledge of these mechanisms could be critical for the treatment and prevention of diseases caused by infectious agents.

Author Contributions

Conceived and designed the experiments: JGM, FS. Performed the experiments: JGM, RM, MSL. Analyzed the data: JGM, FS. Contributed reagents/materials/analysis tools: JGM, RM, MSL. Wrote the paper: JGM, FS.

References

1. Mebius MM, van Genderen PJ, Urbanus RT, Tielens AG, de Groot PG, van Hellemond JJ. Interference with the host haemostatic system by schistosomes. PLoS Pathog. 2013;9: e1003781.
2. Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. Br J Haematol. 2005;129: 307-321.
3. Law RH, Abu-Ssaydeh D, Whisstock JC. New insights into the structure and function of the plasminogen/plasmin system. Curr Opin Struct Biol. 2013;23: 836-841.
4. Yang Z, Eton D, Zheng F, Livingstone AS, Yu H. Effect of tissue plasminogen activator on vascular smooth muscle cells. J Vasc Surg. 2005;42: 532-538.
5. Nicholl SM, Roztocil E, Davies MG. Plasminogen activator system and vascular disease. Curr Vasc Pharmacol. 2006;4: 101-116.
6. Hayashi M, Matsuzaki Y, Shimonaka M. Impact of plasminogen on an in vitro wound healing model based on a perfusion cell culture system. Mol Cell Biochem. 2009;322: 1-13.
7. Syrovets T, Simmet T. Novel aspects and new roles for the serine protease plasmin. Cell Mol Life Sci. 2004;61: 873-885.
8. Bhattacharya S, Ploplis VA, Castellino FJ. Bacterial plasminogen receptors utilize host plasminogen system for effective invasion and dissemination. J Biomed Biotechnol. 2012;2012: 482096.
9. Figuera L, Gómez-Arreaza A, Avilán L. Parasitism in optima forma: Exploiting the host fibrinolytic system for invasion. Acta Trop. 2013;128: 116-123.
10. Simón F, Siles-Lucas M, Morchón R, González-Miguel J, Mellado I, Carretón E, et al. Human and animal dirofilariasis: the emergence of a zoonotic mosaic. Clin Microbiol Rev. 2012;25: 507-544.

11. Adcock JL. Pulmonary arterial lesions in canine dirofilariasis. Am J Vet Res. 1961;22: 655-662.
12. Atwell RB, Sutton RH, Buoro IB. Early pulmonary lesions caused by dead *Dirofilaria immitis* in dogs exposed to homologous antigens. Br J Exp Pathol. 1986;67: 395-405.
13. Hidaka Y, Hagio M, Horii Y, Murakami T, Naganobu K, Miyamoto T. Histopathological and Enzyme Histochemical Observations on Mast Cells in Pulmonary Arterial Lesion of Dogs with *Dirofilaria immitis* Infestation. J Vet Med Sci. 2004;66: 1457-1462.
14. Kawabata A, Nakagaki K, Yoshida M, Shirota K. Histopathological comparison of pulmonary artery lesions between raccoon dogs (*Nyctereutes procyonoides*) and domestic dogs experimentally infected with *Dirofilaria immitis*. J Vet Med Sci. 2008;70: 301-303.
15. Wang JS, Tung KC, Huang CC, Lai CH. Alteration of extracellular collagen matrix in the myocardium of canines infected with *Dirofilaria immitis*. Vet Parasitol. 2005;131: 261-265.
16. McCall JW, Genchi C, Kramer LH, Guerrero J, Venco L. Heartworm disease in animals and humans. Adv Parasitol. 2008;66: 193-285.
17. González-Miguel J, Morchón R, Mellado I, Carretón E, Montoya-Alonso JA, Simón F. Excretory/secretory antigens from *Dirofilaria immitis* adult worms interact with the host fibrinolytic system involving the vascular endothelium. Mol Biochem Parasitol. 2012;181: 134-140.
18. González-Miguel J, Morchón R, Carretón E, Montoya-Alonso JA, Simón F. Surface associated antigens of *Dirofilaria immitis* adult worms activate the host fibrinolytic system. Vet Parasitol. 2013;196: 235-240.
19. de la Torre-Escudero E, Manzano-Román R, Pérez-Sánchez R, Siles-Lucas M, Oleaga A. Cloning and characterization of a plasminogen-binding surface-associated enolase from *Schistosoma bovis*. Vet Parasitol. 2010;173: 76-84.
20. Bendtsen JD, Nielsen H, von Heijne G, Brunak S. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol. 2004;340: 783-795.
21. Eisenhaber B, Bork P, Yuan Y, Loeffler G, Eisenhaber F. Automated annotation of GPI anchor sites: case study *C. elegans*. TIBS. 2000;25: 340-341.

22. Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics. 2006;22: 195-201.
23. Morchón R, González-Miguel J, Mellado I, Velasco S, Rodríguez-Barbero A, Simón F. Adult *Dirofilaria immitis* excretory/secretory antigens upregulate the production of prostaglandin E2 and downregulate monocyte transmigration in an “in vitro” model of vascular endothelial cell cultures. Vet Parasitol. 2010;170: 331-335.
24. Morchón R, Rodríguez-Barbero A, Velasco S, López-Belmonte J, Simón F. Vascular endothelial cell activation by adult *Dirofilaria immitis* antigens. Parasitol Int. 2008;57: 441-446.
25. Yamaguchi M, Ebihara N, Shima N, Kimoto M, Funaki T, Yokoo S, et al. Adhesion, migration, and proliferation of cultured human corneal endothelial cells by laminin-5. Invest Ophthalmol Vis Sci. 2011;52: 679-684.
26. Vandooren J, Geurts N, Martens E, Van den Steen PE, Opdenakker G. Zymography methods for visualizing hydrolytic enzymes. Nat Methods. 2013;10: 211-220.
27. Marangoni NR, Melo GD, Moraes OC, Souza MS, Perri SH, Machado GF. Levels of matrix metalloproteinase-2 and metalloproteinase-9 in the cerebrospinal fluid of dogs with visceral leishmaniasis. Parasite Immunol. 2011;33: 330-334.
28. Prato M, D'Alessandro S, Van den Steen PE, Opdenakker G, Arese P, Taramelli D, et al. Natural haemozoin modulates matrix metalloproteinases and induces morphological changes in human microvascular endothelium. Cell Microbiol. 2011;13: 1275-1285.
29. Lind SE, Smith CJ. Actin accelerates plasmin generation by tissue plasminogen activator. J Biol Chem. 1991;266: 17673-17678.
30. Dudani AK, Ben-Tchavtchavadze M, Porter S, Tackaberry E. Angiostatin and plasminogen share binding to endothelial cell surface actin. Biochem Cell Biol. 2005;83: 28-35.
31. Ramajo-Hernández A, Pérez-Sánchez R, Ramajo-Martín V, Oleaga A. *Schistosoma bovis*: plasminogen binding in adults and the identification of plasminogen-binding proteins from the worm tegument. Exp Parasitol. 2007;115: 83-91.
32. de la Paz Santangelo M, Gest PM, Guerin ME, Coinçon M, Pham H, Ryan G, et al. Glycolytic and non-glycolytic functions of *Mycobacterium tuberculosis* fructose-1,6-

bisphosphate aldolase, an essential enzyme produced by replicating and non-replicating bacilli. J Biol Chem. 2011;286: 40219-40231.

33. Crowe JD, Sievwright IK, Auld GC, Moore NR, Gow NA, Booth NA. *Candida albicans* binds human plasminogen: identification of eight plasminogen-binding proteins. Mol Microbiol. 2003;47: 1637-1651.
34. Stie J, Bruni G, Fox D. Surface-associated plasminogen binding of *Cryptococcus neoformans* promotes extracellular matrix invasion. PLoS One. 2009;4: e5780.
35. Geary J, Satti M, Moreno Y, Madrill N, Whitten D, Headley SA, et al. First analysis of the secretome of the canine heartworm, *Dirofilaria immitis*. Parasit Vectors. 2012;5: 140.
36. Gómez-Arreaza A, Acosta H, Quiñones W, Concepción JL, Michels PA, Avilán L. Extracellular functions of glycolytic enzymes of parasites: Unpredicted use of ancient proteins. Mol Biochem Parasitol. 2014;193: 75-81.
37. Plow EF, Herren T, Redlitz A, Miles LA, Hoover-Plow JL. The cell biology of the plasminogen system. FASEB J. 1995;9: 939-945.
38. Ehinger S, Schubert WD, Bergmann S, Hammerschmidt S, Heinz DW. Plasmin(ogen)-binding alpha-enolase from *Streptococcus pneumoniae*: crystal structure and evaluation of plasmin(ogen)-binding sites. J Mol Biol. 2004;343: 997-1005.
39. Wang H, Doll JA, Jiang K, Cundiff DL, Czarnecki JS, Wilson M, et al. Differential binding of plasminogen, plasmin, and angiostatin4.5 to cell surface beta-actin: implications for cancer-mediated angiogenesis. Cancer Res. 2006;66: 7211-7215.
40. Urano T, Suzuki Y. Accelerated fibrinolysis and its propagation on vascular endothelial cells by secreted and retained tPA. J Biomed Biotechnol. 2012;2012: 208108.
41. Gebbink MF. Tissue-type plasminogen activator-mediated plasminogen activation and contact activation, implications in and beyond haemostasis. J Thromb Haemost. 2011;9: 174-181.
42. Fuhrman B. The urokinase system in the pathogenesis of atherosclerosis. Atherosclerosis. 2012;222: 8-14.
43. Hawley SB, Green MA, Miles LA. Discriminating between cell surface and intracellular plasminogen-binding proteins: heterogeneity in profibrinolytic

plasminogen-binding proteins on monocytoid cells. Thromb Haemost. 2000;84: 882-890.

44. Venco L. Heartworm (*Dirofilaria immitis*) disease in dogs. In: Genchi C, Ronaldi L, Cringoli G, editors. *Dirofilaria immitis* and *D. repens* in dog and cat and human infections. Italy: Rolando Editore; 2007. pp. 117-125.
45. Bou-Gharios G, Ponticos M, Rajkumar V, Abraham D. Extra-cellular matrix in vascular networks. Cell Prolif. 2004;37: 207-220.
46. Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res. 2006;69: 562-573.
47. Klein T, Bischoff R. Physiology and pathophysiology of matrix metalloproteases. Amino Acids. 2011;41: 271-290.

CONCLVSiONES

1. *Dirofilaria immitis* activa el sistema fibrinolítico de su hospedador mediante la acción conjunta de sus antígenos excretores/secretores y de superficie. Esto podría ser utilizado por el parásito para desplazar el equilibrio fibrinolítico hacia la generación de plasmina, lo que supondría un mecanismo de supervivencia para el parásito al controlar la formación de coágulos en su hábitat intravascular inmediato.

2. Se identifican 10 y 11 proteínas fijadoras de plasminógeno, respectivamente, en los compartimentos antigénicos excretor/secretor y de superficie del parásito. De ellas, se han producido en su forma recombinante la actina, fructosa-bifosfato aldolasa, gliceraldehído-3-fosfato deshidrogenasa y galectina de *D. immitis*, demostrando individualmente sus propiedades profibrinolíticas. Todas ellas, en mayor o menor medida, son capaces de fijar plasminógeno y potenciar la generación de plasmina a través de la implicación de sus residuos de lisina. Además, son capaces de estimular la expresión de activadores fibrinolíticos en cultivos de células endoteliales caninas y se localizan en la interfase *D. immitis*/hospedador.

3. La plasmina, producto de la activación fibrinolítica causada por los antígenos de *D. immitis*, participa en la generación de los procesos patológicos descritos en la aparición de la endarteritis proliferativa en la dirofilariosis cardiopulmonar. Esto incluye la proliferación y migración de las células de la pared arterial, así como la degradación de la matriz extracelular, demostrándose estos hechos mediante la utilización tanto de los antígenos excretores/secretores del parásito, como de las dos proteínas con mayor capacidad profibrinolítica entre las estudiadas (actina y fructosa-bifosfato aldolasa) en un modelo *in vitro*.

4. Los resultados obtenidos en la presente Tesis Doctoral contribuyen al conocimiento de una parte muy compleja de las relaciones parásito/hospedador a nivel molecular en la dirofilariosis cardiopulmonar, mostrando por primera vez cómo un proceso relacionado con la supervivencia del parásito puede desencadenar mecanismos patogénicos de gran importancia. Puesto que la capacidad para unir plasminógeno y potenciar la generación de plasmina ha sido demostrada en proteínas de muchos patógenos y debido al alto grado de conservación evolutiva de algunos de los antígenos estudiados, mecanismos similares podrían ocurrir en otras infecciones provocadas por patógenos sanguíneos que desarrollen procesos crónicos.

ABSTRACT

UNIVERSITY OF SALAMANCA

FACULTY OF BIOLOGY

DEPARTMENT OF ANIMAL BIOLOGY, PARASITOLOGY, ECOLOGY, EDAFOLOGY AND AGRICULTURAL CHEMISTRY

DOCTORAL THESIS

The role played by plasmin in the survival of *Dirofilaria inmitis* and in the vascular pathology of the host during cardiopulmonary dirofilariosis

Javier González Miguel

Salamanca, 2015

INTRODUCTION, MAIN THESIS AND KEY GOALS

Dirofilaria immitis is the causing filaroid nematode of canine and feline cardiopulmonary dirofilariosis, a vector-borne transmitted parasitosis with cosmopolitan distribution. *D. immitis* is, furthermore, responsible of human pulmonary dirofilariosis, a clinical entity characterized by the formation of benign pulmonary nodules, which could be wrongly taken for carcinoma during radiology sessions (Simón et al., 2012). The dog acts as definitive host and reservoir of the parasite. In it, adult worms can survive inside the pulmonary arteries and in the right ventricle of the heart for 7 years or more, causing a chronic inflammatory pathology on a vascular level (Venco et al., 2011). One of the key facts about this pathology is the appearance of proliferative endarteritis, which has as a consequence the formation of intravascular microvilli. It has been reported that this process goes hand in hand with the proliferation and migration of cells belonging to the arterial wall towards the interior of the blood vessels, alongside the destruction of the extracellular matrix (Adcook, 1961; Atwell et al., 1986; Hidaka et al., 2004; Wang et al., 2005; Kawabata et al., 2008).

The aforementioned alterations cause the lack of organization of the endothelium and the reduction of vascular lumen in the pulmonary vessels, with the subsequent apparition of hypertension and edema. As a consequence of the damages reported during the late stages of the disease the cardiac function can be affected, leading to the apparition of hypertrophy and cardiac-congestive failure. Besides this chronic development, acute processes involving an immediate life-risk for the infected animals may appear. They emerge when the sudden and simultaneous death of many adult worms occurs, either in a natural way or as a consequence of a filaricide treatment. The massive liberation of antigenic products of parasites and their symbiotic bacteria *Wollbachia* to the circulatory system is responsible of the exacerbation of inflammatory reactions in the vascular endothelium and the formation of thromboembolisms of different entity (Venco, 2007). However, *D. immitis* possesses mechanisms enabling the regulation of these pathological processes, contributing to its survival in the intravascular habitat during several years.

With the aim of maintaining and spreading to the blood vessels, many pathogens not only require adaptations in order to avoid the activity of the host's immune system,

but they must also hinder the coagulation of blood through the interaction with the fibrinolytic system (Mebius et al., 2013). Fibrinolysis is one of the most important anticoagulant mechanisms of the haemostatic system. One of its key elements is plasminogen, a single-chain glycoprotein with a molecular mass of 92 KDa. Plasminogen is produced in the liver, and is present in blood and other extravascular fluids. Plasminogen is a pro-enzyme, which is transformed into plasmin (the serine protease responsible for the degradation of the fibrin present in clots) after its activation. The transformation of plasminogen into plasmin is regulated by its binding to the receptors through its five "kringle" domains with affinity for lysine residues and plasminogen activators [tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA)] (Cesarman-Maus and Hajjar, 2005). The plasminogen receptors are present in the fibrin net and also in diverse types of cells, such as monocytes, macrophages, endothelial and neuronal cells, fibroblasts, platelets and tumor cells (Hawley et al., 2000). These have also been identified on the surface of diverse bacteria, fungi, protozoa and helminths (Bhattacharya et al., 2012; Figuera et al., 2013)

Given that the mechanisms of these alterations are not completely known in cardiopulmonary dirofilariosis, their study is of paramount importance, as its understanding could facilitate the management of these situations by clinical vets, contributing to a considerable improvement in the life quality of the affected animals. Due to the survival capacity of *D. immitis* in its hosts, and also because thromboembolisms appear when the worms die, our first hypothesis was that the parasite controls and modifies the sanguine habitat in order to facilitate its survival through molecules present in its antigenic products, generating a clear anti-thrombotic effect through the use of pro-fibrinolytic products.

On the other hand, the activation of the fibrinolytic system through the participation of the antigenic extracts of *D. immitis* as plasminogen receptors and the subsequent maintenance of the haemostasis, which a priori has beneficial results for both the parasite and the host, could have pathologic consequences. An over-production of the plasminogen/plasmin pathway has been related to cell invasion and to the intra-organic migration of diverse pathogens (Jong et al., 2003; Bernal et al., 2004). Furthermore, cardiovascular research conducted on humans has linked the over-

production of plasmin with the proliferation and migration of vascular cells and the degradation of the extracellular matrix (Nicholl et al., 2005; Yang et al., 2005; Roth et al., 2006; Hayashi et al., 2009). These mechanisms are similar to those observed in the formation of microvilli in cardiopulmonary dirofilariosis, but its molecular aspects have not been hitherto conveniently studied in this parasitic disease. As a result of this, our second hypothesis was to consider the over-activation of the fibrinolytic route by *D. immitis* as directly related to the apparition of such pathologic processes in the vessel wall of the infected animals. In order to demonstrate both hypotheses, we proposed the following objectives of the present doctoral dissertation:

1. Analyze the interaction of the antigens of *D. immitis* with the fibrinolytic system of its host in relation to the survival mechanisms on a vascular level.

2. Study if the activation of the fibrinolytic system by the parasite has an influence on the pathological processes described on the development of proliferative endarteritis in cardiopulmonary dirofilariosis.

RESULTS

1. Collection of excretory/secretory and surface associated extracts of proteins from *D. immitis* adult worms

As a first step, in order to perform the proposed objectives, we start by developing excretory/secretory (DiES) and surface associated (DiSAA) extracts of proteins from *D. immitis* adult worms. DiES and DiSAA extracts were respectively prepared following the methodology described by Morchón et al. (2010) and Wedrychowicz et al. (1994) with minor modifications. Proteins were extracted in saline solution mixed with a cocktail of protease inhibitors and their concentration measured by DC protein assay commercial kit (Bio-Rad).

2. Proteins of DiES and DiSAA extracts bind plasminogen and enhance its activation by tPA

To assess the capability of the antigenic extracts of *D. immitis* to interact with the host fibrinolytic system, their ability to bind plasminogen and stimulate plasmin generation was analyzed. The binding capacity of plasminogen to DiES and DiSAA

extracts was measured by ELISA. Multiwell microplates (Costar) were coated with 1 µg/well of DiES or DiSAA extracts. The wells were blocked with 1% BSA in PBS and incubated successively with increasing amounts (from 0 µg to 3 µg) of human plasminogen (Acris Antibodies), with a sheep anti-human plasminogen IgG and then with a peroxidase-conjugated donkey anti-sheep IgG. Competition assays were performed by including 50 mM of the lysine analogue ε-aminocaproic acid (εACA) during plasminogen incubation. Some wells coated with BSA only were used as negative controls.

The test showed that DiES and DiSAA bind plasminogen obtaining optical densities statistically higher ($p < 0.05$) than those of the control wells (coated only with BSA) (Figure 1). This binding is also directly proportional to the amount of plasminogen. The competition assay showed that the inclusion of 50 mM εACA inhibits the plasminogen-binding (Figure 1), demonstrating that this union is dependent on lysine residues.

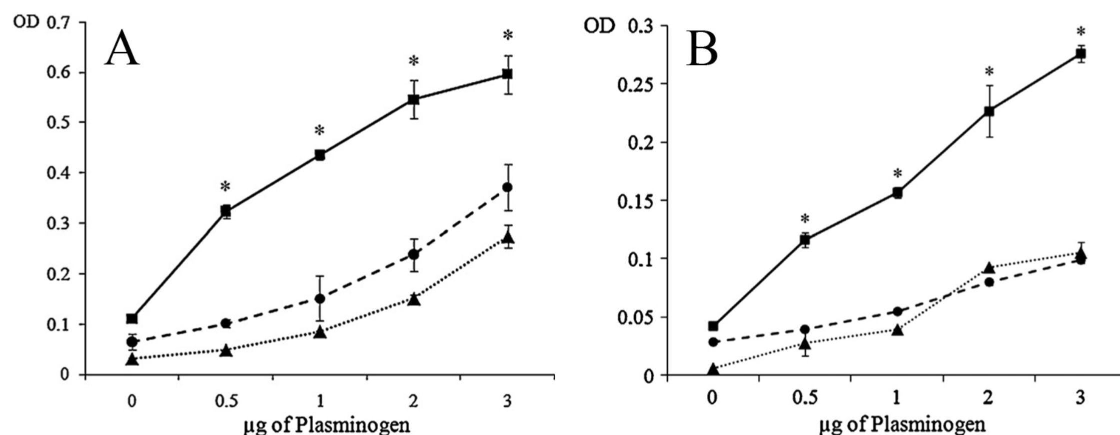

Figure 1. Plasminogen binding to 1 µg of DiES (A) or DiSAA (B) extracts of *D. immitis* measured over a range of plasminogen amounts using a microtiter plate method. (■) Incubation with increasing amounts of plasminogen, 0-3 µg. (●) Competition assay with 50 mM εACA included during plasminogen incubation. (▲) Negative control consisted of wells coated only with BSA. Each point is the mean of three replicates ± SD. The asterisk (*) designates significant ($p < 0.05$) differences.

The ability to activate plasminogen by DiES or DiSAA extracts and to generate plasmin was assessed by measuring the amidolytic activity of plasmin generated in the presence of the antigenic extracts and plasminogen. This effect was measured in the

presence or absence of a physiological activator of the process, tPA, to observe the ability of the DiES and DiSAA extracts proteins of activating plasminogen on their own. Negative controls replacing DiES or DiSAA by BSA or t-PA were also used. As shown in Figure 2, the generation of plasmin by tPA is enhanced by DiES and DiSSA reaching optical density values significative higher ($p < 0.05$) than the negative controls in the presence of tPA. However, DiES and DiSAA extracts are unable to generate plasmin without tPA resulting in optical density values identical to the negative controls.

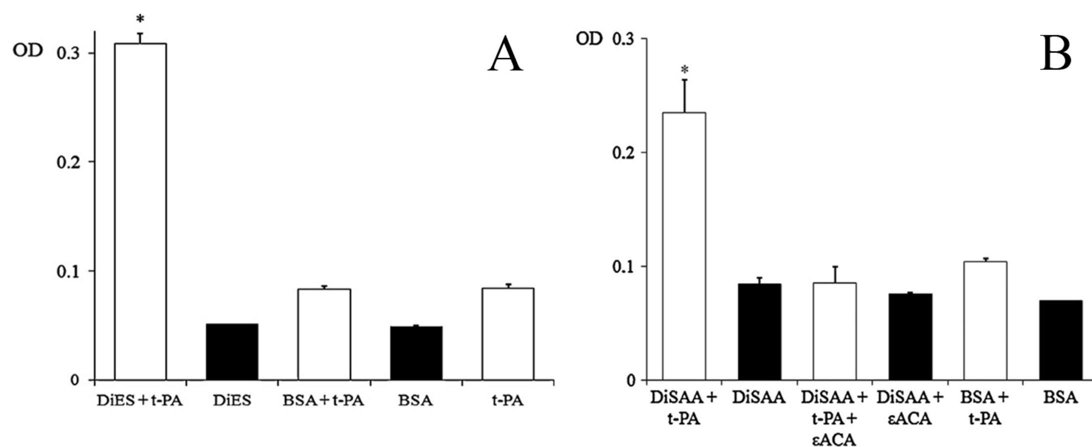

Figure 2. Plasminogen activation and plasmin generation by DiES (A) and DiSAA (B) extracts of *D. immitis*. (□) 15 ng of t-PA was added to mixtures containing 2 μg of human plasminogen, 3 μg of S-2251 (Sigma) and 1 μg of DiES or DiSAA extracts (or BSA as negative control) in the presence or absence of 50mM of εACA in a test volume of 100 μl. (■) No t-PA was added to reaction mixtures. Each point is the mean of three replicates ± SD. The asterisk (*) designates significant ($p < 0.05$) differences.

3. Effect of DiES on the fibrinolytic system components (tPA, uPA, Annexin A2 and PAI-1) expression in CnAOEC and CnAOSMC

The study on the interaction between the antigens from *D. immitis* and the host fibrinolytic system was completed by analyzing the effect of DiES on the expression of the fibrinolytic activators tPA and uPA, the inhibitor PAI-1 and the plasminogen receptor Annexin A2 in vascular cell cultures. For this purpose, we have developed an "in vitro" model of canine endothelial (CnAOEC) and smooth muscle cells (CnAOSMC).

Confluent cultures of CnAOEC and CnAOSMC were previously treated with 1 µg/ml of DiES for 24 h and lysed in ice-cold lysis buffer. Non-stimulated cells were used as controls under the same conditions. Extracted proteins from DiES-treated or untreated CnAOEC and CnAOSMC extracts were separated by SDS–PAGE and analyzed by Western blotting using anti-tPA, anti-uPA, anti-Annexin A2 and anti-PAI-1 antibodies. DiES induced a marked increase in the expression of the main fibrinolytic activators tPA and uPA in cultured endothelial cells ($p<0.05$) (Figure 3A and 3B), as well as a slight decrease in the expression of the main fibrinolytic inhibitor PAI-1 in both types of cultures ($p<0.05$) (Figure 3D). Significant differences in the expression of tPA and uPA in CnAOSMC (Figure 3A and 3B) and in the expression of Annexin A2 in both cell types between DiES-treated or untreated cultures were not found (Figure 3C).

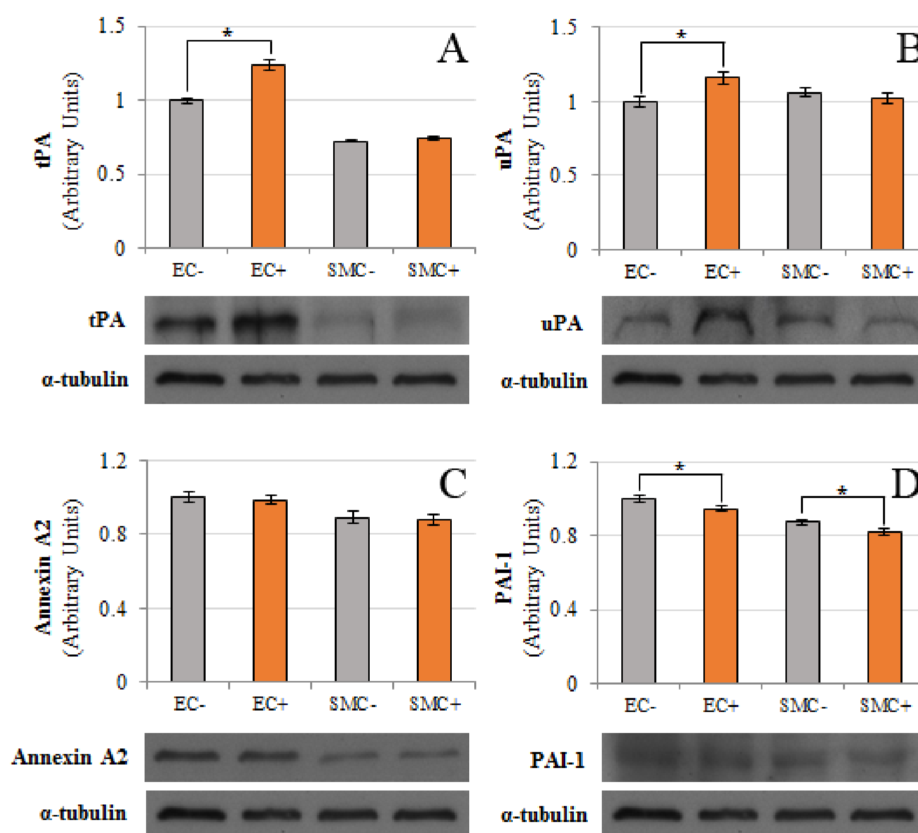

Figure 3. Effect of DiES on the expression of tPA (A), uPA (B), annexin A2 (C) and PAI-1 (D) in canine vascular endothelial (EC) and smooth muscle cells (SMC). Protein extracts from lysed DiES untreated or treated confluent cell cultures were analyzed by Western blot for tPA, uPA annexin A2 and PAI-1. α-tubulin served as a protein control. Results were expressed as the mean ± SD of at least 3 independent experiments. The asterisk (*) designates significant ($p < 0.05$) differences from control cells. (■) Non-treated control cells. (■) Stimulated endothelial or smooth muscle cells with 1µg/ml of DiES.

4. Two-dimensional analysis of DiES and DiSSA extracts and identification of plasminogen-binding proteins by mass spectrometry

Two-dimensional electrophoresis of DiES and DiSAA extracts were performed in order to obtain an overall view of all the proteins of both extracts. In order to improve spot resolution and detection, once the spot MW and pI ranges were determined, both extracts were electrofocused in 5–8 and 7–10 IPG strips. With these conditions, silver staining respectively revealed a total of 636 and 347 spots in the DiES and DiSSA extracts proteomes (Figures 4A, 4B, 5A and 5B).

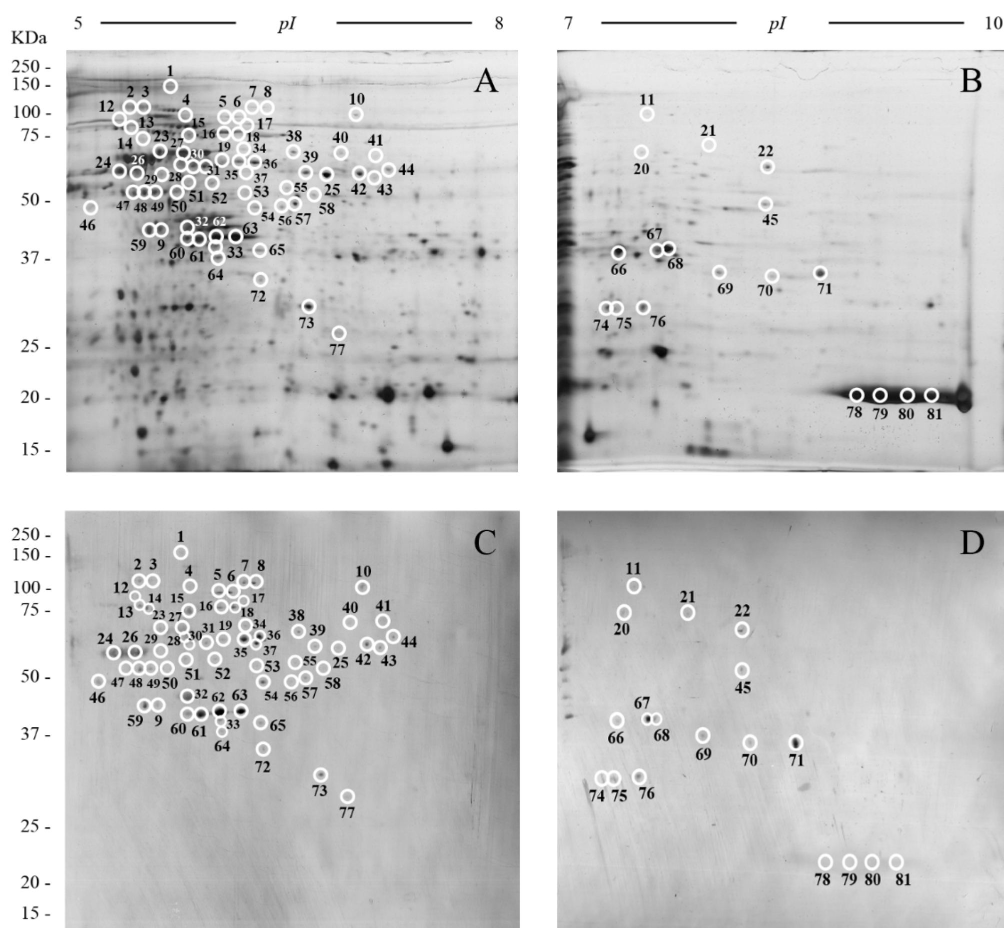

Figure 4. Representative 2-DE of 60 µg of the DiES extract from adult *D. immitis* worms. The gels were in the 5-8 and 7-10 pH ranges, 12% polyacrylamide and silver-stained (A and B). Plasminogen-binding spots revealed on ligand blots from gels A and B (C and D). Reference molecular masses are indicated on the left. The plasminogen-binding spots analyzed by MS are circled and numbered.

Next, to determine which proteins of DiES and DiSAA extracts bind plasminogen an immunoblot was performed. The 2-D gels were transferred to nitrocellulose membranes which were blocked and then incubated with plasminogen. After incubating the membranes with the corresponding antibodies, proteins were revealed with 4-chloro naphthol and spots analyzed using the PDQuest Software v.8.0.1 (Bio-Rad).

Spot number	Accesion code	Protein definition	Species	MW (kDa) theor/exp	pI theor/exp	Sequence coverage (%)	Mascot score
23	ACY25666	Chaperonin-like protein HSP60	*Brugia malayi*	61.4/67.1	5.7/5.6	11	130
27	AF121264_1	Chaperonin protein HSP60	*Onchocerca volvulus*	64.5/67.0	5.7/5.8	17	145
28	AF121264_1	Chaperonin protein HSP60	*Onchocerca volvulus*	64.5/65.2	5.7/5.8	16	130
31	ACT1_CAEEL	Actin-1/3	*Caenorhabditis elegans*	42.1/65.2	5.3/5.9	11	41
32	XP_001894819	Actin	*Brugia malayi*	42.1/43.3	5.3/5.8	4	64
33	NP_508842	ACTin family member (act-4)	*Caenorhabditis elegans*	37.5/39.4	5.4/6.0	17	142
37	AAC24752	Transglutaminase precursor	*Dirofilaria immitis*	57.6/61.0	5.7/6.3	19	91
66	XP_001899850	Glyceraldehyde 3-phosphate dehydrogenase	*Brugia malayi*	32.1/40.8	8.5/7.5	20	207
67	XP_001899850	Glyceraldehyde 3-phosphate dehydrogenase	*Brugia malayi*	32.1/40.7	8.5/7.8	25	292
69	AAD00843	Ov87	*Onchocerca volvulus*	36.7/36.4	8.9/8.2	24	161
71	AAD00843	Ov87	*Onchocerca volvulus*	36.7/36.5	8.9/9.0	16	157
72	XP_003150284	Hypothetical protein LOAG_14743	*Loa loa*	13.3/33.7	6.7/6.3	11	94
73	AAF37720	Galectin	*Dirofilaria immitis*	32.2/30.1	6.0/6.6	11	118
78	AAD11968	P22U	*Dirofilaria immitis*	24.4/22.0	8.9/9.2	66	499
79	AAD11968	P22U	*Dirofilaria immitis*	24.4/22.0	8.9/9.4	62	458
80	AAD11968	P22U	*Dirofilaria immitis*	24.4/22.0	8.9/9.6	62	489
81	AAD11968	P22U	*Dirofilaria immitis*	24.4/22.0	8.9/9.8	54	201

Table 1. Plasminogen-binding protein spots of DiES extract identified by MALDI-TOF MS. Exp, experimental; theo, theoretical.

As shown in Figures 4C, 4D, 5C and 5D, 81 and 61 plasminogen-binding spots were revealed in the DiES and DiSAA membranes. In the control blots, in which plasminogen incubation was omitted, the anti-plasminogen antibody did not reveal any spots (not shown). The matching of spots revealed by ligand-blotting with their homologous in the silver-stained 2-D gels allowed us to select a total of 53 plasminogen-binding spots of *D. immitis*, which were manually excised from 2-D gels and submitted to analysis by mass spectrometry. Seventeen spots corresponded to 10 different proteins and 16 spots corresponded to 11 different proteins were respectively identified in the DiES and DiSAA 2-D gels. Tables 1 and 2 show the identity of these proteins and their MWs and pIs (theoretical and experimental), the number of access to similar information available in the NCBI database, the sequence coverage and the Mascot score.

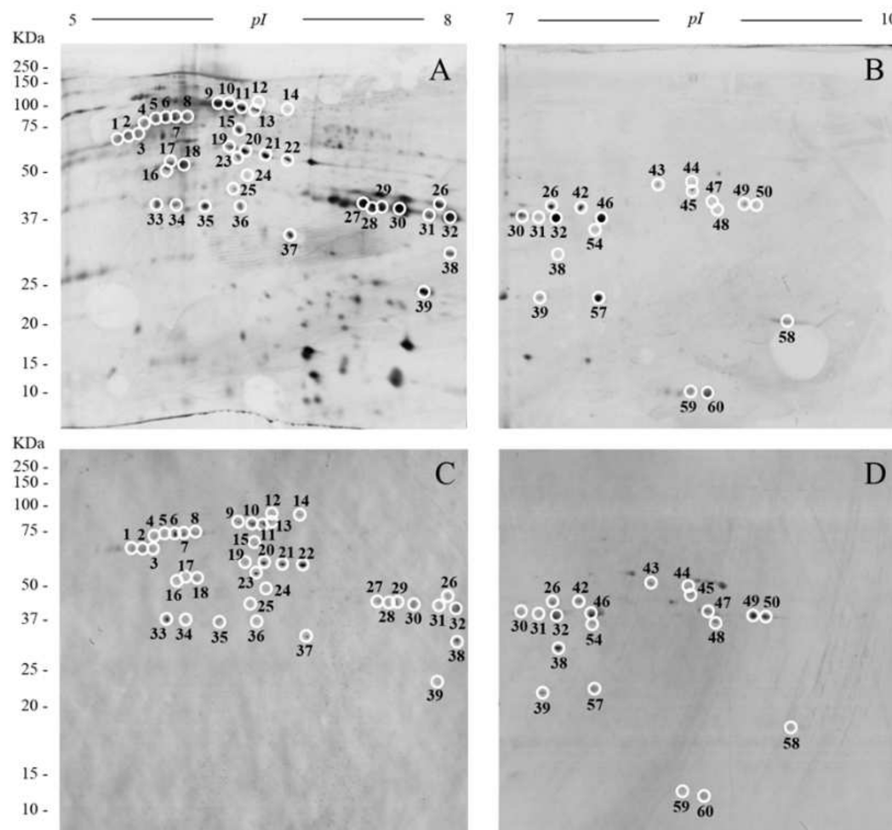

Figure 5. Representative 2-DE of 60 µg of the DiSAA extract from adult *D. immitis* worms. The gels were in the 5-8 and 7-10 pH ranges, 12% polyacrylamide and silver-stained (A and B). Plasminogen-binding spots revealed on ligand blots from gels A and B (C and D). Reference molecular masses are indicated on the left. The plasminogen-binding spots analyzed by MS are circled and numbered.

Spot number	Accesion code	Protein definition	Species	MW (kDa) theor/exp	pI theor/exp	Queries matched	Mascot score
17	EFV54220	Actin-5C	*Trichinella spiralis*	41.8/54.1	5.3/5.7	4	154
18	P02578	Actin-1	*Acanthamoeba castellanii*	41.7/52.8	5.4/5.8	6	190
20	XP_001896281	Enolase	*Brugia malayi*	47.5/59.6	6.0/6.3	6	248
22	Q7YZX3	Enolase	*Onchocerca volvulus*	47.1/55.5	6.0/6.6	8	64
26	AAB52600	Fructose-bisphosphate aldolase	*Onchocerca volvulus*	39.2/40.5	7.7/7.7	8	247
42	AAB52600	Fructose-bisphosphate aldolase	*Onchocerca volvulus*	39.2/39.0	7.7/7.9	2	59
28	P48812	GAPDH	*Brugia malayi*	36.1/40.0	7.7/7.2	13	128
30	P48812	GAPDH	*Brugia malayi*	36.1/39.8	7.7/7.4	17	259
32	P48812	GAPDH	*Brugia malayi*	36.1/37.4	7.7/7.8	11	84
46	P48812	GAPDH	*Brugia malayi*	36.1/36.0	7.7/8.0	9	71
35	XP_001900868	MSP domain protein with Glu-rich domain	*Brugia malayi*	18.1/40.1	5.5/6.0	2	64
60	P13263	Major sperm protein 2	*Onchocerca volvulus*	14.3/15.7	7.8/8.8	18	265
37	AAA20541	Beta-galactosidase-binding-lectin	*Onchocerca volvulus*	32.0/33.6	6.0/6.6	20	143
38	XP_001900812	Galectin	*Brugia malayi*	31.8/30.3	6.4/7.8	3	68
39	XP_003139445	Immunoglobulin I-set domain-containing protein	*Loa loa*	22.5/24.2	6.6/7.6	8	307
58	AAC47233	Cyclophilin Ovcyp-2	*Onchocerca volvulus*	18.5/20.9	8.3/9.4	1	71

Table 2. Plasminogen-binding protein spots of DiSAA extract identified by MALDI-TOF MS. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; Exp, experimental; theo, theoretical.

5. Amplification, cloning, sequencing, and expression of *D. immitis* actin, fructose-bisphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenase and galectin

Among the plasminogen-binding proteins identified by mass spectrometry in the *D. immitis* antigenic extracts, four proteins were selected for the following experiments based on the availability of homologous sequences from other filarial parasites, their evolutionary conservation and if they had been previously related with plasminogen-binding activities. We select actin (ACT), fructose-bisphosphate aldolase (FBAL), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and galectin (GAL).

After designing the primers of the proteins, the genetic material was amplified and isolated. DNA fragments were inserted in a PSC-A cloning vector, and the obtained

sequences were developed in their recombinant form in the TOPO/pDEST expression system.

Amplification of *D. immitis* ACT, FBAL, GAPDH and GAL cDNAs by RT-PCR respectively resulted in 4 PCR products of around 1100, 1000, 1000 and 850 bp. After their cloning into the pSC-A vector, fragments were fully sequenced and their identities demonstrated as actin, fructose-bisphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenase and galectin by BLAST analysis. The ACT, FBAL and GAPDH new sequences were respectively deposited in the Gen-Bank under accession numbers JQ780093.1, JQ780094.1 and JQ780095.1. The full *D. immitis* ACT, FBAL, GAPDH and GAL cDNAs respectively contained 1131, 1092, 1020 and 846 nucleotides, encoded proteins of 376, 363, 339 and 280 amino acids, with theoretical molecular weights of 41820, 39423, 36179 and 32085 Da, and pIs of 5.29, 7.65, 7.11 and 6.08.

The bioinformatics analyses of the deduced amino acid sequences did not reveal a signal peptide, transmembrane helices or glycosyl-phosphatidyl inositol anchors. The percentage identity between recombinant proteins and homologous sequences from other organisms was analyzed using multiple sequence alignment with the ClustalW program. The analysis revealed that DiACT, DiFBAL, DiGAPDH and DiGAL are highly conserved proteins. Additionally, a plasminogen-binding domain to actin within amino acids 56 to 70 (GDEAQSKRGILTLKY) and 19, 12, 7 and 16 conserved lysine residues in the DiACT, DiFBAL, DiGAPDH and DiGAL alignments were respectively found as possible plasminogen-binding sites.

Prediction of the secondary structures and three-dimensional modelling were done with the Swiss-Model server (http://swissmodel.expasy.org/). In silico three-dimensional modelling of the molecules predicted the 3D structures showing in the case of DiACT a monomer with 20 α-helices and 19 β-sheets (Figure 6A). Molecular modelling of DiFBAL showing a homo-tetramer with the presence of 14 α-helices and 13 β-sheets (Figure 6B). DiGAPDH appeared as a homo-tetramer with 15 α-helices and 4 β-sheets (Figure 6C), whereas modellling of DiGAL showed a monomer with the presence of 2 α-helices and 26 β-sheets (Figure 6D). The plasminogen-binding domain

(GDEAQSKRGILTLKY) and the conserved lysine residues were highlighted and were visualized on the outside of the proteins.

Proteins were finally purified under denaturing conditions using nickel affinity chromatography. The purified rDiACT, rDiFBAL, rDiGAPDH and rDiGAL respectively had molecular weights of 43.6 kDa, 41.6 kDa, 38.6 kDa and 34.6 kDa in polyacrylamide gel.

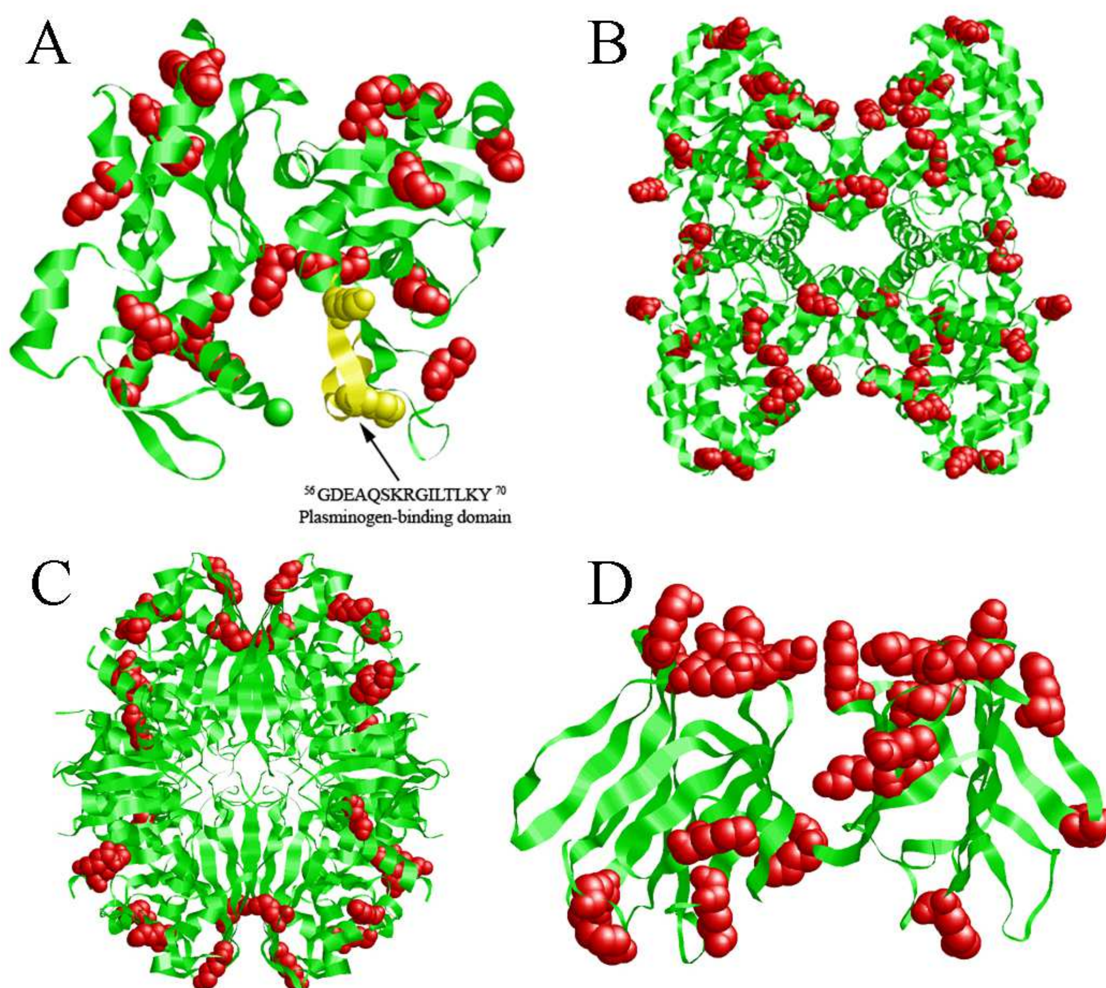

Figure 6. Molecular modelling of *D. immitis* ACT, FBAL, GAPDH and GAL. The secondary structure of the proteins was predicted with the Swiss-Model web server (http://swissmodel.expasy.org/) by analogy with the X-ray crystallography available models. The three-dimensional models of DiACT (A), DiFBAL (B), DiGAPDH (C) and DiGAL (D) were visualized with the RasMol application v. 2.7.5.2. Conserved lysine residues of proteins were highlighted as red balls. The PLG-binding domain (GDEAQSKRGILTLKY) is highlighted in yellow.

6. rDiACT, rDiFBAL, rDiGAPDH and rDiGAL bind plasminogen and enhance its activation by tPA

To analyze the ability of recombinant proteins as plasminogen-binding proteins, experiments performed on antigenic extracts of *D. immitis* described in section 2 of this abstract were repeated. After performing the corresponding ELISAs, these showed that rDiACT, rDiFBAL, rDiGAPDH and rDiGAL bind plasminogen and that this binding is directly proportional to the amount of plasminogen.

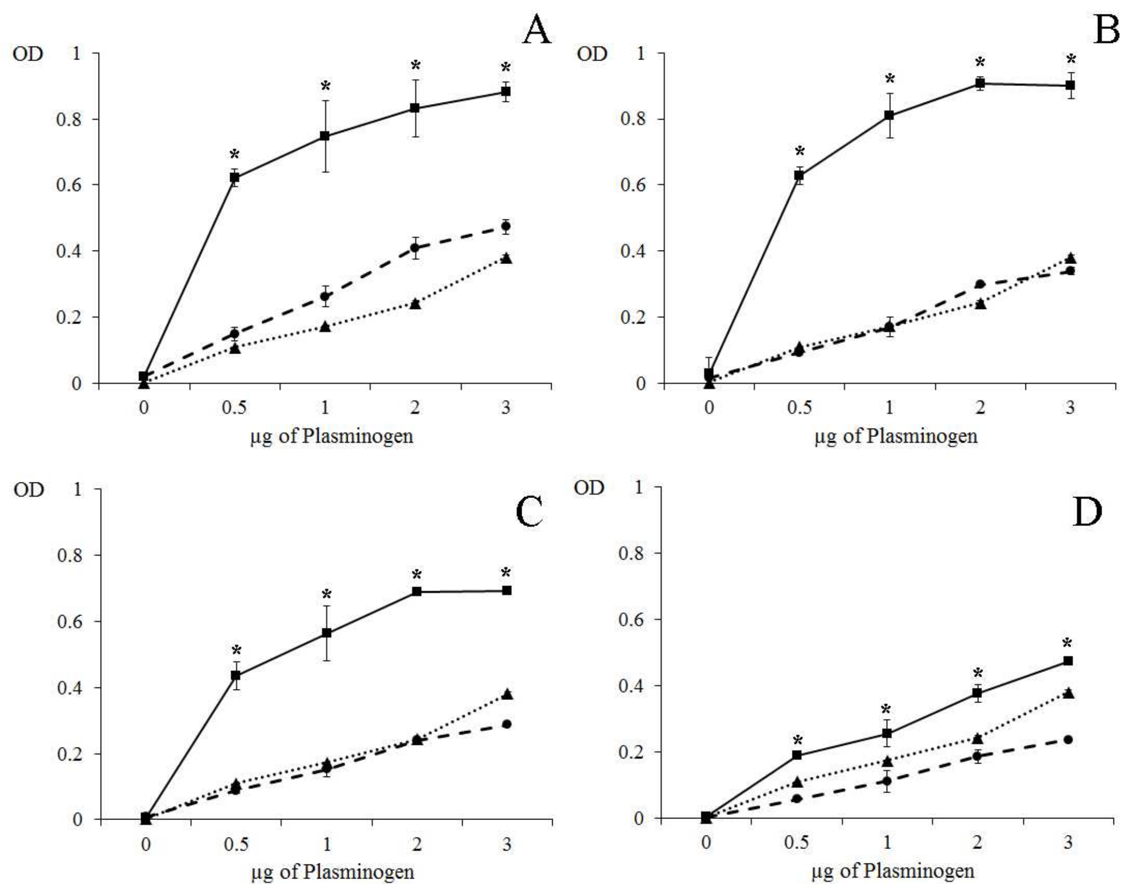

Figure 7. Plasminogen binding to 0.5 μg of rDiACT (A), rDiFBAL (B), rDiGAPDH (C) or rDiGAL (D) analyzed over a range of plasminogen amounts using a microtiter plate method. (■) Incubation with increasing amounts of plasminogen, 0–3 μg. (▲) Competition assay with 50 mM εACA included during plasminogen incubation. (●) Wells coated with BSA used as negative control. Each point is the mean ± SD from three independent experiments. The asterisk (*) designates significant ($p < 0.05$) differences.

Comparing the results obtained, rDiACT and rDiFBAL showed higher plasminogen-binding capacity than rDiGAPDH, being rDiGAL the protein with less binding capacity (Figure 7). The negative control consisting of wells coated only with BSA showed some non-specific binding activity, but always with values significantly lowers than those obtained by recombinant proteins ($p < 0.05$). To determine whether or not lysine residues are involved in binding, a competition experiment including 50 mM εACA was carried out. In this case, the binding was inhibited about 90% in the case of rDiFBAL and rDiGAPDH and approximately 70% in the case of rDiACT and rDiGAL, resulting in slightly higher optical densities than the negative control (Figure 7).

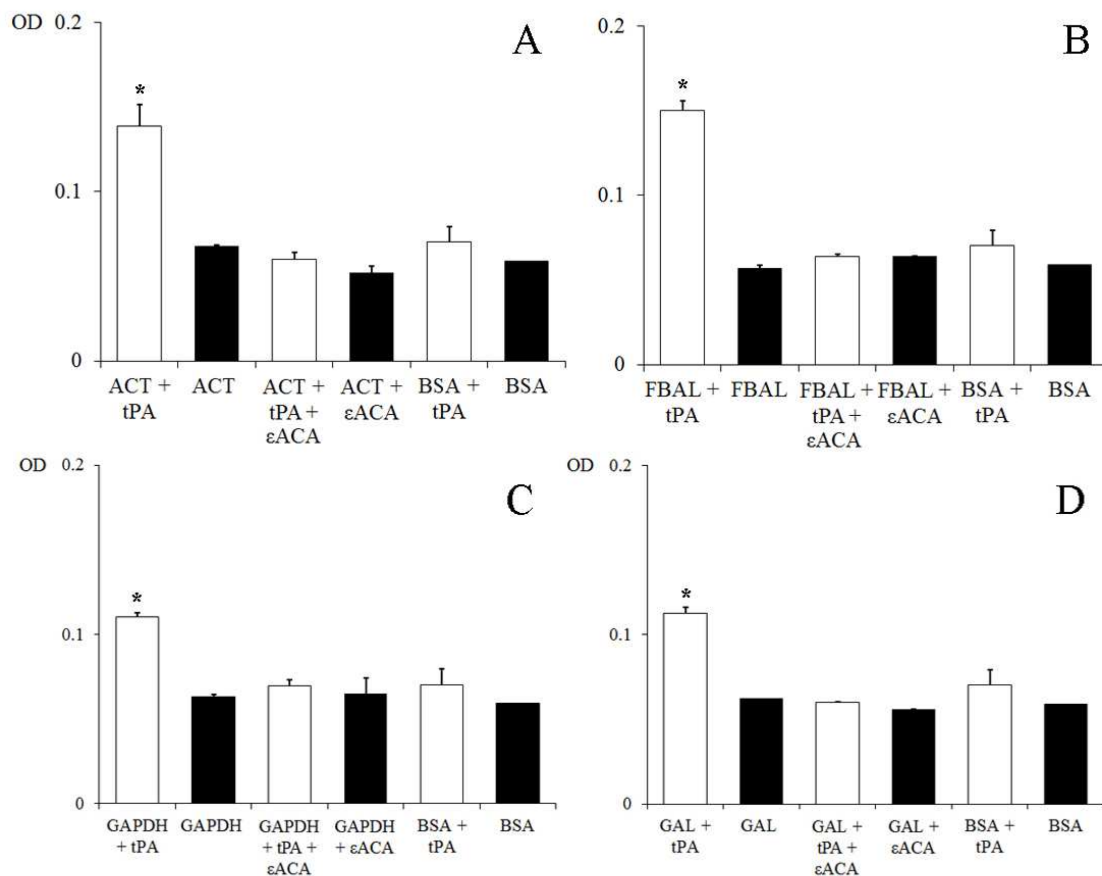

Figure 8. Plasminogen activation and plasmin generation by rDiACT (A), rDiFBAL (B), rDiGAPDH (C) or rDiGAL (D). (□) 15 ng of tPA was added to mixtures which contained 2 µg of human plasminogen, 3 µg of D-Val-Leu-Lys 4-nitroanilide dihydrochloride (Sigma) and 1 µg of each recombinant protein (or BSA as negative control) in the presence or absence of 50 mM of εACA in a test volume of 100 µl. (■) Reaction mixtures in absence of tPA. Each point is the mean ± SD from three independent experiments. The asterisk (*) designates significant ($p < 0.05$) differences.

In order to assess the ability of rDiACT, rDiFBAL, rDiGAPDH and rDiGAL to activate plasminogen and generate plasmin on their own, the amidolytic activity of plasmin generated in the presence or absence of tPA was measured. Negative controls replacing each recombinant protein for BSA or tPA were also used. Figure 8 shows the capacity of rDiACT, rDiFBAL, rDiGAPDH and rDiGAL to stimulate plasmin generation by tPA obtaining optical densities significantly higher than the negative controls ($p < 0.05$). rDiACT and rDiFBAL obtained higher optical densities than those obtained by rDiGAPDH and rDiGAL and plasminogen-activation did not occur in the absence of tPA. Furthermore this effect is inhibited by 50 mM εACA, indicating the involvement of lysine residues in the process.

7. Effect of rDiACT, rDiFBAL, rDiGAPDH and rDiGAL on the fibrinolytic system activators (tPA and uPA) expression in CnAOEC

To study the possible effect of rDiACT, rDiFBAL, rDiGAPDH and rDiGAL on the expression of the main activators of fibrinolysis (tPA and uPA), the parasitic proteins were employed to stimulate CnAOEC in culture. Following a similar procedure to that described in section 3 of this abstract. Proteins from rDiACT/rDiFBAL/rDiGAPDH/rDiGAL-treated or untreated vascular endothelial cell extracts were separated by SDS-PAGE and analyzed by Western blotting using anti-tPA or anti-uPA antibodies. rDiACT and rDiFBAL induced a significant increase in the expression of the main fibrinolytic activators tPA in cultured endothelial cells ($p < 0.05$) (Figure 9A), being this increase slightly higher in the case of stimulation with rDiFBAL. Significant differences in the expression of tPA in CnAOEC between rDiGAPDH/rDiGAL-treated or untreated cultures were not found (Figure 9B). On the other hand, all the proteins induced a marked increase in the expression of uPA in CnAOEC cultures ($p < 0.05$) (Figures 9C and 9D). This increase was greater in the case of the rDiFBAL stimulation, rDiACT and rDiGAPDH showed similar optical density values, whereas rDiGAL showed the lowest differences.

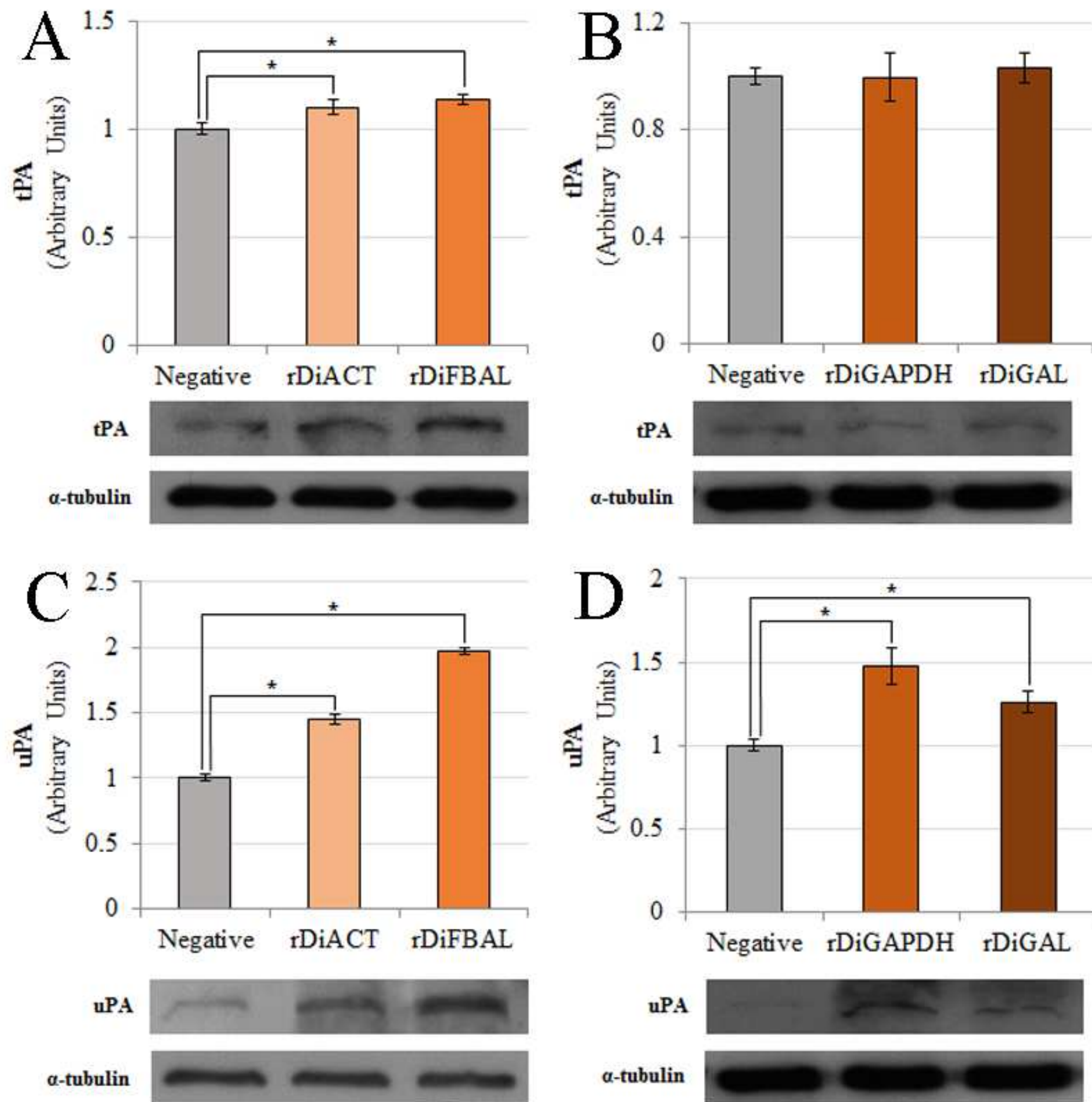

Figure 9. Effect of rDiACT, rDiFBAL, rDiGAPDH and rDiGAL on the expression of the fibrinolytic system activators tPA (A and B) and uPA (C and D) in canine vascular endothelial cells. Protein extracts from lysed rDiACT, rDiFBAL, rDiGAPDH or rDiGAL treated or untreated confluent cell cultures were analyzed by Western blot for tPA and uPA. α-tubulin served as a protein control. Data are shown as representative images or means ± SD from three independent experiments. The asterisk (*) designates significant ($p < 0.05$) differences from control cells. (■) Stimulated cells with 1µg/ml of rDiACT. (■) Stimulated cells with 1µg/ml of rDiFBAL. (■) Stimulated cells with 1µg/ml of rDiGAPDH. (■) Stimulated cells with 1µg/ml of rDiGAL. (■) Non-treated control cells.

8. Immunolocalization of DiACT, DiFBAL, DiGAPDH and DiGAL

The immunolocalization of proteins was conducted in order to know if these were localized in tissues in contact with the blood of the host. This fact is essential, so that the interaction of these proteins with the fibrinolytic system may have relevance "in vivo".

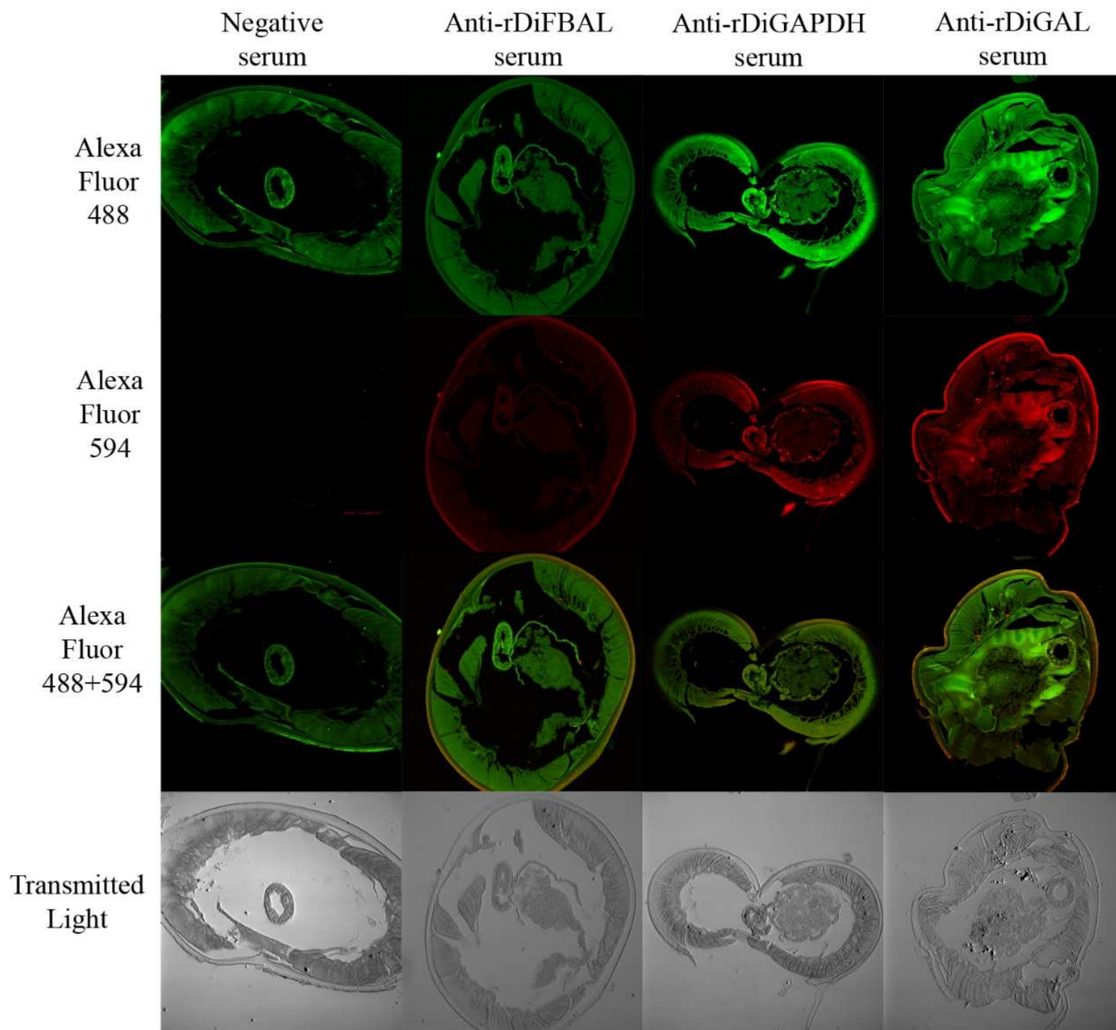

Figure 10. Immunolocalization of DiACT, DiFBAL, DiGAPDH and DiGAL in sections from *D. immitis* adult worms. Representative images from three independent experiments of parasite sections incubated with phalloidin-Alexa Fluor 488 (in green, specific binding to ACT) plus the negative or the anti-rDiFBAL, anti-rDiGAPDH or anti-rDiGAL rabbit serum and an anti-rabbit IgG-Alexa Fluor 594 (in red). Corresponding transmitted light images are also addressed. Magnification 4X.

Thus, the anatomical localization of selected proteins was carried out in histological sections of *D. immitis* adult worms by immunofluorescence using a commercially available high-affinity ligand (in the case of DiACT) and rabbit polyclonal antiserum (in the case of DiFBAL, DiGAPDH and DiGAL). As shown in Figure 10, all sections showed green fluorescence throughout the soma of the parasite, as a result of the binding of phalloidin-Alexa Fluor 488, actin high-affinity ligand which serves also as a positive control of the technique. Sections incubated with the anti-rDiFBAL, anti-rDiGAPDH or anti-rDiGAL antiserum respectively showed, in addition, specific reactivity (in red) against the parasitic FBAL, GAPDH or GAL due to the binding of the anti-rabbit IgG antibody conjugated to Alexa Fluor 594. All proteins are located scattered throughout all the soma, being especially abundant in the cuticle (reflected by an orange color in the overlay of Phalloidin-Alexa Fluor 488 + Alexa Fluor 594 images). Sections incubated with a rabbit negative serum showed no specific red fluorescence from recombinant proteins.

9. Reagents and stimulation of CnAOEC and CnAOSMC

To analyze the effects of plasmin generated by the interaction of plasminogen-binding proteins of *D. immitis* with the fibrinolytic system of the host on the proliferation and cell migration, as well as the destruction of extracellular matrix (ECM), we use the previously optimized CnAOEC and CnAOSMC cultures. In addition, the excretory/secretory extract of proteins from *D. immitis* (DiES) and the two recombinant proteins, whose optical density levels in experiments of binding of plasminogen, enhancement of plasmin generation and stimulation on the expression of the fibrinolytic activators had been higher, were employed.

CnAOEC and CnAOSMC were grown for 4 days to obtain confluent cultures and were treated with 1 µg/ml of DiES, rDiACT or rDiFBAL, 10 µg/ml of plasminogen (PLG) (Acris Antibodies) or with a mixture of both treatments (DiES + PLG, rDiACT + PLG or rDiFBAL + PLG). Untreated cells and cells treated with DiES/rDiACT/rDiFBAL + PLG + 50 mM of εACA as an inhibitor of plasminogen activation were used as control cells under the same conditions.

10. DiES and rDiACT, but not rDiFBAL, produces proliferation of CnAOEC and CnAOSMC via PLG/plasmin system

Cell proliferation was analyzed by crystal violet nuclei staining over 10 days determining the number of viable cells. Cells were plated on 24-well plates to a density of 10^4 CnAOEC/well or 1.5 x 10^4 CnAOSMC/well, allowed to attach overnight, rinsed, fixed and stained. After staining, the absorbance of the samples was measured at 595 nm and transformed to "number of viable cells" using a curve that correlated absorbance and number of endothelial or smooth muscle cells previously determined.

Both cultures showed typical curves of cell growth in all experimental groups with a progressive growth between days 0 and 6 or 8 post-treatment, experiencing cell death and an evident decrease of viable cells from there until the end of the experiment (day 10 post-treatment) (Figure 11). Crystal violet staining showed an increase significantly greater in the number of viable cells in cultures stimulated with DiES + PLG (on day 8 post-treatment, $p < 0.05$) and with rDiACT + PLG [on days 4 and 6 post-treatment (CnAOEC) or day 8 post-treatment (CnAOSMC), $p < 0.05$] than that showed by other experimental groups, indicating that these treatments stimulates the proliferation of CnAOEC and CnAOSMC in culture. Significant differences in cell proliferation between cells stimulated with rDiFBAL + PLG and other experimental groups were not found in both types of cultures (Figure 11).

11. DiES, rDiACT and rDiFBAL produce migration of CnAOEC and CnAOSMC via PLG/plasmin system

A Wound Healing assay was performed to assess migration of endothelial (Figure 12) and smooth muscle cells (Figure 13). The quantification was carried out by measuring the distance of migration in comparison with negative control (untreated cells) to 8 hours post-treatment. In both CnAOEC and CnAOSMC cultures a significant increase of cell migration after stimulation with DiES + PLG, rDiACT + PLG or rDiFBAL + PLG with respect to the other experimental groups ($p < 0.05$) occurred. This increase was higher in cultured endothelial cells in the case of DiES + PLG treatment and in cultured smooth muscle cells in the case of rDiACT/rDiFBAL + PLG treatment. After comparing the effect of both parasitic proteins, rDiACT showed higher

values of migration ability in both types of cell cultures with respect to those obtained by rDiFBAL.

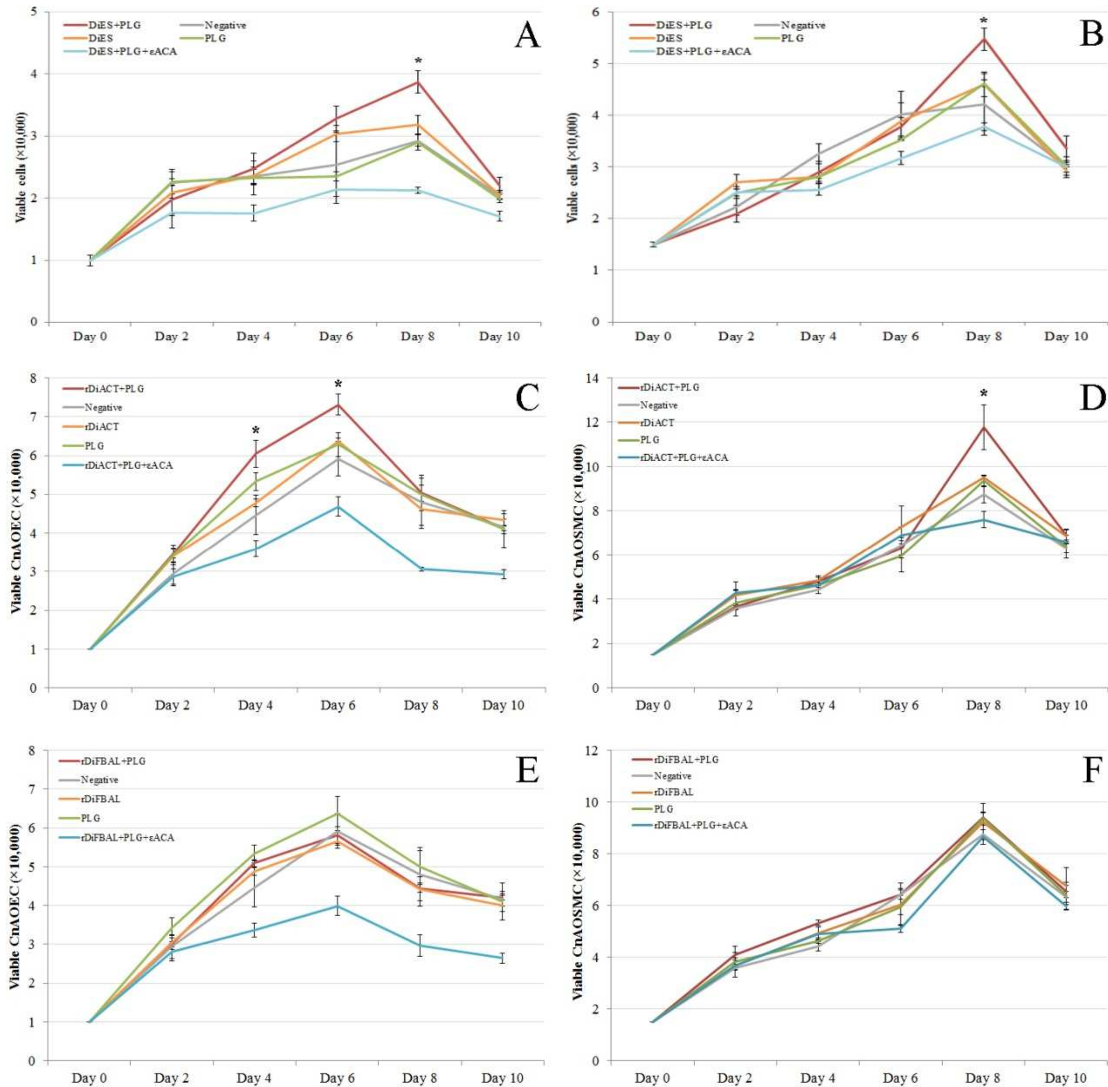

Figure 11. Cell proliferation assay performed by the crystal violet technique measuring cell viability over a 10 days period. The experiment was carried out in canine endothelial (A, C and E) and smooth muscle cells (B, D and F) untreated (▬) or treated with 1 µg/ml of DiES, rDiACT or rDiFBAL + 10 µg/ml of PLG (▬), 1 µg/ml of DiES, rDiACT or rDiFBAL (▬), 10 µg/ml of PLG (▬), or with 1 µg/ml of DiES, rDiACT or rDiFBAL + 10 µg/ml of PLG + 50 mM of the εACA (▬). Results were expressed as number of viable cells (x 10,000). Each point is the mean ± SD from three independent experiments. The asterisk (*) designates significant ($p < 0.05$) differences between DiES/rDiACT + PLG treatment and control groups.

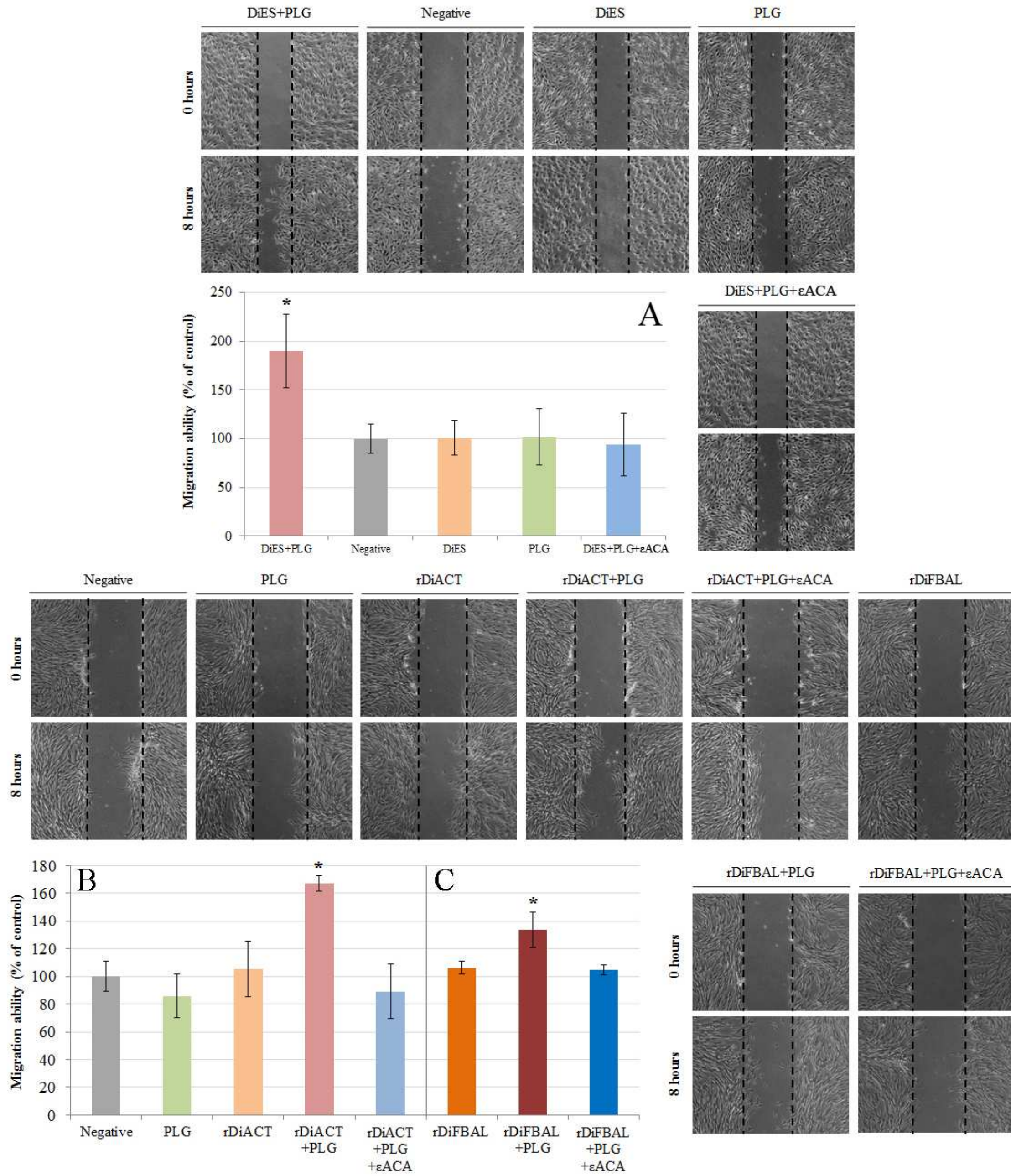

Figure 12. CnAOEC migration by Wound-Healing assay. Confluent cell cultures were wounded post-treatment and migration distances were measured at 8 hours. The experiment was carried out in canine endothelial cells untreated or treated with 10 µg/ml of PLG, 1 µg/ml of DiES/rDiACT/rDiFBAL (A/B/C), 1 µg/ml of DiES/rDiACT/rDiFBAL + 10 µg/ml of PLG (A/B/C) or with 1 µg/ml of DiES/rDiACT/rDiFBAL + 10 µg/ml of PLG + 50 mM of the εACA (A/B/C). The results were expressed as percentage of the migration ability of the negative control cells (100%). Data are shown as representative images or means ± SD from three independent experiments. The asterisk (*) designates significant ($p < 0.05$) differences between DiES/rDiACT/rDiFBAL + PLG treatment and control groups.

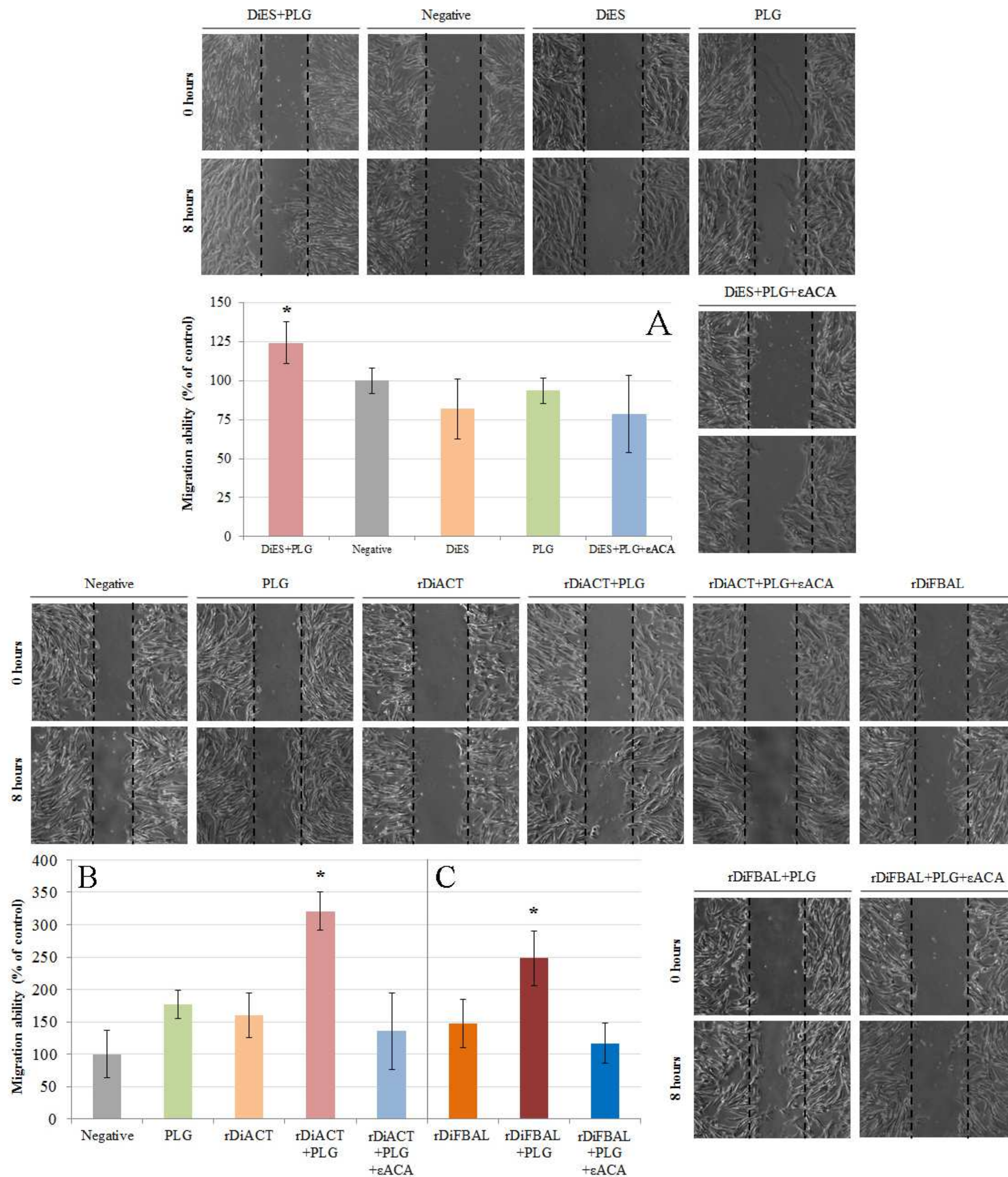

Figure 13. CnAOSMC migration by Wound-Healing assay. Confluent cell cultures were wounded post-treatment and migration distances were measured at 8 hours. The experiment was carried out in canine endothelial cells untreated or treated with 10 µg/ml of PLG, 1 µg/ml of DiES/rDiACT/rDiFBAL (A/B/C), 1 µg/ml of DiES/rDiACT/rDiFBAL + 10 µg/ml of PLG (A/B/C) or with 1 µg/ml of DiES/rDiACT/rDiFBAL + 10 µg/ml of PLG + 50 mM of the εACA (A/B/C). The results were expressed as percentage of the migration ability of the negative control cells (100%). Data are shown as representative images or means ± SD from three independent experiments. The asterisk (*) designates significant ($p < 0.05$) differences between DiES/rDiACT/rDiFBAL + PLG treatment and control groups.

12. DiES, rDiACT and rDiFBAL produce ECM degradation of CnAOEC and CnAOSMC via PLG/plasmin system

To examine ECM degradation, Type I Collagen in the culture supernatant of treated and untreated CnAOEC and CnAOSMC were measured by ELISA (Figure 14). A lower concentration of Type I Collagen and therefore a further degradation of the secreted collagen by the cells was observed in the CnAOEC and CnAOSMC stimulated with DiES + PLG, rDiACT + PLG or rDiFBAL + PLG than that obtained by the control cells ($p < 0.05$). There were no large differences between these treatments (DiES/rDiACT/rDiFBAL + PLG) but always a further degradation of Type I Collagen in cultured smooth muscle cells (Figures 14B and 14D) than in endothelial cells (Figures 14A and 14C) was found.

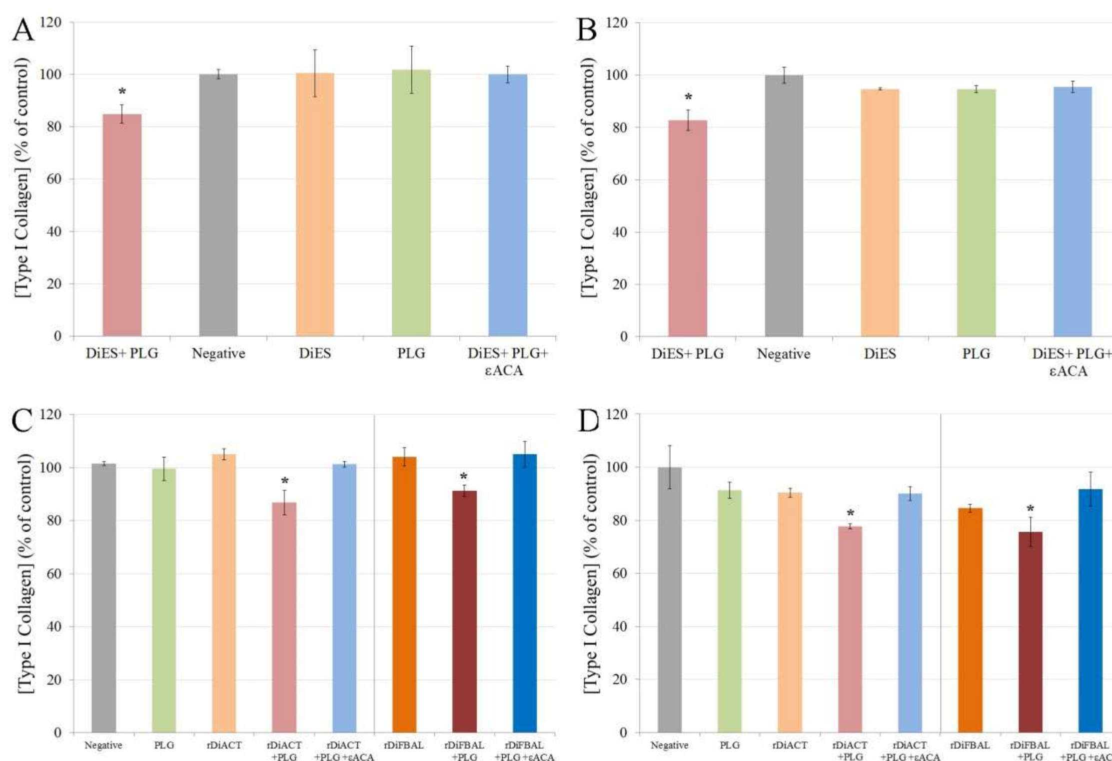

Figure 14. Type I Collagen degradation measured in culture supernatants from canine endothelial (A and C) and smooth muscle cells (B and D) untreated or treated with 10 µg/ml of PLG, 1 µg/ml of DiES/rDiACT/rDiFBAL, 1 µg/ml of DiES/rDiACT/rDiFBAL + 10 µg/ml of PLG or with 1 µg/ml of DiES/rDiACT/rDiFBAL + 10 µg/ml of PLG + 50 mM of the εACA. The results were expressed as percentage of the Type I Collagen concentration in the culture supernatant from negative control cells (100%). Each point is the mean ± SD from three independent experiments. The asterisk (*) designates significant ($p < 0.05$) differences between DiES/rDiACT/rDiFBAL + PLG treatment and control groups.

In addition, the same culture media from treated and untreated cells was analyzed with gelatin zymography for metalloproteinase-2 (MMP-2) and metalloproteinase-9 (MMP-9) levels (Figure 15). Media samples employed in the collagen degradation assays were electrophoresed on polyacrylamide gel copolymerized with gelatin. The gels were washed in 2.5% Triton X-100, incubated at 37 °C in agitation in an enzymatic activation buffer and stained with Coomassie blue.

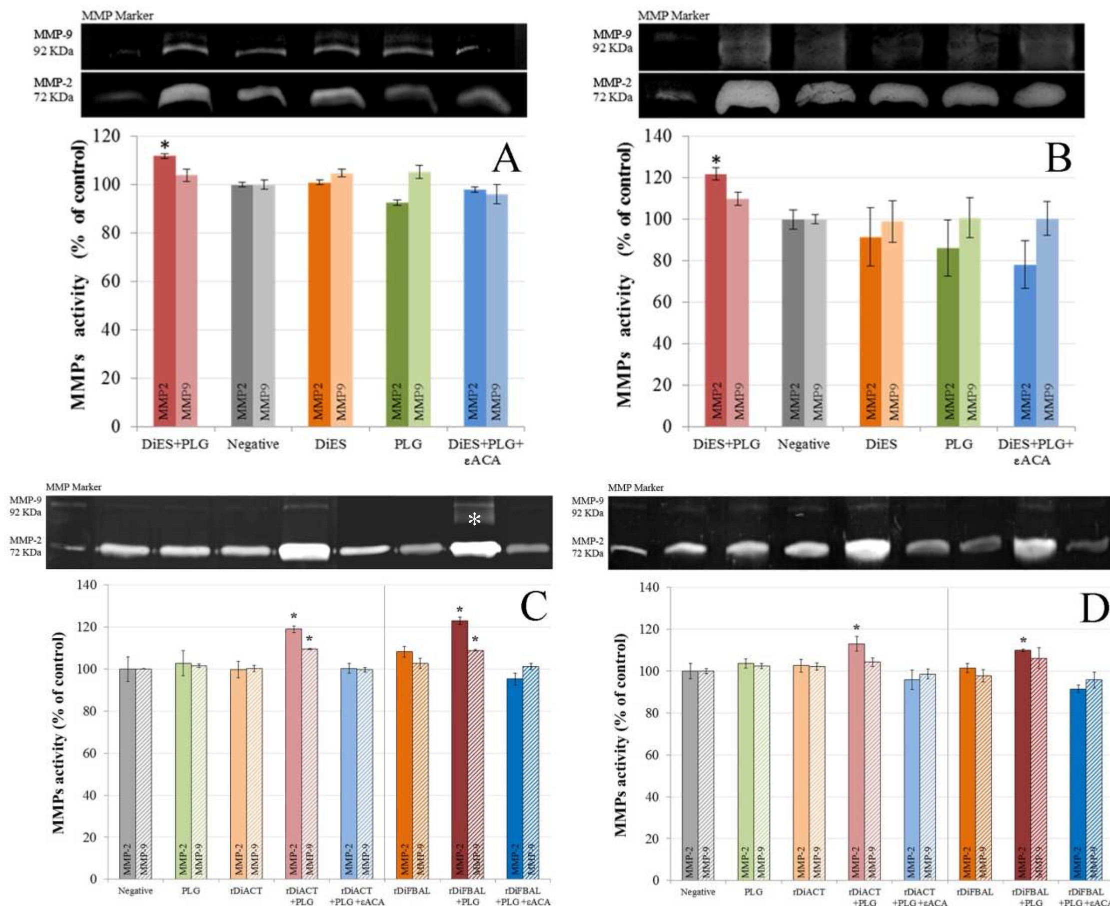

Figure 15. Representative zymography of MMP-2 (solid bars) and MMP-9 (hatched bars) levels in the culture supernatants from canine endothelial (A and C) and smooth muscle cells (B and D) untreated or treated with 10 µg/ml of PLG, 1 µg/ml of DiES/rDiACT/rDiFBAL, 1 µg/ml of DiES/rDiACT/rDiFBAL + 10 µg/ml of PLG or with 1 µg/ml of DiES/rDiACT/rDiFBAL + 10 µg/ml of PLG + 50 mM of the εACA. Note the gelatinolytic bands associated with MMP-2 (72 KDa) and MMP-9 (92 KDa) levels, as well as with the MMP-9 activated form (marked with a white asterisk at 82KDa. The results were expressed as percentage of the MMPs levels in the culture supernatant from negative control cells (100%). Data are shown as representative images or means ± SD from three independent experiments. The asterisk (*) designates significant ($p < 0.05$) differences between DiES/rDiACT/rDiFBAL + PLG treatment and control groups.

The positivity was assessed as appearance of clear bands on a dark background with molecular weights of 72 kDa (MMP-2) and 92 kDa (MMP-9). The radius of the MMPs levels was calculated after measuring the density of the existing bands, which is directly proportional to the amount of gelatin degraded into the gel. Treatment with DiES, rDiACT or rDiFBAL + PLG shows a significantly higher MMP-2 level in the CnAOEC and CnAOSMC culture media ($p < 0.05$) (Figure 15). The results also show a significantly higher level of MMP-9 in the CnAOEC treated with rDiACT or rDiFBAL + PLG than that obtained in the control cells ($p < 0.05$) (Figure 15C). In addition, treatment with rDiFBAL + PLG shows an activation of the latent form of the MMP-9 in the CnAOEC culture media (show by a clear band of 82 kDa), which does not appear with other treatments (Figure 15C). No significant differences in the MMP-9 levels in the culture media of both types of cultures in the case of DiES experiment (Figures 15A and 15B) or in smooth muscle cells cultures in the case of recombinant proteins experiments (Figure 15D) were observed.

CONCLUSIONS

D. immitis produces chronic infections characterized by the persistence of its adult worms in the vascular system of its host. There, the parasites are exposed to a wide range of defense mechanisms which are highly aggressive for their integrity. One of these mechanisms is the generation of thromboembolisms, a process physiologically regulated by the fibrinolytic system, whose final product, plasmin, is capable of degrading fibrin clots.

In the present doctoral dissertation, we demonstrate how *D. immitis* is capable of modifying this route towards the generation of plasmin through the use of different antigens of the host/parasite interface. This fact would imply a benefit for both the host and the parasite, as it would enable them to maintain an antithrombotic state in the immediate vascular medium of *D. immitis*. Due to this fact and given that plasmin has been related with the lysis of extra-cytoplasmic matrices, a fact which has been interpreted as a mechanism related to cell invasion and intra-organic migration, the activation of fibrinolysis has been historically considered as a beneficial mechanism for the survival of blood-borne pathogens and also for its invasive capacity.

Nevertheless, the great number of substrata on which plasmin can carry out its proteolytic function has shown, in other contexts, the implication of the activation of the

fibrinolytic system in different pathological processes on a vascular level. We demonstrate a direct relationship between the plasmin derived from the pro-fibrinolytic capacity of the antigens of *D. immitis* with the proliferation and migration of the host's cells located in the arterial wall; as well as the degradation of the extracellular matrix in an "in vitro" model. These mechanisms are directly related with proliferative endarteritis, a key pathological process in the development of the subsequent pulmonary and cardiac pathology in cardiopulmonary dirofilariosis, resulting in the formation of intravascular microvilli.

From the findings obtained in the present doctoral dissertation, we derive the following conclusions:

1. *D. immitis* activates the fibrinolytic system of its host through the joint action of its excretory/secretory and surface antigens. This could be used by the parasite to displace the fibrinolytic balance towards the generation of plasmin, which would imply a change of the survival mechanism of the parasite in order to control the formation of clots in its immediate intravascular habitat.

2. Ten and 11 plasminogen-binding proteins are respectively identified in the excretory/secretory and surface antigenic compartments of the parasite. Of these, actin, fructose-bisphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenase and galectin of *D. immitis* have been produced in their recombinant form, individually demonstrating their pro-fibrynolitic properties. All of them, in a greater or lesser extent, are capable of binding plasminogen and enhancing the generation of plasmin through the implication of its lysine residues. Furthermore, they are capable of stimulating the expression of fibrinolytic activators in cultures of canine endothelial cells, and are located in the host/*D. immitis* interface.

3. Plasmin, a product of the fibrinolytic activation caused by the antigens of *D. immitis*, participates in the generation of the pathologic processes described in the apparition of proliferative endarteritis in cardiopulmonary dirofilariosis. This includes the proliferation and migration of the arterial wall cells, as well as the degradation of the extracellular matrix, all these facts being demonstrated

through the use of both the parasite's excretory/secretory antigens and the two studied proteins with a higher pro-fibrinolytic capacity in an "in vitro" model.

4. The results obtained in the present doctoral dissertation contribute to the understanding of a very complex part of the relationships between parasite and host on a molecular level in cardiopulmonary dirofilariosis, demonstrating for the first time how a process related to the survival of the parasite can unleash pathogenic mechanisms of great importance. Given that the capacity of binding plasminogen and enhancing the generation of plasmin has been demonstrated in the proteins of many pathogens, and taking into account the high degree of evolutionary conservation of some of the studied antigens, similar mechanisms could occur in other infections caused by blood-borne pathogens capable of developing chronic processes.