Genética en la inflamación, Apoptosis y otras vías de Muerte Celular en el Desprendimiento de Retina y Vitreorretinopatía Proliferante

Proyecto Retina-4

Salvador Pastor Idoate

Tesis Doctoral

Directores:
Dra. María Jimena Rojas
Dr. Rogelio González Sarmiento
Dr. José Carlos Pastor Jimeno

SEPTIEMBRE 2015

a mi familia,

A EVA,

En dos palabras puedo resumir cuanto he aprendido acerca de la vida: Sigue adelante!

– Robert Frost –

Universidad de Valladolid

INSTITUTO UNIVERSITARIO DE OFTALMOBIOLOGÍA APLICADA DE LA UNIVERSIDAD DE VALLADOLID

TESIS DOCTORAL:

GENÉTICA EN LA INFLAMACIÓN, APOPTOSIS Y OTRAS VÍAS DE MUERTE CELULAR EN EL DESPRENDIMIENTO DE RETINA Y LA VITREORRETINOPATÍA PROLIFERANTE. PROYECTO RETINA 4

(Genetics of Inflammation, Apoptosis Pathway and Other Cell Death Mechanisms in Retinal Detachment and Proliferative Vitreoretinopathy. The Retina 4 Project)

Presentada por D. SALVADOR PASTOR IDOATE para optar al grado de Doctor por la Universidad de Valladolid

Dirigida por:
DR. JOSÉ CARLOS PASTOR, DRA. JIMENA ROJAS, DR. ROGELIO GONZALEZ SARMIENTO

2015

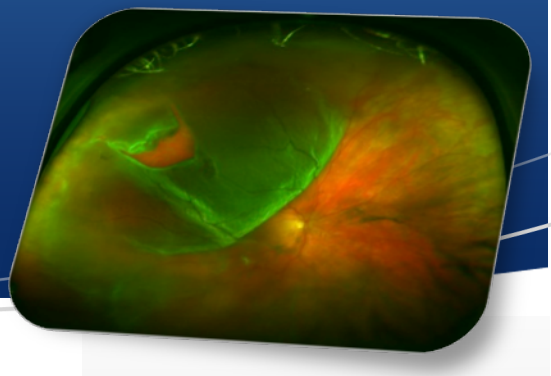
TESIS DOCTORAL SEPTIEMBRE 2015

Genética en la Inflamación, Apoptosis y otras vías de Muerte Celular en el Desprendimiento de Retina y la Vitreorretinopatía Proliferante

Proyecto Retina-4

DO NOT ONLY PRACTICE YOUR ART,
BUT FORCE YOUR WAY INTO ITS SECRETS,
FOR IT AND KNOWLEDGE CAN
RAISE MEN TO THE DIVINE

LUDWIG VAN BEETHOVEN



Certifican:

Que el licenciado Don. **Salvador Pastor Idoate**, alumno del programa de doctorado en Ciencias de la Visión del I.O.B.A, ha realizado bajo la dirección la presente Tesis Doctoral por la cual opta al título de Doctor en Medicina, titulada "Genética en la Inflamación, Apoptosis y Otras Vías de Muerte Celular en el Desprendimiento de Retina y la Vitreorretinopatía Proliferante. Proyecto Retina-4". Y que una vez revisado el presente manuscrito autorizan la presentación del mismo considerando que es apto para su defensa.

Conforme	Conforme	Conforme
María Jimena Rojas	**Rogelio González Sarmiento**	**José Carlos Pastor Jimeno**

Valladolid, 30 Septiembre de 2015

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Instituto de Oftalmobiología Aplicada

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Todo esto empezó, a finales del 2010, cuando transmití mi deseo y mi inquietud de formar parte de una línea de investigación llamada "Retina 4 Project". Durante estos últimos 5 años mis directores de tesis han confiado en mi, me han transmitido toda su sabiduría y han puesto todos los medios a su alcance para que yo pudiese formarme y terminase esta aventura.

Los tres han "tirado" de mi y me han ayudado en las ocasiones en las que a todo doctorando, a pesar de estar disfrutando del trabajo que esta llevando a cabo, le ronda en la cabeza la idea de abandono.

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Al Dr. José Carlos Pastor Jimeno

«Padre, Amigo y Tutor de Tesis»

Tendría que darte las gracias por multitud de cosas, además sería injusto resumir todo lo que me has ayudado desde que nací en unas pocas líneas. Sin embargo aprovecharé la ocasión para darte las gracias por algo muy especial y que nunca antes te había agradecido. Gracias por ser «*mi maestro*» al igual que "D. Diego" lo fue para ti en una ocasión. Gracias por ser mi referente. Y gracias por ayudarme a "colarme" en la Liga de los "Dres. Pastor" (sé que el abuelo estaría orgulloso de todos nosotros).

"He´s the best physician that knows the worthlessness of the most medicines"

Benjamin Franklin

Saltar de la clínica al laboratorio y empezar a trabajar con las pipetas, campana de flujo, termoclicladores y PCRs no es tarea fácil sin el apoyo de compañeros de alegrías y fatigas. Gente que comparte contigo su conocimiento, que te entiende, te apoya y te aconseja.

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"Coming together is a beginning; keeping together is Progress; working together is success "

Henry Ford

TESIS DOCTORAL **SEPTIEMBRE 2015**

"IT IS ONE OF THE BLESSINGS OF OLD FRIENDS THAT YOU CAN AFFORD TO BE STUPID WITH THEM."

RALPH W. EMERSON

A los compañeros/as residentes y adjuntos en Oftalmología del Hospital Clínico Universitario de Valladolid y en especial a aquellos que me han apoyado, ayudado y han sabido entenderme durante mi años de formación y durante los años de la tesis. Con especial cariño a los **Dres. Enrique de la Rua, Lucia Manzanas y Maribel López** y a los **médicos Lucia González Buendía, Santiago Delgado Tirado, Yrbani Lantigua y David Peña.**

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Retina 4 Project
PVR Study Group
Colaboraciones Internacionales

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«Y con especial cariño a mi Madre» Gracias!

"A MOTHER IS NOT A PERSON TO LEAN ON BUT A PERSON TO MAKE LEANING UNNESSARY "

DOROTHY CANFIELD

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Este proyecto se ha desarrollado en el seno del Red Temática de Investigación Cooperativa Sanitaria 03/13 del Instituto de Salud Carlos III (Madrid, Spain)

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Publicaciones Científicas

- Pastor-Idoate S, et al. Adrenocortical adenoma and central serous chorioretinopathy: a rare association? Case Report Ophthalmol.2011;2(3):327-32.
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SERV Sociedad Española de Retina y Vítreo

ARVO The Association for Research in Vision and Ophthalmology

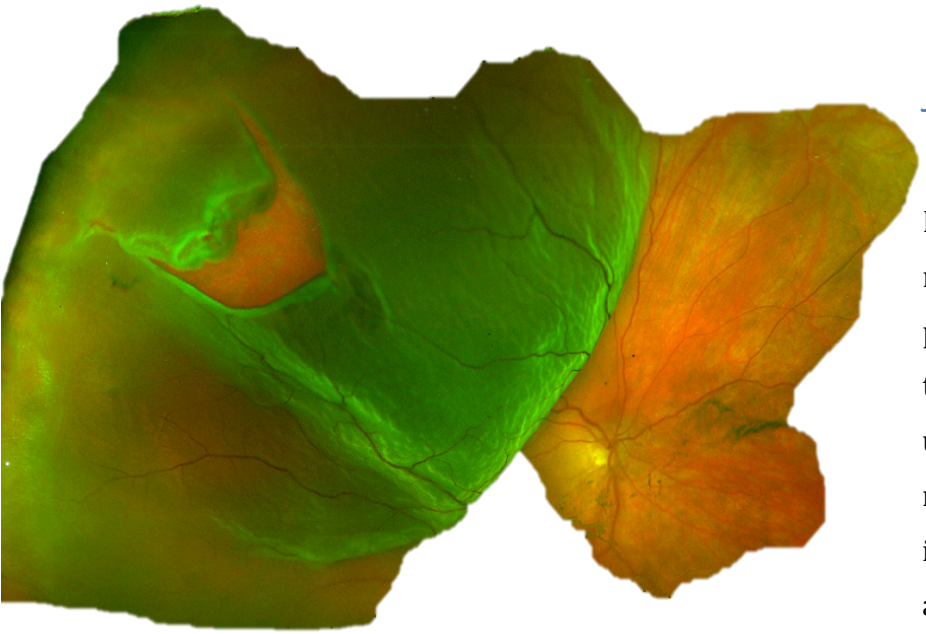
Allergan

Abreviaturas

- **VRP:** *vitreorretinopatía proliferante*
- **DRR:** *desprendimiento de retina regmatógeno*
- **DR:** *desprendimiento de retina*
- **ERP:** *epitelio pigmentario de la retina*
- **BCL-2:** familia de células de linfoma B-2
- **NF-κB:** *factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas*
- **TNFA:** *factor de necrosis tumoral alfa*
- **BHR:** *barreras hemato-retinianas*
- **IL:** *interleuquinas*
- **INF-γ:** *interferón gamma*
- **MCP-1:** *proteína quimioatrayente de monocitos 1*
- **MEC:** *matriz extracelular*
- **PDGF:** *factor de crecimiento derivado de plaquetas*
- **TGF-β:** *factor de crecimiento transformante-β*
- **EGF:** *factor de crecimiento epidérmico*
- **CTFG:** *factor de crecimiento de tejido conectivo*
- **MMP:** *metaloproteinasas*
- **SMADs:** *madres contra decapentaplegic homólogos*
- **TIMP1:** *inhibidor tisular de la metaloproitenasa-1*
- **TNFR:** *receptores de la familia TNF*
- **ROS:** *especies reactivas de oxigeno*
- **HB-EFG:** *factor de crecimiento ligado a heparina*
- **RIP:** *proteína de interacción con receptor*
- **RE:** *retículo endoplasmático*
- **ERSMP:** *vía programada de estrés del retículo endoplasmático*
- **HGF:** *factor de crecimiento hepatocitario*
- **FGF:** *factor de crecimiento fibroblástico*
- **IGF:** *factor de crecimiento insuíinico*
- **VEGF:** *factor de crecimiento vascular endotelial*
- **MDM2:** *gen homologo murino duplo minuto-2*
- **SF6:** *hexafluoruro de azufre*
- **SNPs:** *polimorfismo de nucleótido simple*
- **LTA:** *linfotoxina alfa*
- **RNAm:** *ácido ribonucleico mensajero*
- **RNA:** *ácido ribonucleico*
- **ADN:** *ácido desoxirribonucleico*
- **ADNc:** *ácido desoxirribonucleico complementario*
- **BAX:** *gen regulador apoptótico BAX*
- **DMAE:** *degeneración macular asociada a la edad*
- **NK:** *células natural killers*
- **RT-PCR:** *reacción en cadena de polimerasa con transcriptasa inversa*
- **PIK3C-γ:** *Fosfoinositol 3 Kinasa subunidad catalítica-γ*
- **pCEFL-Flag:** *vector de expresión plasmídico con proteína bandera de etiquetado*
- **COS:** *fibroblast-like cell derivada de riñón de mono*
- **GAPDH:** *Gliceraldehído-3-fosfato deshidrogenasa*
- **PCR-RFLP:** *reacción en cadena de polimerasa con polimorfismos de longitud de fragmentos de restricción*
- **AIC:** *criterio de información de Akaike*
- **UV:** *Luz ultravioleta*
- **FITC:** *conjugados con isotiocianato de fluoresceína*

INDICE

INDICE



JUSTIFICACIÓN

El desprendimiento de retina regmatógeno (DDR) es un proceso agudo que necesita tratamiento quirúrgico y urgente, y que en estos momentos se presenta con incidencia anual de aproximadamente, de 1 a 12 casos sobre 100.000 individuos, con un riesgo individual de padecerlo en el curso de la vida del 0.6% (Mitry D et al 2010).

Su incidencia está en continuo aumento, debido a diversos factores que predisponen a su aparición, entre los que destacan: el envejecimiento poblacional y el incremento en el número de cirugías de cataratas (Erie JC et al 2006, Pastor JC et al 2008). Así por ejemplo, se calcula que el riesgo de desarrollar un DRR se incrementa hasta 5,5 veces a los 10 años de haber sido intervenido de cirugía de cataratas (Erie JC et al 2006, Pastor JC et al 2008).

La vitreo-retinopatia proliferante (VRP), sigue siendo la principal causa de fracaso en la cirugía del desprendimiento de retina (DR) (Machemer R et al 1991) y se calcula que cerca de un 5-10% de los pacientes con DRR desarrollarán VRP, representando aproximadamente el 75% de los casos del fracaso de la cirugía (Machemer R et al 1991, de la Rúa E et al 2008). Identificada como una entidad clínica independiente en 1983 (Retina Society Terminology Committee Classification. 1983), todavía no ha sido posible, todavía, comprender de forma adecuada su fisiopatología, no se ha conseguido disminuir su incidencia, ni existe

un tratamiento efectivo que prevenga de recidivas, a pesar de los numerosos estudios realizados en busca de posibles dianas terapéuticas (Asaria RH et al 2006, Pennock S et al 2011, Pennock S et al 2014), durante más de 40 años de investigación. Por ello, su manejo implica en la mayoría de los casos una repetición de los procedimientos quirúrgicos, con un incremento significativo de los costes sanitarios (Patel NN et al 2004) y sobre todo con pobres resultados anatómicos y funcionales (Silicone Study Group. 1991a, Silicone Study Group. 1991b, Lewis H et al 1991a, Lewis H et al 1991b, Pastor JC et al 2002, Aaberg T.M Sr. 2010, Sadaka A et al 2012).

Por otro lado, el diagnóstico de la VRP sigue siendo eminentemente clínico y en estadios que se consideran tardíos. Son numerosos los estudios previos realizados para predecir el riesgo de desarrollo de VRP basándose en las características clínicas de los pacientes con DR habiéndose desarrollado incluso formulas predictivas. Desafortunadamente, los resultados obtenidos hasta ahora no son consistentes entre si, y las fórmulas basadas en estos factores han demostrando una escasa utilidad para su uso clínico diario (Rojas J et al 2009). De hecho, solamente cuando se añaden otras variables, tales como factores genéticos o biomarcadores, el rendimiento predictivo de los modelos mejora (Rojas J et al 2013, Rojas J et al 2015). Teóricamente, la identificación de estos factores podría ser útil para identificar aquellos pacientes con alto riesgo, reduciendo el tamaño de la muestra de nuevos ensayos clínicos y además permitiría a la selección de una población de sujetos mucho más homogénea. Además ayudaría a elucidar alguno de los mecanismos de su patogénesis, pudiendo así proporcionar datos útiles para el manejo del DR y la VRP (Rojas J et al 2013).

Durante los últimos años, se han identificado diversos factores implicados en pérdida de función visual tras un DR. Así, se reconoce claramente que la muerte de los fotorreceptores tras el DR (Cook B et al 1995, Fisher SK et al 2005, Rosenbaum D et al 2010, Lo AC et al 2011, Dong K et al 2014), es un evento fundamental para la pérdida de agudeza visual que sufren estos pacientes, así como la existencia de otras modificaciones celulares, que afectan significativamente a la recuperación funcional tras un DR (Fisher SK et al 2005). Por lo que en estos momentos, los

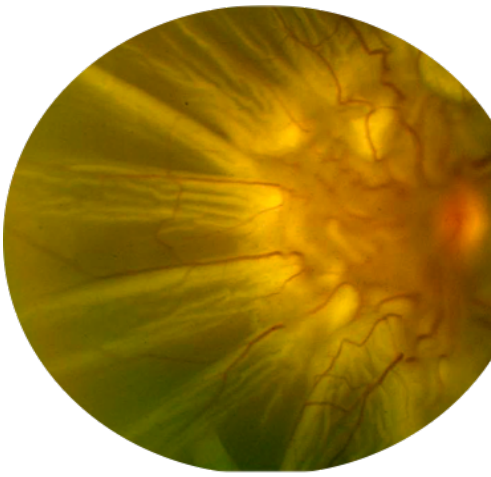
esfuerzos de la investigación se centran en el desarrollo de tratamientos farmacológicos, basados fundamentalmente en la neuroprotección, como adyuvantes del procedimiento quirúrgico del DR, con la finalidad de reducir los fenómenos de degeneración y de muerte celular (Sadaka A et al 2012). Sin embargo, no parecería justificado en términos económicos y de seguridad la administración sistemática de un fármaco neuroprotector en todos los pacientes con DR, ni con el fin de prevenir un posible desarrollo de VRP, cuya prevalencia habitual es de un 10% de los DR (Machemer R et al 1991), ni con el fin de prevenir una pérdida importante de visión tras la cirugía de un DR. Por el contrario, un sistema diagnóstico, basado en biomarcadores y factores clínicos podría detectar aquellos pacientes de alto riesgo tras una cirugía de DR, restringiendo los casos en los que asociar un tratamiento adyuvante a la cirugía.

Dentro de esta segunda etapa del *Proyecto Retina-4*, a la que se ha incorporado la Unidad de Medicina Molecular del I.B.S.A.L de la Universidad de Salamanca, se han analizado las posibles implicaciones funcionales del polimorfismo de nucleótido simple (SNPs): rs222909 dentro del gen de *la Linfotoxina alfa* (LTA), previamente asociaciado con la enfermedad (Rojas J et al 2010), y se han conseguido identificar nuevos marcadores de riesgo para la VRP, relacionados con en el control del ciclo celular y en la apoptosis, incluyendo: el SNP rs1042522, ubicado en el gen p53, el rs2279744, ubicado en el gen MDM2 y los SNPs rs4645878 y rs2279115, ubicados en los genes BAX y BCL-2, respectivamente.

Estos nuevos biomarcadoes, no sólo han aportado nuevos datos sobre los mecanismos de la patogénesis, de la VRP (Pastor JC et al 2015), si no que además pueden permitir, tras su validación, formar parte del sistema diagnostico, mejorando la identificación de aquellos pacientes con alto riesgo de desarrollar VRP tras un DR, y abriendo nuevos caminos hacia posibles dianas terapéuticas tanto para el DR como para la VRP.

ESTADO ACTUAL DEL TEMA

Concepto de VRP



La VRP es una complicación de los DDR, considerada actualmente como un proceso de *cicatrización exagerado* de la retina que es inducido por una rotura retiniana, en donde los procesos de muerte celular, inflamatorios y/o fibróticos parecen tener un papel fundamental en el desarrollo de la patogénesis de la misma (Pastor JC. 1998, Pastor JC et al 2002).

Este cuadro clínico se puede producir espontáneamente después de un DRR (causado por una rotura retiniana ej. desgarro o agujero retiniano) y/o mas frecuentemente, tras su reparación quirúrgica, y está caracterizado por la formación de membranas pre-retinianas y sub-retinianas, cambios glioticos intra-retinianos, y la posterior contracción de las membranas, lo que se asocia a resultados anatómicos y funcionales pésimos (Pastor JC. 1998, Pastor JC et al 2002). Sigue constituyendo la principal causa de fracaso en la cirugía reparadora del desprendimiento de retina (DR) (Machemer R et al 1991), con una prevalencia del 5% al 10% de los casos de DDR, representando aproximadamente el 75% de los casos del fracaso de la cirugía del DR (Machemer R et al 1991, de la Rúa E et al 2008). A pesar de ser un proceso dinámico se sabe muy poco acerca de su cronobiología, pudiendo aparecer antes de la cirugía, aunque como se ha dicho esta complicación suele ocurrir fundamentalmente tras cualquier procedimiento quirúrgico utilizado para reparar el DR (Pastor JC et al 2002, Pennock S et al 2014).

Presentación clínica y diagnóstico de la VRP

La VRP puede aparecer en ojos, en los que el desprendimiento permanece sin tratar durante semanas o meses, aunque, típicamente suele presentarse de 4 a 12 semanas después de cualquier procedimiento quirúrgico (Pastor JC et al 2002, Pennock S et al 2014). El primer signo de VRP es la aparición de células que proliferan en la cavidad vítrea (Pennock S et al 2014). Este proceso es seguido por la proliferación de esas células tanto en la interfase vitreorretiniana como en la

superficie de la retina, y por cambios en las neuronas y en las células de la glia de la neuroretina (Pastor JC et al 2006), creando membranas epi, intra o sub-retinianas y una gliosis reactiva que afecta a la propia retina. Estas membranas pueden eventualmente contraerse dando lugar a pliegues y arrugas de la retina. Al progresar la VRP, se ejerce un aumento en la tracción retiniana, conduciendo a un incremento en la rigidez del tejido, a mayores pliegues y a un acortamiento de la superficie y volumen retiniano (Pastor JC et al 2002). Estos cambios generalmente conducen a nuevos DR, ya sea, "re-abriendo" roturas previamente tratadas o por la generación de nuevas roturas en zonas distintas de la retina. En el caso de la denominada VRP posterior el proceso suele terminar con una configuración de "DR en embudo", mientras que la denominada VRP anterior suele conducir a la formación de membranas ciclíticas e hipotonía (Pennock S et al 2014). En cualquiera de los casos, el riesgo de desarrollar una *ptisis bulbi* es elevado.

Actualmente, el diagnóstico de la VRP sigue siendo eminentemente clínico mediante la visualización directa de la retina en la biomicroscopía posterior con lámpara de hendidura o con el oftalmoscopio invertido o con pruebas indirectas como la tomografía de coherencia óptica, la retinografía o la ultrasonografía modo B. Estas ultimas pruebas son útiles para delimitar la extensión y localización del DR, de los pliegues retinianos, valorar la tracción de las membranas y la rigidez retiniana, sobre todo en los casos en los que hay opacidad de medios (Pennock S et al 2014).

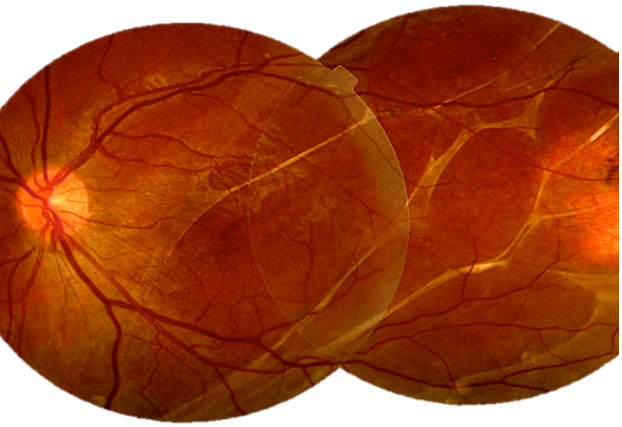
Patogénesis de la VRP

A pesar de que fue identificada como una entidad clínica independiente en 1983 (Retina Society Terminology Committee Classification. 1983), y de los avances tecnológicos que ha vivido la Oftalmología en las últimas décadas, aún no ha sido posible comprender de forma adecuada la fisiopatología de esta entidad, ni se ha conseguido disminuir la incidencia de la misma con un tratamiento profiláctico adecuado. Por lo que su manejo implica en la mayoría de los casos una repetición de los procedimientos quirúrgicos con un incremento significativo de los costes sanitarios (Patel NN et al 2004) y sobre todo la obtención de pobres resultados

anatómicos y funcionales (Silicone Study Group. 1991a, Silicone Study Group. 1991b, Lewis H et al 1991a, Lewis H et al 1991b).

De acuerdo con la descripción original, la consecuencia final de este proceso patológico es: la *formación de membranas contráctiles* en la interfase vitreorretiniana y en la superficie de la retina. La posterior contracción de estas membranas causa distorsión retiniana, lo que conduce en la mayoría de los casos a un nuevo desprendimiento de retina y aparición de nuevas roturas, transformando un DRR en un DR de tipo traccional.

La patogénesis de esta enfermedad podría resumirse en los siguientes pasos:



1) Migración de células, principalmente células gliales y del epitelio pigmetario de la retina (ERP);
2) proliferación de las células migratorias;
3) desarrollo de membranas;
4) contracción de las membranas celulares;
5) producción de colágeno extracelular;
6) creación de pliegues “fijos” en la retina.

(Cuadro patogénico modificado del artículo: Pastor JC et al 2015)

Los autores de esta clasificación hicieron hincapié en que la proliferación celular parecía ser el mecanismo patogénico clave de esta entidad (Retina Society Terminology Committee Classification. 1983). Basándose en la idea de que la proliferación es la principal característica de la VRP, muchos investigadores han intentado evitar durante más de 40 años esta entidad, fundamentalmente Inhibiendo la proliferación celular, pero a día de hoy la VRP, sigue siendo un problema sin resolver.

Proceso de curación-reparación en la retina

El proceso de formación de la VRP muestra similitudes con el proceso dinámico de curación y reparación de las heridas, que tiene lugar en otros tejidos y órganos de la economía. Aunque muchos de los mecanismos involucrados tienen lugar de forma simultánea y se superponen, su desarrollo puede ser dividido en tres etapas: *inflamación, proliferación y modulación del tejido cicatricial* (Weller M et al 1990a, Weller M et al 1990b, Pastor JC. 1998, Garweg JG et al 2013).

Sin embargo, las causas que inician exactamente una normal curación, reparación y posterior remodelación retiniana tras una rotura retiniana o el desarrollo de la VRP, incluso después de una cirugía de DR exitosa, es todavía una cuestión sin resolver. Ya que además de situaciones clínicas mas o menos identificadas, hay cada vez más estudios que sugieren una predisposición genética en el desarrollo de la VRP que podría ser una de las piezas claves en el desarrollo de diferentes respuestas reparadoras tras una rotura retiniana (Sanabria Ruiz-Colmenares MR et al 2006, Rojas J et al 2010, Rojas J et al 2013, Pastor-Idoate S et al 2013a, Pastor-Idoate S et al 2013b, Pastor-Idoate S et al 2015).

Mecanismos de daño tisular desencadenados por el DR

En un DRR, el trauma creado por la rotura retiniana y la posterior separación de la neuro-retina de la capa de células del EPR, inicia una respuesta de curación-reparación, principalmente ejercida a niveles bioquímicos y celulares, con liberación de citoquinas y migración celular (Asaria RHY et al 2006).

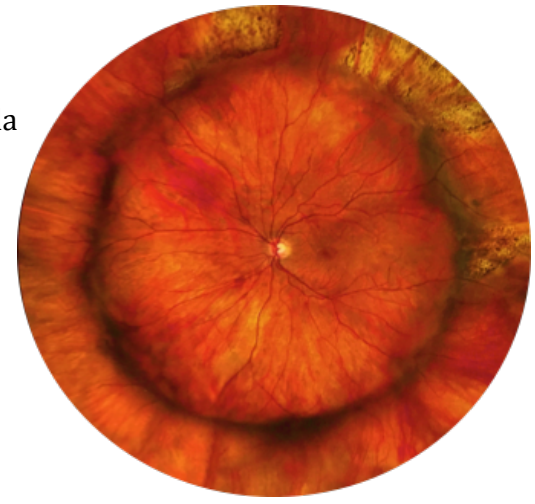


Foto modificada del Atlas de Retina (The Retinal Atlas. Yannuzzi. Elsevier 2010)

Etapa de isquemia, muerte celular e inflamación:

Como se ha dicho los procesos desencadenados por el DR no ocurren de manera consecutiva, sino que en realidad ocurren de manera simultanea solapándose unos con otros. Hay estudios que sugieren que la desregulación en alguno o varios, de estos tres procesos podría ser la clave en la patogénesis y desarrollo de la VRP.

La separación de la neuro-retina de la capa de células del EPR causada por el liquido subretiniano, de origen vítreo produce un stress mecánico en el área afectada, produciendo un estado de relativa hipoxia en las capas más externas de la retina debido a su separación del lecho vascular coroideo, induciendo la muerte de los fotoreceptores por necrosis y apoptosis, lo cual contribuye a la perdida de visión (Erikson PA et al 1983, Cook B et al 1995, Abouzeid H et al 2006, Sadaka A et al 2012).

Histológicamente, las primeras manifestaciones microscópicas incluyen la acumulación de líquido sub-retiniano con desorganización y pérdida de los segmentos externos de los foto-receptores, edema intra-retiniano (formación de quistes mayoritariamente en la capa nuclear interna) con la subsiguiente migración hacia las capas más externas a medida que persiste el DR, alargamiento de las células del EPR separadas de la membrana de Bruch y reactividad de las células de Müller y de otras células gliales de la retina (Geller SF et al 2001, Ghazi NG et al 2002, Abouzeid H et al 2006). En este proceso, las células gliales tienen un papel importante, y la remodelación tisular implica la hipertrofia de las celulas de Müller. Se han descrito cambios en estas células tan solo un día después de producirse el DR (Wickham L et al 2009). Dentro de los 3 primeros días, los principales cambios se caracterizan por la migración de los cuerpos de las células de Müller hacia las capas plexiforme y nucleares externas, ocupando los espacios dejados por los foto-receptores, extendiendo sus procesos hacia el espacio sub-retiniano (Wickham L et al 2009). Estas células, junto con células del EPR, la microglia y los macrófagos, contribuyen a crear los cambios intrarretinianos de la VRP (Pastor JC et al 2006). Aunque estos cambios no son específicos de la VRP,

conducen a un acortamiento de la retina lo que constituye la más forma grave de VRP (Pastor JC et al 2003, Sethi CS et al 2005).

En casos de desprendimiento crónico, los cambios más importantes incluyen: quístes y degeneración macroquística de la retina, adelgazamiento y acortamiento retiniano, alteraciones del EPR, líneas de demarcación, drusas grandes, neovascularización coroidea en la ora serrata (Kaba F et al 1987) y en el iris cambios secundarios a la isquemia retiniana por el DR.

Las capas externas de la retina obtienen su nutrición de la capa coriocapilar. La separación del soporte nutritivo al interrumpirse el complejo foto-receptor-EPR tras un DR impide la difusión de importantes recursos metabólicos conduciendo a respuestas degenerativas y apoptóticas, principalmente en los foto-receptores. Aunque hoy en día, no existe un método específico para valorar y cuantificar la isquemia en las capas externas de la retina, hay estudios que han descrito que la hipoxia y la hipoglucemia provocada tras el DR están entre las causas de las modificaciones apoptóticas y degenerativas de la misma (Mervin K et al 1999). Y cómo la creación de condiciones de hiperoxia, tras la inducción de un DR experimental, permite una reducción en la proliferación, en los cambios morfológicos de células de Müller y en la desregulación del ciclo del glutamato (Lewis G et al 1999). También, se ha publicado que la isquemia retiniana es uno de los principales factores activadores de genes implicados en la muerte celular como es el p53 (factor regulador de la apoptosis) (Rosenbaum DM et al 1998, Lo AC et al 2011), así como de otros mediadores involucrados en la muerte celular tras la isquemia retiniana, como son: las endo-nucleasas (Rosenbaum DM et al 1997), las caspasas (Singh M et al 2001) y la familia de células de linfoma B-2 (Bcl-2)(Kaneda K et al 1999, Hahn P et al 2003, Yang L et al 2004, Zhang C et al 2002), y cuyo papel en desarrollo de la VRP aún no ha sido completamente estudiado.

Sorprendentemente, a pesar del las altas demandas metabólicas que tiene la neuro-retina, no se produce una inmediata muerte neuronal debido sobre todo a la activación de mecanismos intrínsecos de defensa. Especialmente, los genes implicados en la supervivencia y determinadas vías de señalización celular, como

por ejemplo: la vía *factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas* (NF-κB), desencadenadas en situaciones de estrés celular, que logran la supervivencia de los fotoreceptores y células neuronales en las fases agudas del DR (Zacks DN et al 2006). Y existen mecanismos inherentes de resistencia del EPR frente a estímulos desencadenantes de apoptosis como los bajos niveles intracelulares de caspasa-8 o la resistencia a las señales inducidas por *el factor de necrosis tumoral alfa* (TNFA) (principal activador de la vía extrínseca de la apoptosis) y que podrían jugar un papel clave en la supervivencia y posterior migración y proliferación de estas células en condiciones oculares como la VRP o la retinopatía diabética proliferante (Yang P et al 2005). Sin embargo, los factores responsables de la indeseable supervivencia, migración hacia la cavidad vítrea y proliferación de células del EPR en la VRP no han podido ser, todavía, claramente definidos. La persistencia de los procesos isquémicos y del estrés oxidativo conduce a un fracaso de estos mecanismos protectores con la consiguiente muerte celular, principalmente mediante apoptosis (Cook B et al 1995, Ghazi NG et al 2002).

El DDR, además provoca la rotura de las barreras hemato-retininas (BHR), lo que activa la migración y proliferación celular, involucrando principalmente a las células del EPR pero también a otras células como: astrocitos, células de Müller, fibroblastos y mio-fibroblastos (Vinores SA et al 1990). Esto origina también la atracción de células inflamatorias como los macrófagos, los linfocitos y los polimorfonucleares, así como de células implicadas en el proceso de curación-reparación de heridas como las plaquetas (Wiedemann P. 1992, Pastor JC. 1998), con la consiguiente liberación de citoquinas inflamatorias como las *interleuquinas* (IL-1, IL6, IL8, IL-10), el *interferón gamma* (INF-γ) o la *proteína quimioatrayente de monocitos-1* (MCP-1), así como diversos factores de crecimiento al espacio vítreo (Rouberol F et al 2014, Pennock S et al 2014). Se ha descrito que la rotura de las BHR continua una vez re-aplicada la retina mediante cirugía (Oshika T. 1990a, Oshika T. 1990b). La presencia de células inflamatorias en el vítreo, en especial los macrófagos, se ha asociado con un mayor riesgo de desarrollo de la VRP. En 2003, el grupo de investigación del I.O.B.A demostró que los macrófagos eran más frecuentes en el vítreo de pacientes que habían desarrollado VRP después de una

cirugía de DR (Martin F et al 2003). También se ha descrito la presencia de estas células en las cercanías de los vasos retinianos y entre las diferentes capas de la retina en los pacientes que desarrollaron VRP (Pastor JC et al 2006). Además, se ha publicado que los macrófagos no sólo participan en la génesis y mantenimiento de la inflamación liberando factores pro-inflamatorios sino que también participan en los procesos de muerte celular de los foto-receptores mediante la apoptosis a través de la MCP-1 (Nakazawa T et al 2007).

La presencia y concentración de diversas citoquinas se han propuesto como biomarcadores para predecir la gravedad y desarrollo de la VRP (Kon CH et al 1999, Kon CH et al 2000, Rusnak S et al 2013). Sin embargo, la presencia de estas citoquinas no es un fenómeno especifico de la VRP ya que por ejemplo se han encontrado en DR sin VRP, así como en otras patologías intraoculares como la retinopatía proliferante (Rusnak S et al 2013, Pennock S et al 2014).

Tras un desprendimiento que evoluciona a VRP, las células del EPR migran hacia la cavidad vítrea y se de-diferencian (Anderson DH et al 1981, Heriot WJ et al 1992), dentro de una matriz extracelular (MEC) provisional que contiene colágeno, fibronectina, tromboplasmina y otras proteínas de la matriz (Wiedemann P. 1992). Se han identificado numerosas señales responsables de esta migración y proliferación de las células del EPR, incluyendo la perdida de contacto celular, señales de procedentes de los fotoreceptores, factores presentes en el vítreo y otras señales procedentes de las células inflamatorias.

La rotura de la membrana limitante interna permite a las células gliales migrar hacia los dos lados de la retina desprendida, que también sufren un proceso de des-diferenciación (Mandelcorn MS et al 1975, Mueller-Jensen K et al 1975) y de transformación hacia miofibroblastos (Iandiev I et al 2010), cuya adherencia se ve favorecida por la secreción de elastina, fibronectina y fibrina.

Durante esta primera fase, como ya se ha mencionado, se liberan al vítreo diversos factores de crecimiento, los cuales ejercen sus funciones durante la fase inflamatoria, siendo algunos de los más importantes: el *factor de crecimiento*

derivado de plaquetas (PDGF), el *factor de crecimiento transformante-β* (TGF-β), el *factor de necrosis tumoral alfa* (TNFA), el *factor de crecimiento epidérmico* (EGF) *ó el CCN2 ó* el *factor de crecimiento de tejido conectivo* (CTGF).

El PDGF posee una marcada acción quimiotáctica y mitogénica para las células mesenquimales y de la glía. También induce la migración de las células del EPR (Cui JZ et al 2007) y a su vez estimula la síntesis y depósito de colágeno (Wiedemann P. 1992). Muchos grupos de investigación han asociado su presencia al desarrollo de la VRP. Los niveles de PDGF en vítreo se mantienen constantes durante todo el proceso de VRP (Cui JZ et al 2007) y además es superior al de otros pacientes con patologías oculares distintas al de la VRP (Baudouin C et al 1993, Cassidy L et al 1998, Kon CH et al 1999, Cui JZ et al 2007, Lei H et al 2009, Pennock S et al 2011).

Además, también se ha descrito que los niveles de PDGFs se incrementan en los modelos experimentales que desarrollan VRP (Lei H et al 2009, Pennock S et al 2011), y que se encuentran en las membranas de VRP así como receptores de PDGF, que están activados (Robbins SG et al 1994, Cui J et al 2009, Lei H et al 2010). De todos los factores de crecimiento y citoquinas medidas hasta la fecha, el PDGFs es el que ha mostrado los niveles más altos tanto en pacientes con VRP como en los modelos experimentales (Lei H et al 2007, Pennock S et al 2011).

El TGF-β es liberado por las plaquetas y por los macrófagos activados, aunque también es sintetizado por las células del EPR. Este factor, es activado por la Trombospondina-1, constituyente de la MEC (Crawford SE et al 1998) y por las *metaloproteinasas-2* (MMP2) y *9* (MMP9) (Yu Q et al 2000), enzimas responsables de la degradación de la MEC. El TGF-β, actúa como un factor autocrino negativo e induce la expresión de PDGF (Nagineni CN et al 2005). Además es un potente agente quimiotáctico para monocitos y fibroblastos e induce la formación de la MEC (Wiedemann P. 1992). Los efectos del TGF-β estan mediados por unas proteínas reguladoras llamadas *madres contra decapentaplegic homólogos* (SMADs), que actúan como mediadores directos de la activación o supresión de la transcripción producida por esta citoquina (Böttner M et al 2000).

Las más implicadas en los mecanismos pro y antifibroticos son la *3* (SMAD-3) (media la mayoría de las acciones profibroticas del TGF-β (Flanders KC. 2004)) y la *7* (SMAD-7). La proteína SMAD-7 ha sido implicada en la degradación del receptor del TGF-β (Kavsak P et al 2000) y su gen ha sido identificado como potencial diana terapéutica en la VRP (Saika S et al 2007). Este gen ha sido estudiado por nuestro grupo, encontrando una fuerte asociación entre el *SMAD-7* y los pacientes que desarrollan VRP tras un DR (Rojas J et al 2013).

El TNFA, es una citoquina multifuncional, producida por muchas células, pero principalmente por los macrófagos activados, con efectos en el metabolismo de los lípidos, la coagulación, la resistencia a la insulina y la función endotelial (Goetz FW et al 2004). Además es un mediador central en la respuesta inflamatoria por su capacidad de aumentar las moléculas de adhesión, incrementar la quimiotaxis y el reclutamiento de células inflamatorias como los leucocitos y de desarrollo de la fibrosis, ya que estimula la proliferación de miofibroblastos y la inhibición de la degradación del colágeno vía *inhibidor tisular de la metaloproitenasa-1* (TIMP1) (Goetz FW et al 2004). Además, interactúa con alta afinidad con los *receptores de la familia TNF* (TNFR1 y TNFR2), hiper-regulados a su vez por el *Interferón gamma* (INF-γ), activando dos vías de señalización: la dependiente del complejo JNK-AP1, que activará las colágenasas, y la formación de *especies reactivas de oxigeno* (ROS), y la activación de la vía NF-κB (Corda S et al 2001). Mediante esta última vía se induce la expresión de reactantes de fase aguda, citoquinas, proteínas anti-apoptóticas y auto-reguladoras de la apoptosis como p53 ó familia bcl-2. También se ha asociado el TNFA con la activación de otras vías no apoptóticas de muerte celular relacionadas con la inflamación, isquemia o estrés oxidativo como la necrosis programada ó necroptosis (Declercq W et al 2009, Huckfeldt RM et al 2013, Chinskey ND et al 2014a, Chinskey ND et al 2014b, Newton K et al 2014). Se ha demostrado niveles elevados de TNFA y del TNFR2 en los pacientes con VRP (El-Ghrably et al 1999, Charteris DG et al 2007) y se ha sugerido que el TNFA médiate la vía NF-κB, favorece la expresión de factores protectores de apoptosis en las células del EPR (Yang P et al 2005), proceso que también tiene lugar durante la VRP (El-Ghrably IA et al 2004). Nuestro grupo ha encontrado una fuerte asociación

entre los pacientes con VRP y el gen del TNF, así como asociaciones positivas con el TNFR2. (Rojas J et al 2010, Rojas J et al 2013).

El EFG, constituye una familia de mitógenos junto con el TNFA y el *factor de crecimiento ligado a heparina* (HB-EFG) capaz de inducir la proliferación de las células epiteliales, los fibroblastos y las células gliales (Arrindell EL et al 1992) y se ha implicado en la VRP, al encontrarse su expresión aumentada en retinas con esta complicación (Hollborn M et al 2005).

El CTGF es una proteína de matriz extracelular, que promociona la migración y la proliferación de células e incrementa la expresión de proteínas de la matriz extracelular (Kita T et al 2007a, He S et al 2008, Zhu J et al 2013). El CTGF parece actuar promoviendo la unión y activación del TGF-β a su receptor (Khankan R et al 2011). También sus niveles se encuentran elevados en las membranas de VRP y en los pacientes con VRP (Hinton DR et al 2002, He S et al 2008, Kita T et al 2007b). Además aumenta la fibrosis de las membranas epi-retinianas en los modelos experimentales de VRP (He S et al 2008).

Los factores de crecimiento generalmente actúan sobre vías de señalización relacionadas que dan lugar a un menú variado de respuestas celulares. Sería lógico pensar, que cada factor contribuyera de manera equivalente, incrementando entre todos ellos las posibilidades para el desarrollo de procesos fibróticos y proliferativos en la VRP. Sin embargo, esto no parece ser el caso en la VRP. Ya que parece que existe un subconjunto de factores especializados en los aspectos fibróticos e inflamatorios de la VRP (ej: TGF-β, CTGF o TNFA) mientras que otros (ej: PDGF, NF-κB,…) parecen promover la supervivencia de las células localizadas en la cavidad vítrea (Pennock S et al 2014). La supervivencia, es por tanto una pieza clave en el comienzo de la progresión de la VRP, ya que las células desplazadas de su sitio original, sin contacto celular y/o expuestas a una diferente MEC son particularmente susceptibles a morir (Pennock S et al 2014).

Apoptosis y otras vías de muerte celular

Se ha descrito que un proceso inadecuado de apoptosis es un importante factor en la patogénesis de muchas enfermedades incluyendo procesos neuro-degenerativos, daño isquémico, trastornos autoinmunes y muchos tipos de canceres (Hetts SW et al 1998, Elmore S. 2007).

Además, se ha descrito que una alteración en la regulación de la apoptosis durante proceso de curación-reparación de heridas podría conducir a formas patológicas de la curación tales como cicatrización excesiva y fibrosis (Elmore S. 2007).

Estudios actuales han puesto de relieve la participación de las vías extrínseca e intrínseca de la apoptosis, en células de la retina después de DR (Lo AC et al 2011). La respuesta apoptótica puede estar desencadenada por múltiples factores, entre los que se encuentran: la isquemia y la liberación de citoquinas desde los tejidos dañados. A su vez las citoquinas, debido a sus propiedades quimiotácticas, pueden atraer y activar macrófagos y células de la microglia. La activación de estos tipos de células puede generar entonces un mayor estrés oxidativo y un mayor daño tisular contribuyendo al efecto citotóxico sobre los fotorreceptores después del DR (Lo AC et al 2011).

A pesar de que la apoptosis es el principal mecanismo de pérdida de fotoreceptores después de un DR, estos estudios también han puesto de manifiesto la existencia de otras vías de muerte celular, como la necrosis programada o necroptosis, mediada por la *proteína de interacción con receptor* (RIP) kinasa, que son vías más inflamatorias que la apoptosis e independientes de las caspasa (principales moléculas efectoras de la apoptosis). En situación normal, la necrosis programada aparece con menor frecuencia que la apoptosis tras un DR, sin embargo está aumenta cuando se inhiben las caspasas (Rosenbaum DM et al 2010, Lo AC et al 2011, Murakami Y et al 2011). Otras vías no-apoptóticas de muerte celular también puede ser activadas por el estrés oxidativo prolongado o excesivo, como la que actúa sobre el *retículo endoplasmático* (RE). Esta vía (*vía programada de estrés del retículo endoplasmático* (ERSMP)) juega un importante papel en la

muerte neuronal en trastornos neurodegenerativos y también se ha identificado su activación en los DR experimentales (Lo Ac et al 2011).

La autofagia, proceso catabólico similar a un "reciclaje celular" también se ha encontrado aumentado tras un DR (Cook B et al 1995, Rosenbaum D et al 2010) Estas vías favorecen la supervivencia celular inhibiendo temporalmente la apoptosis bajo condiciones de estrés oxidativo hasta que la célula finalmente muere (Besirli CG et al 2011). Además en un modelo experimental se ha demostrado que la necroptosis o necrosis programada de los foto-receptores se asocia con la activación de la autofagia (Dong K et al 2014).

Es obvio que tener una mejor comprensión de las vías de autoprotección y autodestrucción neuronales es esencial para el desarrollo de nuevas estrategias neuro-protectoras como terapia complementos para la reparación quirúrgica de DR (Lo AC et al 2011). Probablemente hace falta un mejor manejo de la técnica quirúrgica y la adición de enfoques innovadores para prevenir el desarrollo de la PVR por completo.

PUNTOS CLAVE

i. La apoptosis es el principal mecanismo de muerte celular de la retina en números diferentes modelos de enfermedad, y cuya prevención sigue siendo la clave en la neuroprotección de la retina.
ii. La prevención de la muerte celular en enfermedades oculares humanas requerirá la inhibición de la apoptosis y la necrosis programada o necroptosis.
iii. La autofagia, que parece ser una forma independiente de muerte celular, cumple una función protectora, al menos en etapas precoces de la enfermedad.
iv. La inhibición de la apoptosis parece cumplir un papel profiláctico en modelos de distrofias retinianas hereditarias.
v. El papel de la inflamación en las enfermedades de la retina se está empezando a comprender mejor, así como el papel de moléculas anti-inflamatorias, como los esteroides, en la prevención de la muerte celular de la retina.

Etapa de proliferación y modulación de los tejidos:

En la segunda etapa del proceso de curación-reparación de los tejidos se produce la proliferación y metaplasia de las células el EPR (Rodriguez de la Rua E et al 2000). Después del daño retiniano, ya sea por la rotura de la neuro-retina o por la separación de las células del EPR, estas células pierden su polaridad, retraen sus microvellosidades y comienzan a desprenderse de su membrana basal, migrando hacia la cavidad vítrea a través de las roturas de la neuro-retina, exponiéndose así a los factores procedentes de la rotura de BHR (Chiba C. 2014). Este proceso implica una transición epitelial-mesenquimal de las células del EPR, un proceso biológico mediante el cual las células migradas y fuera de contacto con su membrana basal pierden sus características epiteliales. Al hacerlo, las células del EPR adquieren un fenotipo mesenquimal que incluye capacidad mejorada migratoria, capacidad invasiva, resistencia a la apoptosis, y la producción de matriz extracelular (Chiba C. 2014).

En este punto juega un papel importante el *factor de crecimiento hepatocitario* (HGF), liberado por las propias células del EPR y las células gliales y que alcanza sus niveles máximos en las etapas intermedias de este proceso (Cui JZ et al 2007). Estas células son las que formaran parte de las membranas sub-retinianas junto con las células de Müller (El-Gharably IA et al 2001). Por otra parte, algunas células del EPR migradas se des-diferencian hacia mio-fibroblastos ó macrófagos (trans-diferenciación), depositándose en la superficie interna de la retina, lo que dará lugar a la formación de membranas epi-retinianas, compuestas por fibroblastos, células gliales y células del EPR diferenciadas a fibroblastos o a macrófagos (Briggs MC et al 2000, Shediran CM et al 2002, Chiba C. 2014). Aquí juegan un papel importante el PDGF y el TGF-β, principales responsables de la trans-diferenciación de esas células (Ando A et al 2000, Gamulescu MA et al 2006).

La MEC también participa de manera activa en estos procesos ya que a través de su capacidad de modificar la adhesión celular, controla la respuesta celular a los diferentes factores de crecimiento. La fibronectina, potencia la acción de determinados factores estimulando la quimiotaxis de los fibroblastos, macrófagos

y las células del EPR (Campochiaro PA et al 1984, Hogg PA et al 2002). Además, favorece la actividad fagocítica de los monocitos y la síntesis del citoesqueleto (Campochiaro PA et al 1984). La síntesis de la membrana extracelular es regulada principalmente por el TGF-β (Wiedemann P et al 1992). Además, otras moléculas como la cadherina, la trombospondina-1 y la osteonectina modulan la adhesión celular, por lo que podrían ser también responsables de la disminución de la adherencia de las células del EPR a su membrana basal (Hisscott P et al 2002, Chen HJ et al 2007). Muchos de estos cambios pueden ser reversibles tras la reaplicación de la retina (Chen HJ et al 2007).

Existen numerosos estudios acerca de la capacidad de proliferación de las células del EPR en respuesta a diferentes factores de crecimiento (PDGF, *factor de crecimiento fibroblástico* (FGF), EGF, *factor de crecimiento insuíinico* (IGF), *factor de crecimiento vascular endotelial* (VEGF), HGF ó el TGF-β), sin embargo, el mecanismo por el cual se inicia la migración y proliferación del EPR no ha llegado a ser entendido completamente por el momento (Chiba C. 2014).

Una de las principales conclusiones de estos estudios es que la pérdida de contacto con las células contiguas permite al EPR responder a factores mitóticos. La pérdida del contacto célula a célula, junto con una alterada supervivencia podrían ser por tanto, uno de los pasos desencadenantes e importante en el desarrollo de la VRP. Recientemente nuestro grupo ha publicado la asociación entre la VRP y genes que están involucrados en la supervivencia y proliferación celular en procesos inflamatorios y tumorales, tales como el p53, el *gen homologo murino duplo minuto-2* (MDM2) y la familia BCL-2 (Pastor-Idoate S et al 2013a, Pastor-Idoate S et al 2013b, Pastor-Idoate S et al 2015).

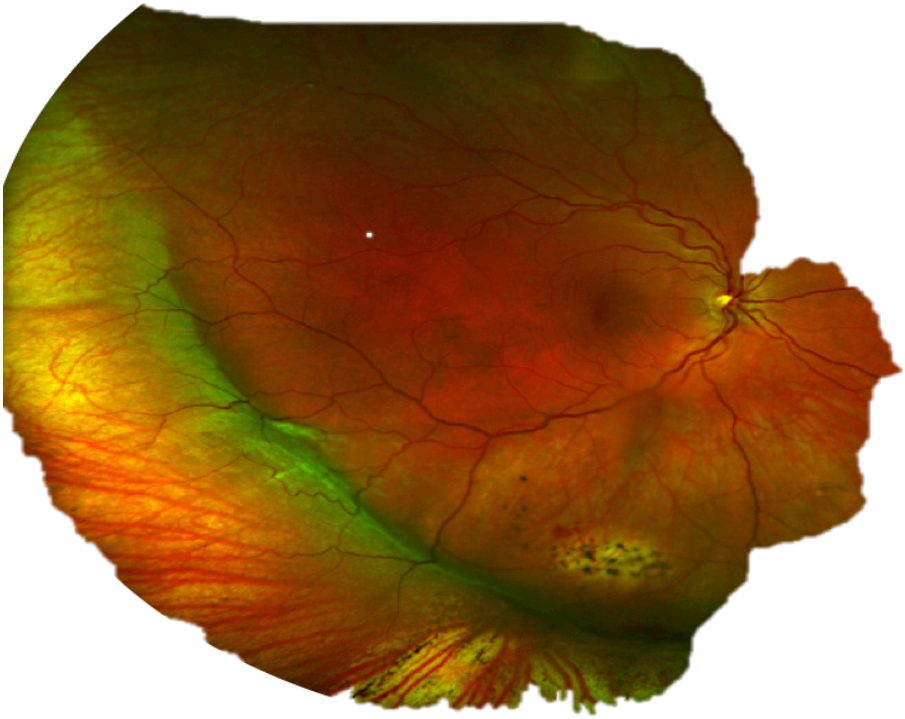
En la tercera etapa del proceso de curación-reparación el tejido se organiza y el número de células disminuye. El PDGF y el TGF-β, mediante el receptor alpha del PDGF (PDGFRα) inducen la contracción de las membranas previamente formadas que provoca la distorsión de la retina y la formación de nuevas roturas (Grisanti S et al 1995, Ikuno Y et al 2002, Bando H et al 2006).

Diferencias entre el DRR y la VRP

Como ya se ha mencionado, la VRP es un proceso complejo que implica no sólo el un daño tisular isquémico, sino que también inflamación, muerte celular, migración y proliferación de varios tipos de células junto con la producción de factores locales (Garweg JG et al 2013, Pennock S et al 2014).

Tanto en el DRR como la VRP el punto de inicio es el trauma retiniano originado por una rotura retiniana, lo que provoca la separación de la neuro-retina de las células del EPR. Esto, conduce a un procesos de isquemia, muerte celular fundamentalmente en los foto-receptores y a un proceso que engloba a toda la retina como parte de una respuesta de reparación tisular inespecífica que conduce a una remodelación de la misma (Garweg JG et al 2013) en el que las células gliales van a jugar un papel fundamental. Como se ha descrito, poco después de un DR, las células del EPR son estimuladas hacia una transición epitelial-mesenquimal adquiriendo capacidad migratoria, producción de moléculas de la matriz extracelular, invasividad y resistencia a la apoptosis (Chiba C. 2014). Por otra parte, algunas células del EPR migradas se des-diferencian hacia mio-fibroblastos o macrófagos (trans-diferenciación) (impulsado por factores que aún no se han identificado completamente), depositándose en la superficie interna de la retina, lo que dará lugar a la formación de membranas epi-retinianas (Briggs MC et al 2000, Shediran CM et al 2002, Chiba C. 2014). Estas membranas han sido consideradas el rasgo más característico de la VRP (Garweg JG et al 2013). Sin embargo, excepto por la formación de membranas peri-retinianas y la hiper-reactividad glial no existen rasgos histológicos distintivos con respecto a los provocados por cualquier DR. Y en cuanto a las características clínicas que promueven la aparición de esta complicación ya se ha visto que estos factores clínicos por sí solos no proporcionan suficiente poder predictivo como para identificar a los pacientes con alto riesgo de desarrollar VRP tras un DR.

Desde nuestros conocimientos de la VRP, las principales hipótesis que plantea nuestro grupo de investigación en cuanto a las diferencias entre el DR y la VRP incluirían:



1. Mayor afectación de la BHR
2. Aumento de la inflamación
3. Mayor grado de isquemia
4. Proceso de remodelación intra-retiniano exagerado
5. Desregulación de los procesos normales de apoptosis
6. Incremento de los procesos reactivos de oxidación (ROS)
7. Activación de las vías de muerte celular secundarias
8. Aumento de la supervivencia y de los procesos de migración proliferación celular

Factores clínicos de riesgo de desarrollo de VRP

Los factores de riesgo clínicos asociados al desarrollo de VRP tras un DR han sido evaluados desde que se empezó a estudiar este proceso. Teóricamente, la identificación de estos factores podría ser útil para elucidar los mecanismos de su patogénesis, pudiendo así proporcionar datos útiles para el manejo del DR, y quizás también para la toma de decisiones a la hora de añadir tratamientos co-adyuvantes (Cowley M et al 1989). Pero también permitiría identificar aquellos pacientes con alto riesgo de desarrollar VRP y así tratar de evitar los factores que fuesen desencadenantes, o para modificar las opciones quirúrgicas (Tolentino FI et al 1967, Chignell AH et al 1973, Yoshida A et al 1984, Bonnet M. 1984, Bonnet M. 1988, Cowley M et al 1989).

La identificación de factores de riesgo es un aspecto cada vez más importante, debido a que continuamente aparecen más opciones terapéuticas, al menos teóricas, para prevenir la aparición de la VRP, lo que permitiría seleccionar con

mayor precisión a los mejores candidatos a recibir un posible tratamientos farmacológico profiláctico (Asaria RH et al 2002, Kon CH et al 2000).

Se han realizado numerosos estudios para predecir el riesgo de desarrollo de VRP basándose en las características clínicas de los pacientes con DR (Rodríguez de la Rúa E et al 2005). Desafortunadamente, los resultados obtenidos hasta ahora no son consistentes, y las fórmulas desarrolladas basadas en estos factores han demostrando una escasa utilidad para su uso clínico diario (Kon CH et al 2000, Asaria RH et al 2001, Rodríguez de la Rúa E et al 2005, Wickham L et al 2011, Sala-Puigdollers A et al 2013). La mayoría de estos estudios se han realizado de forma retrospectiva, y los resultados son a menudo contradictorios y no concluyentes (Kon CH et al 2000). Además, la existencia de diferencias entre los estudios en cuanto al diseño, metodología, definiciones y el análisis estadístico no permite su comparación directa (Asaria RH et al 2002). Otros estudios se han centrado en discernir la influencia de un solo factor específico individual o en la identificación de factores de riesgo asociados con circunstancias específicas como afaquia, VRP pre-operatoria o VRP sub-retiniana. Incluso algunos fueron diseñados específicamente para probar una sola hipótesis en particular con respecto a la origen patológico de la VRP, una decisión que implica un sesgo importante por parte de los investigadores (Asaria RH et al 2006), ya que cualquier factor de riesgo debe ser identificado como un parámetro independiente.

Además, la alta incidencia de VRP encontrada en algunos de estos estudios sugiere que las muestras analizadas estuvieron formadas por casos complejos, y que por lo tanto no serian representativos de la población de DR no complicado, que es la mas importante (Rodríguez de la Rúa E et al 2005).

Además, estos enfoques no parecen ser los más apropiados para una enfermedad multifactorial como es la VRP, ya que la mayoría de los factores de riego están claramente relacionados entre sí, y no es posible aislarlos para su análisis en solitario (Rodríguez de la Rúa et al 2005). Sin embargo, estos estudios han contribuido aportando una valiosa información, ya que han ayudado a desentrañar la compleja naturaleza de la enfermedad. La mayoría de factores de riesgo clínicos

son consistentes con la naturaleza inflamatoria de la VRP. Pero aunque la inflamación juega un papel importante en su génesis y desarrollo, en todos los casos de VRP, no es el único factor desencadenante (Pastor JC et al 2002, Rodríguez de la Rúa E et al 2005).

Factores de riesgo pre-operatorios, intra-operatorios y post-operatorios:

Los factores de riesgo se pueden clasificar en: *pre-operatorios*, *intra-operatorios* o *post-operatorios*. Los factores *pre-operatorios* asociados con un mayor riesgo de VRP incluyen: trauma ocular (Girard P et al 1994), antecedente de inflamación intraocular prolongada (uveítis anterior), retinitis infecciosa, y la baja presión intraocular (IOP) secundaria a la inflamación intraocular (Girard P et al 1994, Yoshino Y et al 1989, Rodríguez de la Rúa E et al 2000, Pastor JC et al 2002, Rodríguez de la Rúa E et al 2005, Wickham L et al 2011). Las características del desgarro retiniano en cuanto al tamaño y el número o la extensión del DR son también algunos de los factores clínicos casi constantemente asociados con el desarrollo de VRP (Yoshino Y et al 1989, Rodríguez de la Rúa E et al 2000, Rodríguez de la Rúa E et al 2005, Wickham L et al 2011). Otros factores de riesgo pre-operatorios incluyen: DR asociados con hemorragia vítrea (Duquesne N et al 1996), afaquia, cirugía intraocular anterior, desprendimiento coroideo anterior y VRP pre-operatoria grado A o B. Todos estos factores de riesgo preoperatorios presentan un importante componente inflamatorio (Bonnet M. 1984, Bonnet M. 1988, Yoshino Y et al 1989, Asaria RH et al 2001).

Dentro de los factores de riesgo *intra-operatorios* se encuentran: las hemorragias vítreas o sub-retinianas, fracaso de la retinopexia, desprendimientos coroideos intra-operatorias (Cowley M et al 1989), la liberación de pigmento durante el endo-drenaje del fluido sub-retiniano (Lleo Pérez A et al 2000), la crioterapia o el endo-láser excesivos (Bonnet M. 1988, Cowley M et al 1989, Rodríguez de la Rúa E et al 2000). Ambas estrategias de retinopexia inducen un ambiente pro-inflamatorio que podría explicar el alto riesgo de desarrollar VRP, tal y como se ha mostrado no solo en modelos experimentales (Pinon RM et al 1992, Garcia-Layana A et al 1997; Goldaracena MB et al 1997), sino también en estudios clínicos, como

el realizado por Jonas JB y col. en el año 2000 o el de Koerner F y col. en el año 2012, en los que muestran un efecto positivo de los corticoides en la atenuación de la VRP. Estos resultados refuerzan la idea de que la estrategia anti-inflamatoria podría ser una de las mejores opciones para la prevención de la VRP. Idea que esta siendo estudiada por otros investigadores mediante ensayos clínicos (Banerjee PJ et al. 2013a, Banerjee PJ et al. 2013b, Khan MA et al 2015).

En cuanto a los factores de riesgo *post-operatorios* se incluyen: la inflamación o uveítis prolongada, hemorragia vítrea nueva o persistente, desprendimiento coroideo postoperatorio, el uso o el aire o *hexafluoruro de azufre* (SF6), numero de procedimientos quirúrgicos, pérdida de vítreo durante el drenaje del líquido, sub-retiniano (Asaria RH et al 2001, Pastor JC et al 2002) y la tracción persistente de los desgarros retinianos. La mayoría de estos factores, al igual que los anteriores están claramente relacionados con la inflamación.

Problemas de diseño y de las fórmulas de predicción de la VRP

Como se ha mencionado, el diseño de muchos estudios clínicos retrospectivos para identificar factores que predisponen a la aparición de la VRP no es el adecuado. A menudo los factores que contribuyen al desarrollo VRP están estrechamente relacionados unos con otros, y como se ha mencionado, no pueden ser analizados de manera independiente. Por ejemplo: en el estudio realizado por Duquesne N y col. en el año 1996 que incluyó 409 ojos y en el que se analizó la influencia de 14 categorías dentro las variables clínicas, tras el análisis de región simple y múltiple, tan sólo cuatro variables de forma independiente y en conjunto se encontraban asociadas con el riesgo de VRP post-operatoria. De todas estas variables, tan sólo dos han sido confirmadas posteriormente por estudios adicionales: extensión circunferencial del desgarro retiniano de 90° o más y el uso de crio-coagulación transescleral (Sadaka A et al 2012).

En el año 2005, nuestro grupo realizó un estudio observacional de casos y controles entre 335 pacientes (201 controles y 134 casos) con DR no complicados (Rodríguez de la Rúa E et al 2005). Los factores de riesgo para el desarrollo de la

VRP fueron identificados y analizados mediante análisis multivariante, y la influencia de variables se analizó de acuerdo con el abordaje quirúrgico en cada caso. Una vez más, los desgarros retinianos mayores de un huso horario y la extensión del DR resultaron ser factores de riesgo para la VRP. Además la edad y la presión intraocular más baja, la utilización de técnicas quirúrgicas esclerales, la afaquia o pseudofaquia cuando se asocian con cirugía escleral y las reintervenciones fueron también factores de riesgo para la VRP. Nuestro grupo propuso un modelo estadístico para estimar la probabilidad de desarrollar VRP para cualquier paciente independiente de la intervención quirúrgica empleada para la reparación del DR. Los mejores valores de sensibilidad y especificidad que se obtuvieron con este modelo fueron de 78,0% y 75,6%, respectivamente, siendo el área bajo la curva ROC de 0,86 que aunque eran valores más altos que los obtenidos por otros estudios (Asaria RH et al 2001, Kon CH et al 2000), no fueron suficientemente altos como para significar una ventaja en la práctica clínica diaria (Rodríguez de la Rúa E et al 2005). Más recientemente Wickham L y col. en el año 2011 idearon una fórmula simplificada basada en datos clínicos pre-operatorios para estimar el riesgo de VRP después de la reparación primaria del DR mediante vitrectomía. La hemorragia vítrea, el grado C de VRP, y la extensión del desprendimiento estaban relacionados con un fallo de la cirugía secundario al desarrollo de VRP. A pesar de que hubo una buena concordancia entre las estimaciones de riesgo mediante el sistema de puntos y los calculados directamente por un modelo de regresión multivariante y que área bajo la curva fue de 0,84 (Wickham L et al 2011), de nuevo, la baja sensibilidad y especificidad de estas fórmulas las hace inadecuadas para uso clínico práctico. (Kon CH et al 2000, Asaria RH et al 2001, Rodríguez de la Rúa E et al 2005, Wickham L et al 2011).

Existen otros problemas relacionados con las fórmulas de predicción que no son específicos de la VRP. Este tipo de modelos se tienden a obtener mejores resultados con los datos con los que fueron construidos que con datos nuevos (Bleeker SE et al 2003). Además, las fórmulas predictivas requieren una validación externa, tanto geográfica como temporalmente, con muestras diferentes antes de ser plenamente validadas e implantadas en la práctica clínica diaria (Bleeker SE et

al 2003, Terrin N et al 2003). Nuestro grupo realizó una validación externa de las cuatro fórmulas publicadas para predecir el desarrollo de VRP después de la cirugía del DR, cada una de los cuales se desarrollaron con diferentes criterios (Sala-Puigdollers A et al 2013). Las cuatro fórmulas (Kon CH et al 2000, Asaria RH et al 2001, Rodríguez de la Rúa E et al 2005, Wickham L et al 2011) tenían una capacidad limitada para identificar prospectivamente a los pacientes que desarrollarían VRP, y por lo tanto, eran poco fiables para uso general en la clínica diaria (Sala-Puigdollers A et al 2013).

Es evidente que los factores clínicos por sí solos no proporcionan el poder predictivo suficiente como para identificar a los pacientes con alto riesgo de desarrollar VRP. De hecho, solamente cuando se añaden las variables adicionales, tales como los factores genéticos u otros biomarcadores, el rendimiento predictivo de los modelos mejora (Ricker LJ et al 2012, Rojas J et al 2014).

Este es uno de los puntos clave en el *proyecto Retina-4*: identificar biomarcadores asociados al desarrollo de VRP. Estas nuevas fórmulas, después de su validación podrían proporcionar nuevas herramientas para identificar pre-operatoriamente a los pacientes con alto riesgo de desarrollar VRP. Este es un paso crucial para el diseño de nuevos ensayos clínicos. Ya que la mejora de las fórmulas permitirá la inclusión en los ensayos clínicos de sólo aquellos pacientes con un alto riesgo de VRP, reduciéndose el tamaño muestral (punto fundamental, ya que la prevalencia de la VRP es de tan solo del 5-10% de los DRR) y aumentando el poder de los resultados.

Proyecto Retina-1

¿Qué es?

El *Proyecto Retina-1* constituyó una de las principales líneas de investigación del grupo de retina del I.O.B.A, centrada

en la identificación de factores de riesgo clínicos implicados en el desarrollo de VRP tras un DDR.

¿Cómo nació y que pretendía?

Basándose en los trabajos anteriores realizados por nuestro grupo (Rodriguez de la Rua E et al 2000, Rodríguez de la Rua E et al 2003), en la identificación de factores de riesgo clínicos asociados a la VRP, surgió en el año 2005 el *Proyecto Retina-1* con la idea de identificar las variables, pre, intra y post-operatorias asociadas al desarrollo de VRP postquirúrgica, con el fin de mejorar el modelo predictivo de VRP tras un DR, publicado anteriormente por ese mismo grupo (Rodríguez de la Rua E et al 2005) y proponer diferentes aproximaciones quirúrgicas o tratamientos complementarios a la cirugía del DR para reducir el riesgo de esta complicación.

¿Qué ha conseguido hasta ahora?

El *proyecto Retina-1* ha conseguido hasta la fecha las siguientes publicaciones:

1. de la Rúa ER, Pastor JC, Fernández I, Sanabria MR, García-Arumí J, Martínez-Castillo V, Coco R, Manzanas L, Miranda I.Non-complicated retinal detachment management: variations in 4 years. Retina 1 project; report 1. Br J Ophthalmol. 2008;92:523-5.

2. Pastor JC, Fernández I, Rodríguez de la Rúa E, Coco R, Sanabria-Ruiz Colmenares MR, Sánchez-Chicharro D, Martinho R, Ruiz Moreno JM, García Arumi J, Suárez de Figueroa M, Giraldo A, Manzanas L. Surgical outcomes for primary rhegmatogenous retinal detachments in phakic and pseudophakic patients: the Retina 1 Project--report 2. Br J Ophthalmol. 2008;92:378-82.

3. Sanabria MR, Fernández I, Sala-Puigdollers A, Rojas J, Alfaiate M, Elizalde J, Navea A, Cordoves L, Ruiz-Miguel M, Diaz-Llopis M, Coco RM, De La Rúa ER, Pastor JC. A propensity score matching application: indications and results of adding

scleral buckle to vitrectomy: The Retina 1 Project: Report 3. Eur J Ophthalmol. 2012;22:244-53.

4. Pastor JC, Fernández I, Coco RM, Sanabria MR, Rodríguez de la Rúa E, Piñon RM, Martinez V, Sala-Puigdollers A, Gallardo JM, Velilla S. Variations in Functional and Anatomical Outcomes and in Proliferative Vitreoretinopathy Rate along a Prospective Collaborative Study on Primary Rhegmatogenous Retinal Detachments: The Retina 1 Project-Report 4. ISRN Ophthalmol. 2012, 8;2012:206385.

5. Sala-Puigdollers A, Fernández I, Coco RM, Sanabria MR, Rodríguez de la Rúa E, Ruiz-Moreno JM, Navea A, Suárez de Figueroa M, Pastor JC. External validation of existing formulas to predict the risk of developing proliferative vitreoretinopathy: the Retina 1 Project; report 5. Retina. 2013;33:1519-27.

6. Pastor JC, Fernandez I,Castrejon M, et al. Visual loss in retinal detachment and macula-on after successful surgery, Retina-1 project. ARVO Seattle 2013. POSTER

Proyecto Retina-4

¿Qué es?

El *Proyecto Retina-4* constituye otra de las principales líneas de investigación del grupo de Retina del I.O.B.A, centrada en la genética de la inflamación, apoptosis y otras vías de muerte celular en el DR y en la VRP. Esta constituido por un equipo multidisciplinar cuyo objetivo último es la investigación translacional.

¿Cómo nació y que pretendía?

La mayoría de las investigaciones en la patogénesis de la VRP han centrado sus esfuerzos en la identificación de los factores de riesgo clínicos asociados al desarrollo de la VRP, sin embargo, estas variables no terminan de explicar

completamente su probabilidad de aparición (Rojas J et al 2009). Partiendo de la hipótesis de que el DR podía evolucionar de diferentes maneras dependiendo del perfil genético de cada paciente (Sanabria Ruiz-Colmenares MR et al 2006), se inició un proyecto denominado "*Proyecto Retina-4*", en respuesta a las limitaciones obtenidas en trabajos previos realizados en la identificación de factores de riesgo para el desarrollo de la VRP, y con el objetivo de evaluar la posible contribución genética en la patogénesis de la VRP.

¿Quién ha participado hasta ahora?

-Identificación y envío de muestras: durante su desarrollo en estos años, ha involucrando no solo diferentes centros nacionales (*Hospital Vall d´Hebron, Clínica Barraquer, Instituto Vissum, Hospital de Donostia, Hospital Clínico de Valladolid, Clínica Universitaria de Navarra, Fundación Jiménez Díaz, Fundación Oftalmológica del Mediterráneo (FOM), Hospital General de Valencia, Hospital Germans Trias I Pujol, Hospital Ramón y Cajal, Hospital la Fe y el hospital San Millán*) sino también diferentes centros internacionales como: el *Rotterdam Eye Hospital, Erasmus Medical Center* en Holanda, el *Moorfields Eye Hosptal* y el *Nuffield Laboratory of Ophthalmology*, *Oxford University* en Inglaterra y el *San Joao Hospital* y el *Department of Sense Organs, Medical School* en Portugal.

-Tratamiento y análisis de muestras: junto con el I.O.B.A han participado dos centros nacionales en el análisis de las muestras. El Instituto de Medicina Xenómica de la Facultad de Medicina de la Universidad de Santiago de Compostela, y el instituto de Investigación Biomédica de la Universidad de Salamanca.

Todos estos centros han participado no sólo en el reclutamiento de pacientes, envío, tratamiento y análisis de las muestras, sino que también en la elaboración y discusión de los diferentes trabajos publicados.

¿Qué ha conseguido el proyecto Retina-4 hasta ahora?

El *proyecto Retina-4* ha conseguido hasta la fecha:

-1. Poner de relieve no sólo la importancia de la inflamación en la génesis y desarrollo de la VRP, sino que también señala la contribución genética en la patogénesis de la enfermedad (Sanabria Ruiz-Colmenares MR et al 2006, Rojas J et al 2010, Rojas J et al 2013).

-2. Identificar biomarcadores asociados al desarrollo de la VRP, que permiten no sólo mejorar, junto con determinados factores clínicos, la identificación de pacientes de alto riesgo en los modelos predictivos (Rojas J et al 2009, Sala-Puigdollers A et al 2013, Rojas J et al 2014, Rojas J et al 2015), Estos modelos han sido validados externamente, tanto geográfica como temporalmente, mostrando una capacidad predictiva aceptable y dando lugar a una patente ("Método para la detección del riesgo de desarrollar vitreoretinopatía proliferante" WO 2009040461A3). Además permiten también identificar posibles dianas terapéuticas para la profilaxis y tratamiento de la VRP (Fernández-Bueno I et al 2013).

-3. Señalar la importancia de otros factores como la isquemia y la activación de las distintas vías de señalización de muerte celular como posibles factores adicionales en el mecanismo fisiopatogénico de esta enfermedad (Pastor-Idoate S et al 2013a, Pastor-Idoate S et al 2013b, Pastor-Idoate S et al 2015).

Genética de la VRP

Enfermedades monogénicas y complejas

Las enfermedades hereditarias humanas pueden ser clasificadas como monogénicas o complejas. Son enfermedades hereditarias monogénicas las causadas por la mutación o alteración en la secuencia del ADN de un solo gen.

Estas enfermedades son relativamente raras, pero tienen una alta penetrancia a pesar de una baja frecuencia de los alelos. Por el contrario, las enfermedades hereditarias complejas, son multifactoriales en su naturaleza. Son producidas por la combinación de múltiples factores ambientales y mutaciones en varios genes, generalmente de diferentes cromosomas. Son relativamente comunes y tienen baja penetrancia y una frecuencia de alelo común, ocurriendo en al menos un 5% de la población (Lobo I. 2008).

Las enfermedades multifactoriales pueden ocurrir de forma aislada pero las influencias ambientales pueden hacer aumentar o disminuir el riesgo aparición de la enfermedad. En este sentido, una misma enfermedad puede inducir diferentes respuestas en diferentes individuos. Por lo tanto, perfil genético de un individuo podría determinar si una persona tiene una mayor o menor susceptibilidad a una enfermedad bajo las influencias del mismo entorno (Dempfle A et al 2008). Un ejemplo de ello, sería la *degeneración macular asociada con la edad* (DMAE), en la que el perfil genético ha demostrado un importante papel no sólo en el desarrollo de la enfermedad sino también en la respuesta al tratamiento (Gemenetzi M et al 2014, Horie-Inoue K et al 2014, SanGiovanni JP 2014, Schramm EC et al 2014).

Utilidad de los estudios genéticos: patogénesis y biomarcadores de riesgo

Los *polimorfismos de nucleótido simple* (SNPs), son los polimorfismos más frecuentes en la naturaleza. Tienen una amplia variación en su frecuencia entre diferentes poblaciones y puede ocurrir en cualquier parte del genoma: exones, intrones, o regiones intergénicas. Aunque son relativamente pocos los SNPs que se producen en los exones, estos son de gran interés, ya que pueden alterar el producto final del gen y predisponer a un cambio en el fenotipo o en la susceptibilidad a una enfermedad (SNP funcional). Sin embargo, en muchos de los casos, los SNPs no suelen tener un significado funcional real, sino más bien sirven como marcadores, ya que se suelen heredar junto con genes asociados a enfermedades como consecuencia de su proximidad física.

El SNP y el factor genético causal están en desequilibrio de ligamiento, y el SNP puede tener un carácter de identificación cuando se busca el gen causante de la enfermedad (Kumar V et al.1994).

El estudio genético de cualquier enfermedad multifactorial puede ayudar a clarificar la patogénesis de diferentes maneras, mediante la identificación de los genes causantes, los SNPs funcionales o los SNPs en desequilibrio de ligamiento con genes causantes, pudiendo señalar genes que nunca habían sido implicados con la enfermedad, o podrían confirmar el papel de genes previamente identificados (Rojas J et al 2013). Además los SNPs pueden servir como biomarcadores, sirviendo como indicadores de riesgo para las enfermedades poligénicas y también como marcadores biológicos de la respuesta al tratamiento.

Polimorfismos más relevantes en el proyecto Retina-4

Partiendo de la idea de que el DR podía evolucionar de diferentes maneras dependiendo del perfil genético de cada paciente (Sanabria Ruiz-Colmenares MR et al 2006), en la primera etapa se realizó un estudio multicentrico de asociación de genes candidatos, involucrando la participación de 8 centros oftalmológicos españoles. Se analizaron muestras de ADN de 450 pacientes (138 casos y 312 controles). Los participantes fueron pacientes que habían sido sometidos a cirugía de DRR primario. Los que desarrollaron VRP grado C1 ó superior fueron incluidos como casos. Los que no desarrollaron VRP después de 3 meses de seguimiento se incluyeron en el grupo de controles. Para lograr una clasificación fenotípica más estricta, se excluyeron los pacientes con DR secundarios.

Inicialmente, se evaluó la asociación de 197 SNPs pertenecientes a 30 genes relacionados con la inflamación, identificándose marcadores dentro de cuatro de estos genes: TNF, TNF2R, SMAD7 y PICK3 (Rojas J et al 2010), reforzando la idea de que el componente genético podría tener un papel en el riesgo de VRP tras el DR.

Dentro de los SNPs que mostraron asociación con la enfermedad, destaca, por sus posibles implicaciones funcionales, el *rs2229094* (T→C), ubicado dentro del gen de *la Linfotoxina alfa* (LTA) y dentro del bloque del TNFA. Este SNP, mostró una fuerte asociación con el desarrollo de VRP, tanto en el análisis simple como en el haplotípico, en donde se encontró presente en el 100% de las asociaciones significativas y no encontrándose presente en ninguna de las no significativas (Rojas J et al 2010). El SNP *rs2229094* (T→C) se localiza en el cromosoma 6, en el exon 2, codón 13, del gen LTA, región que codifica para el péptido señal de la proteína LTA. Es un polimorfismo de tipo no sinónimo, produciendo un cambio del aminoacído Cisteína por Arginina (Rojas J et al 2010).

Tanto el carácter de hidrofobicidad del péptido señal, la distribución asimétrica de los potenciales de los residuos hidrofóbicos e hidrofílicos, como la calidad del "splicing" constituyen unos de los factores más influyentes en el correcto transporte de la proteínas (Goldstein J et al 1990, Talmud P et al 1996), regulación de expresión ó de cualquiera de sus funciones (Hoyt DW et al 1991), así como cambios en la región trans-membrana que podrían tener efectos sobre la topología de la proteína o de sus interacciones. Por lo que se pensó, que este cambio en el péptido señal podría estar implicado en alteraciones tanto de localización como del correcto funcionamiento de proteína LTA (Wahl MC et al 2009). Se analizó el SNP mediante herramientas bioinformáticas (Rojas J et al 2010), y se observó que el cambio del aminoácido del polimorfismo produciría cambios tanto en la calidad del "splicing", en el perfil hidropático de la proteína como de la posible localización en la región trans-membrana. Además en un trabajo posterior en el que se ajustaron modelos de predicción de riesgo de VRP, basándose en las asociaciones encontradas en el Proyecto Retina 4 (Rojas J et al 2009), este SNP se identifico siempre en todos los modelos que fueron más predictivos, siendo a su vez el mejor clasificador de pacientes con VRP. Ambas razones (su posible implicación funcional y su grado predictivo) han motivado la realización de la primera parte de este trabajo.

Los estudios de asociación permiten establecer las diferentes frecuencias de ciertos SNPs en poblaciones afectas y en las controles con el fin de identificar aquellos marcadores genéticos que se asocian al desarrollo de una determinada patología. El mayor problema que presenta este tipo de estudios es el hallazgo de falsos positivos (Crawford DC et al 2005). Hay numerosas razones que pueden dar lugar a falsas asociaciones. Unas de las más importantes: es la que resulta de una clasificación inadecuada de los sujetos en estudio (alteraciones en el fenotipado). Otra, la derivada del propio tratamiento estadístico de los datos. Además ciertos marcadores pueden tener un efecto en ciertas poblaciones y no en otras. Por todo eso, ocurre en ocasiones que las asociaciones encontradas en algunas poblaciones no son definitivas (Ioannidis JP et al 2001) y muchos investigadores consideran que la replicación de estos estudios es fundamental (Patterson M et al 2005).

Los modelos predictivos se utilizan con el fin de estimar las probabilidades de resultados en diversos individuos. Estos modelos se desarrollan utilizando las características de los datos de pacientes con resultados conocidos y se ajustan de manera que son capaces de predecir de la forma más precisa posible, utilizando estos mismos conjuntos de datos. Antes de aplicar estos modelos en la práctica clínica cotidiana es necesario establecer si éstos son capaces de dar estimaciones realistas. Como ya se ha dicho, uno de los principales problemas de los modelos predictivos es el optimismo de las estimaciones, es decir, su capacidad predictiva en nuevos pacientes es muchas veces peor de lo esperado en base a las estimaciones obtenidas con los datos utilizados en el desarrollo del modelo (Harrel FE et al 1996). Este optimismo puede ser estimado mediante técnicas de validación interna basadas en remuestreo (Steyerberg EW et al 2001, Chatfield C et al 1995, Efron B et al 1993). Todo esto es muy importante en los estudios de genética lo que implica que es importante probar la generalización del modelo, es decir, la validación externa en muestras independientes (König IR et al 2007, Hukkelhoven CW et al 2006; Bleeker SE et al 2003; Terrin N et al 2003).

Todas estas premisas motivaron la realización de una segunda etapa del *proyecto Retina-4*, involucrando esta vez centros europeos (Reino Unido, Holanda, Portugal y España) para la obtención de muestras de ADN y para la realización de la

validación externa. Se analizaron 564 muestras con 134 casos de VRP tras DDR, confirmando la implicación del gen SMAD7 y del locus del TNF en la patogénesis de la VRP (Rojas J et al, 2013).

Además, la relación del TNF con los cambios intraretinianos que se producen a consecuencia de la gliosis reactiva de las células de Müller y los astrocitos tras un DR se han demostrado, por nuestro grupo, mediante experimentos *in-vitro* de cultivos organotípicos de neuro-retina de cerdo (Fernández-Bueno I et al 2008, Fernández-Bueno I et al 2012, Fernández-Bueno I et al 2013).

En la segunda etapa del *Proyecto Retina-4*, se han conseguido identificar nuevos marcadores de riesgo para la VRP. Basándose en la hipótesis de que la muerte celular por apoptosis podía evolucionar de diferentes maneras y dar lugar a diferente resultados funcionales en SNC dependiendo del perfil genético de cada paciente (Gómez-Sánchez JC et al 2011), se analizaron SNPs funcionales relacionados con en el control del ciclo celular y en la apoptosis, incluyendo: el SNP *rs1042522*, ubicado en el gen p53.

El gen p53 localizado en el brazo corto del cromosoma 17, codifica una fosfoproteína de 393 aminoácidos (Levine AJ. 1992, Walker KK et al 1996). Esta proteína ejerce su función como supresor de tumores mediante dos mecanismos, regulando de forma negativa el ciclo celular en la fase de transición de G1 a S, o regulando de manera positiva la transcripción de genes relacionados con la regulación del ciclo celular. Además, desempeña un papel determinante en el proceso de apoptosis (Levine AJ. 1997).

El SNP *rs1042522* localizado en el exón número cuatro, codón 72 del gen (Shepherd T et al. 2000), consiste en una transversión de G por C, lo cual da como resultado el cambio de un aminoácido en la secuencia de la proteína (arginina por prolina). Dicho cambio tiene efectos bioquímicos y biológicos importantes en cuanto a la acción de la proteína (Thomas M et al 1999, Vousden KH et al 2002, Dumont P et al 2003, Bonafe M et al 2004). La variante arginina es altamente pro-apoptotica mientras que la variante prolina tiene efectos opuestos.

Numerosos estudios han reportado el posible papel de este SNP con el riesgo a desarrollar diversos tumores, así como su papel en diferentes procesos inflamatorios (Sun Y et al 1999, Vousden KH et al 2002, Van Heemst D et al 2005, Frank AK et al 2011), en donde la apoptosis parece tener un papel fundamental. Recientemente, nuestro grupo ha demostrado una asociación positiva entre este polimorfismo y los pacientes con que desarrollan VRP tras un DDR (Pastor-Idoate S et al 2013a).

El SNP *rs2279744,* ubicado en el gen MDM2, localizado en el brazo largo del cromosoma 12. Este gen codifica una proteína (mdm2) implicada en la degradación del p53 (Moll UM et al 2003, Iwakuma T et al 2003), siendo también un gen regulador del ciclo celular y de la apoptosis.

El SNP rs2279744 localizado en el intron número uno, en la posición 309 (Moll UM et al 2003, Iwakuma T et al 2003, Alhopuro P et al 2005, Bougeard G et al 2006), consiste en una transversión de T por G, lo cual da como resultado cambios importantes en cuanto a la acción de la proteína (Moll UM et al 2003, Iwakuma T et al 2003). La variante alélica G/G, asociada a este polimorfismo, determina un incremento de los niveles de MDM2, responsable de una disminución de la actividad del p53 y consecuentemente de su actividad apoptótica (Bond GL et al 2005, Alhopuro P et al 2005, Bougeard G et al 2006, Di Vuolo V et al 2011).

Al igual que el SNP del p53, numerosos estudios han publicado su posible papel en el desarrollado de diversos tumores, así como en diferentes procesos inflamatorios (Bond GL et al 2005, Alhopuro P et al 2005, Bougeard G et al 2006, Assmann G et al 2010, Di Vuolo V et al 2011), en donde la apoptosis parece tener un papel fundamental. Además se ha comunicado un papel aditivo y por lo tanto un mayor riesgo en los portadores de ambos SNPs Recientemente, nuestro grupo no sólo ha demostrado una asociación positiva entre este polimorfismo y los pacientes con VRP, sino además su papel aditivo en el riesgo de desarrollar VRP tras un DR en los portadores de ambos SNPs (Pastor-Idoate S et al 2013b). Además, se ha publicado que la supresión de la expresión de p53 podría ser un evento necesario el desarrollo del DR y la VRP, y que el mantenimiento de los niveles de p53 con

agentes tales como la Nutlina-3, que impiden la interacción entre el p53 y MDM2, podrían suponer agentes eficaces en la profilaxis del DR y en la VRP (Lei H et al 2012).

Y por último, los SNPs *rs4645878* y *rs2279115,* ubicados en los genes BAX y BCL-2, localizados en el brazo corto de los cromosomas 19 y 18 respectivamente. Estos genes codifican una proteínas (bax y bcl-2) implicadas en la regulación del p53 y la apoptosis (Chen HJ et al 2007).

La familia Bcl-2, se divide en dos clases de miembros que ejercen acciones opuestas sobre la muerte celular. Miembros como BCL-2 y BCL-xL con una acción anti-apoptótica y miembros como BAX y BAK con acciones contrarias (pro-apoptóticas) (Adams JM et al 2001). La activación de BAX y/o BAK desencadena una permeabilización de la membrana mitocondrial externa por mecanismos aún desconocidos. Esto conduce a la liberación de citocromo C y a proteínas reguladoras pro-apoptóticas en el citoplasma celular, que resultan en una activación de las caspasas y a la muerte celular. Por el contrario, BCL-2 y/o BCL-xL bloquean este proceso, inhibiendo la muerte celular programada (Knudson CM et al 1997).

El gen BAX se compone de seis exones y una región promotora con cuatro sitios de unión a p53 (Saxena A et al 2002). Las variaciones en la región promotora y en la región codificante pueden abolir su función pro-apoptótica. El SNP G(-248)A (rs4645878) en la región promotora de BAX se asocia con disminución en los niveles de expresión celular y por lo tanto con una disminución en la apoptosis (Saxena A et al 2002; Starczynski EW et al 2005). Recientemente, nuestro grupo ha demostrado una asociación positiva entre este polimorfismo y los pacientes con VRP (Pastor-Idoate S et al 2015).

El gen BCL-2 consta de tres exones y dos regiones promotoras (P1 y P2). El polimorfismo rs2279115 se encuentra en la región promotora inhibitoria P2 (Park BL et al 2004). Este segunda región promotora P2, funciona como un regulador negativo de la actividad de la región promotora P1(Nückel H et al 2007). El

polimorfismo C (-938) A (rs2279115) en la región promotora de P2 de BCL-2 se asocia con un aumentó significativo de la inhibición en la actividad de promotor P1 de BCL-2 y en la unión de proteínas nucleares (Nuckel H et al 2007). Por lo tanto, el genotipo AA está asociado con un aumento de expresión de BCL-2 y por lo tanto con una mayor actividad anti-apoptotica. Nuestro grupo ha demostrado un efecto protector entre este SNP y los pacientes con VRP (Pastor-Idoate S et al 2015), pudiendo ser una posible diana terapéutica como tratamiento coadyuvante en el manejo de la VRP.

PUNTOS CLAVE

vi. La VRP es una enfermedad compleja (multifactorial), en la que participan factores clínicos, genéticos y ambientales.
vii. El perfil genético puede actuar como factor de riesgo en el desarrollo de la VRP, sin embargo no parece existir todavía un patrón de herencia definido
viii. La inflamación, así como sus mediadores (como el TNFA) favorecen la génesis y desarrollo de la VRP.
ix. La isquemia que se produce en las capas externas de la retina tras un DR también parece tener un papel importante en la génesis de la VRP.
x. La alteración de la apoptosis parece cumplir un papel clave en la supervivencia de las células del EPR, así como en la ulterior creación de un ambiente pro-inflamatorio que facilita la des-diferenciación, migración y proliferación celular.
xi. El papel de la activación de las vías de muerte celular en las enfermedades de la retina se está empezando a comprender mejor, así como el papel de moléculas anti-inflamatorias, en la prevención de la muerte celular de la retina por estas vías.

RELACIÓN TEMÁTICA ENTRE LOS TRABAJOS PRESENTADOS

Como se ha comentado, 4 de los trabajos presentados en la presente tesis forman parte de la producción científica de la segunda etapa del proyecto denominado *Retina-4*, en la que se incorporó el I.B.S.A.L de la Universidad de Salamanca. Además se presenta un trabajo adicional, recientemente publicado, que recoge todos los resultados de las dos grandes líneas de investigación del grupo de Retina del I.O.B.A (proyecto *Retina-1* y *Retina-4*).

Todos ellos tienen en común el intento de aportar más información acerca de la patogénesis de la VRP, así como la posibilidad de identificar potenciales biomarcadores para el diagnostico de los pacientes con alto riesgo e incluso para futuras dianas terapéuticas. Los esfuerzos se han orientado a investigar dos fenómenos importantes en el desarrollo de la VRP: *la inflamación* y *la muerte celular*.

Trabajos presentados en esta tesis:

Evaluación de inflamación y VRP

Trabajo 1.

Pastor-Idoate S, Rodríguez-Hernandez I, Rojas J, Gonzalez-Buendia L, Lopez JC, González-Sarmiento R, Pastor JC. (2015). Functional Characterization of rs2229094 (T>C) polymorphism and LTA Expression in Human Retina. The Retina 4 project. Paper has already submitted to ACTA Ophthalmol Journal. 2015.

En este primer trabajo, se ha pretendido estudiar la funcionalidad del SNP rs2229094 (T>C) del gen LTA y se ha determinado la expresión y la localización de

la LTA en muestras de retina humana. El rs2229094 es un polimorfismo de tipo no sinónimo, situado en el péptido señal del gen LTA y dentro del locus del TNF, y que como ya se ha mencionado, este SNP mostró una fuerte asociación con el desarrollo de VRP (Rojas J et al 2009, Rojas J et al 2010).

La superfamilia de citoquinas del TNF regulan muchos procesos fisiológicos, incluyendo la inflamación, la proliferación, la diferenciación y la muerte celular (Locksley RM et al 2001). La LTA, miembro de la familia del TNF, es una citoquina pro-inflamatoria que juega un papel importante en la respuesta inflamatoria e inmunológica. (Aggarwal BB. 2003). El TNFA y la LTA, comparten un 40% de homología entre ellas. La LTA desarrolla un papel clave en la comunicación entre los linfocitos y las células estromales, provocando efectos citotóxicos sobre las células. También tiene la capacidad de inducir la expresión del *vascular cell-adhesion molecule 1* (VCAM-1) en las células endoteliales vasculares, facilitando el reclutamiento de células *natural killers* (NK) hacia el parénquima de los órganos o áreas de lesión (Fogler WE et al 1996). El TNFA y la LTA ejercen sus efectos biológicos en gran medida a través de la activación de los receptores TNF (TNFR1 y TNFR2) (Naoum JJ et al 2006), pero parece haber diferencias en cuanto a su capacidad de activación (Etemadi N et al 2013). La LTA tiene menor capacidad de promover la muerte celular inducida por el TNFR1 o de activar el factor nuclear-kB (NF-kB) (Chaturvedi MM et al 1994), la expresión de marcadores de superficie celular (Andrews JS et al 1990) o la producción de citoquinas (Oster W et al 1987, Broudy VC et al 1987), que el TNFA.

Por otro lado, también se han descrito efectos protectores en las neuronas cuando actúan a través de TNFR2, especialmente en condiciones de isquemia tanto en el cerebro como en la retina (Shohami E et al 1999, Fontaine V et al 2002). Actualmente, debido a la diversidad de sus funciones, se desconoce bajo que circunstancias produce un efecto beneficioso o nocivo sobre las neuronas. Parece obvio que son necesarios más estudios para avanzar en la comprensión del papel de la LTA en células neuronales de la retina después de un DR y sobre cual es su implicación en el desarrollo de la VRP, ya que se ha demostrado ampliamente la participación de las citoquinas pro-inflamatorias, especialmente, el TNFA, en la

patogénesis de la VRP. Y asi por ejemplo, se han publicado niveles altos en el vítreo de TNFA y de sus receptores (TNFR1 y TNFR2) de ojos de pacientes con VRP (El-Ghrably IA et al 1999, Limb GA et al 2001). Y además, como ya se ha mencionado, la relación entre el TNF con los cambios intraretinianos tras un DR ya han sido demostrados por nuestro grupo (Fernández-Bueno I et al 2008, Fernández-Bueno I et al 2012, Fernández-Bueno I et al 2013).

Evaluación de muerte celular y VRP

Trabajo 2.

Pastor-Idoate S, Rodriguez-Hernández I, Rojas J, Fernández I, García-Gutierrez MT, Ruiz-Moreno JM, Rocha-Sousa A, Ramkissoon Y, Harsum S, Maclaren RE, Charteris D, van Meurs J, González-Sarmiento R, Pastor JC; Genetics on PVR Study Group. (2013). The p53 codon 72 polymorphism (rs1042522) is associated with proliferative vitreoretinopathy: The Retina 4 Project. Ophthalmology. 120(3):623-628.

Trabajo 3.

Pastor-Idoate S, Rodríguez-Hernández I, Rojas J, Fernández I, García-Gutiérrez MT, Ruiz-Moreno JM, Rocha-Sousa A, Ramkissoon Y, Harsum S, MacLaren RE, Charteris D, VanMeurs JC, González-Sarmiento R, Pastor JC; Genetics on PVR Study Group. (2013). The T309G MDM2 gene polymorphism is a novel risk factor for proliferative vitreoretinopathy. The Retina 4 Project. PLoS One. 9;8(12):e82283.

Trabajo 4.

Pastor-Idoate S, Rodríguez-Hernandez I, Rojas J, Fernandez I, Garcia-Gutierrez MT, Ruiz-Moreno JM, Rocha-Sousa A, Ramkisson Y, Harsum S, MacLaren RE, Charteris DG, VanMeurs JC, González-Sarmiento R, Pastor J.C on behalf of the Genetics on PVR Study Group. (2015). BAX and BCL-2 polymorphisms, as predictors of proliferative vitreoretinopathy development in patients suffering

retinal detachment: The Retina 4 Project. Acta Ophthal Dec 2014 (accepted for publication 18/02/2015.

En estos tres trabajos, se evaluó papel de los SNPs rs1042522 en gen p53, el rs2279744 en el gen MDM2, y los polimorfismos rs4645878 y rs2279115 en los genes BAX y BCL-2 respectivamente, y por tanto el papel de estos genes en el desarrollo de la VRP.

Hay estudios actuales que han puesto de relieve la participación, tanto de las vías extrínseca e intrínseca de la apoptosis, principal mecanismo de pérdida de fotoreceptores después de un DR (Lo AC et al 2011), como la existencia de otras vías de muerte celular, tales como la necrosis programada o necroptosis también implicadas en la patogenia de los DR. Son vías más inflamatorias que la apoptosis e independientes de las caspasas, que aumentan cuando la principal vía esta inhibida o esta alterada (Rosenbaum DM et al 2010, Lo AC et al 2011, Murakami Y et al 2011). Además, se ha publicado que la supresión de la expresión de p53 podría ser un evento necesario el desarrollo del DR y la VRP, y que el mantenimiento de los niveles de p53, podrían suponer un fenómeno eficaz en la profilaxis del DR y en la VRP (Lei H et al 2012).

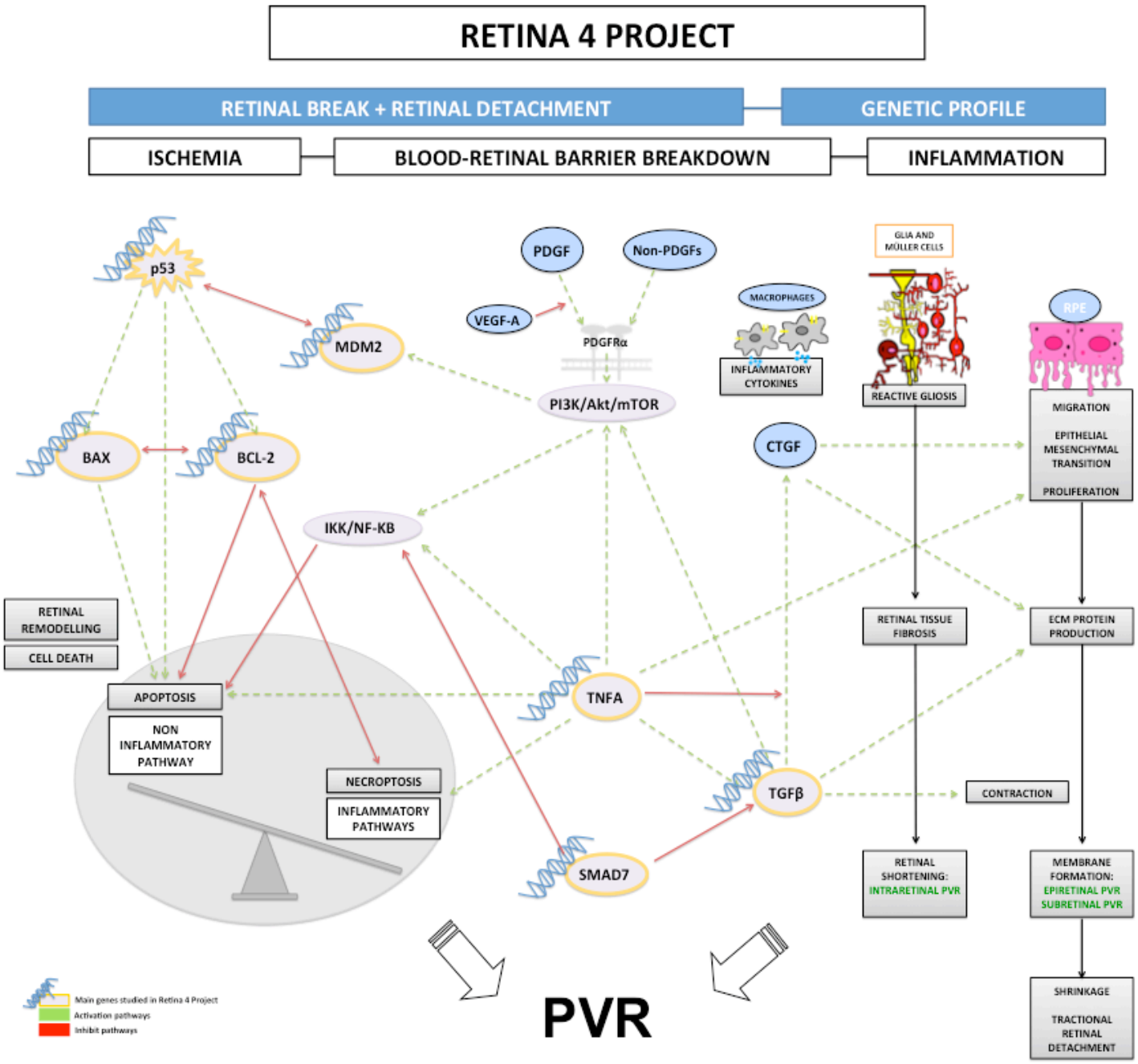
Estos SNPs fueron analizados no sólo por estar relacionados entre si, sino porque además lo están con el gen p53 (Moll UM et al 2003, Iwakuma T et al 2003, Saxena A et al 2002, Bond GL et al 2005, Alhopuro P et al 2005, Bougeard G et al 2006) y con el TNFA en la activación de las vías de muerte celular secundarias (Tamatani M et al 1999, Irrinki KM et al 2011, Lei H et al 2012). Además estos SNPs, están asociados con la disminución o el aumento en los niveles de apoptosis, y con respuestas proliferativas celulares. Como ya se ha comentado, un proceso inadecuado de apoptosis se considera un importante factor en la patogénesis de muchas enfermedades (Hetts SW et al 1998, Elmore S. 2007) y de alteraciones en la regulación del proceso de curación-reparación de heridas que podrían conducir a formas patológicas de cicatrización o de fibrosis (Elmore S. 2007).

Sin embargo, a pesar de su asociación con procesos tumorales e inflamatorios (Sun Y et al 1999, Adams JM et al 2001, Vousden KH et al 2002, Lin HJ et al 2002, Bond GL et al 2005, Van Heemst D et al 2005, Alhopuro P et al 2005, Bougeard G et al 2006, Kang MH et al 2009, Assmann G et al 2010, Irrinki KM et al 2011, Frank AK et al 2011, Gomez-Sanchez JC et al 2011, Di Vuolo et al 2011), su papel en el DR y en el desarrollo de la VRP no ha sido todavía objeto de estudio.

Trabajo 5.

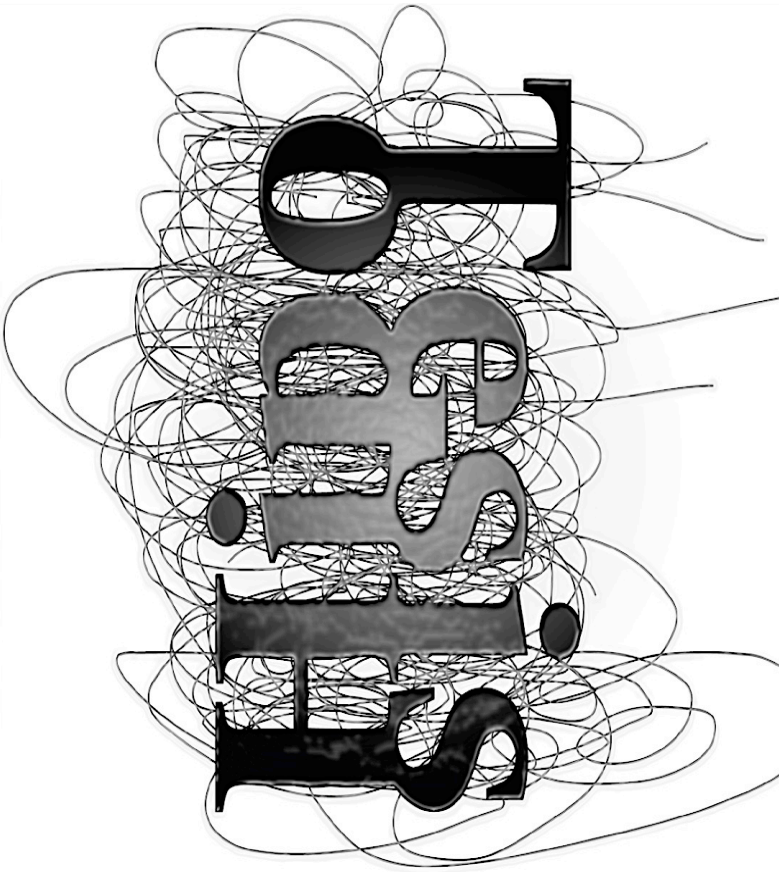
Pastor JC, Rojas J, **Pastor-Idoate S**, Di Lauro S, Gonzalez-Buendia L, Delgado-Tirado S. Proliferative vitreoretinopathy: A new concept of disease pathogenesis and practical consequences. Prog Retin Eye Res. 2015 Jul 21. EPUB ahead of print]

En este último trabajo, se han recogido los conocimientos y las ideas del grupo de retina del I.O.B.A sobre la patogénesis de la VRP, procedentes de las líneas de investigación proyecto *Retina-1* y *Retina-4.* Y aunque no cumple con los requisitos de autoría para formar parte del compendio de los trabajos, enlaza los hallazgos del resto de los trabajos ampliando la información de las investigaciones de los últimos 20 años sobre esta complicación.



El presente gráfico, muestra la relación entre los diferentes trabajos realizados dentro del proyecto *Retina-1* como del proyecto *Retina-4*. Este gráfico, ha sido actualizado y modificado del gráfico presentado en el trabajo-5 (Pastor JC et al 2015), en el que se identifican los genes implicados en los trabajos de la tesis.

La hipótesis general de este trabajo es que tanto modificaciones en la respuesta inflamatoria como en el principal proceso de muerte celular (apoptosis), ambas inducidas por variaciones genéticas, están asociados con el desarrollo de la VRP tras un DR.



Esta hipótesis general está basada en las siguientes sub-hipótesis derivadas de los hallazgos del grupo de trabajo:

Evaluación de inflamación y VRP

El SNP *rs2229094*, que está localizado en el péptido señal del gen LTA podría ser funcional y alterar la producción ó localización de la proteína LTA. Además, los niveles de LTA, asociada previamente a la VRP y a la inflamación, podrían estar alterados (incrementados o disminuidos)en retinas de pacientes que han sufrido un DR.

Evaluación de muerte celular y VRP

Los SNPs *rs1042522*, *rs2279744*, *rs4645878* y *rs2279115,* localizados en los genes p53, MDM2, BAX y BCL-2 están asociados con la disminución o el aumento en los niveles de apoptosis, y con respuestas proliferativas celulares, y podrían estar asociados con una mayor frecuencia en el desarrollo de la VRP.

OBJETIVOS

El objetivo general de este trabajo ha sido relacionar la presencia de estos SNPs en muestras de DNA procedentes de muestras sanguíneas de pacientes que han sufrido un DR con la presencia de VRP y establecer si existen asociaciones estadísticamente significativas con el desarrollo de esta complicación. Además se ha intentado caracterizar funcionalmente uno de los SNP del LTA.

La muestras de DNA utilizadas para este propósito proceden de la colección de muestras biológicas obtenidas en el proyecto denominado *Retina-4*.

El objetivo principal se puede subdividir en los siguientes objetivos secundarios:

Evaluación de Inflamación y VRP

- Caracterización funcional del polimorfismo rs2229094 (T>C) del gen LTA y su expresión y localización de la LTA en retina humana. (**Trabajo. 1**).

Evaluación de muerte celular y VRP

- Evaluación de la asociación del polimorfismo rs1042522 en el codon 72 del gen p53 y la VRP. (**Trabajo. 2**).

- Evaluación de la asociación del polimorfismo rs2279744 en el gen MDM2 y la VRP y la posible asociación entre los polimorfismos rs1042522 del gen p53 y el polimorfismo rs2279744 el gen MDM2 (**Trabajo. 3**).

- Evaluación de la asociación de los polimorfismos rs2279115 y rs4645878 en los genes BAX y BCL-2, respectivamente, y la VRP (**Trabajo. 4**).

Además se ha añadido un quinto trabajo (**Trabajo. 5**) que resume las ideas del Proyecto *Retina -1* y *Retina-4* y los conocimientos de nuestro grupo sobre la patogénesis de la VRP.

METODOLOGÍA

Aspectos éticos y normativos

Las muestras incluidas en todos los trabajos presentados, han sido utilizadas de acuerdo con las leyes aplicables para la investigación con tejidos y muestras humanas (14/2007 de la ley es Española en Investigación Biomédica) y de acuerdo con los postulados de la declaración de Helsinki.

El **trabajo 1** fue aprobado por la Comisión de Investigación del I.O.B.A, de la Universidad de Valladolid y por el Comité de Ética del Hospital Clínico Universitario Valladolid. Además se firmo un acuerdo especifico de cesión de muestras humanas (Real Decreto 1716/2011, del 18 de Noviembre) entre el Hospital Clínico Universitario de Valladolid y el I.O.B.A a fecha de 22-Abril de 2014.

Para los **trabajos 2, 3 y 4** se utilizo la colección biológica de muestras de ADN procedente de la primera etapa proyecto *Retina-4* (Rojas J et al 2010, Rojas J et al 2013), habiendo solicitado la pertinente aprobación por parte del Comité de Investigación del I.O.B.A, de la Universidad de Valladolid y de los colaboradores participantes en la primera etapa del proyecto *Retina-4*. Esta primera etapa del proyecto, fue aprobada por el Comité de Investigación del IOBA y por el comité Ético y de Investigación de cada centro participante (España, Portugal, Holanda y Reino Unido) (Rojas J et al 2010, Rojas J et al 2013). Todos los pacientes firmaron y otorgaron su consentimiento tras haber sido informados antes de entrar en el estudio, para la extracción sanguínea y envío de muestras de ADN.

Todos los análisis estadísticos de los **trabajos 2, 3,** y **4** se llevaron a cabo por la Dra. Itziar Fernández (Sct, PhD), de la unidad de estadística del I.O.B.A.

Trabajo 1:

En el **trabajo 1**, se estudió la funcionalidad del polimorfismo rs2229094 (T>C) del gen LTA y se determinó la expresión y localización de la LTA en muestras de retina humana.

Funcionalidad del polimorfismo rs2229094 (T>C) del gen LTA:

La funcionalidad de los alelos T y C se investigó mediante la clonación de estos alelos en el vector pCEFL-Flag y análisis de su expresión (pCEFL-Flag-LTA-T y pCEFL-Flag-LTA-C) tanto a nivel del RNA como a nivel de proteínas. También se analizó la su localización sub-celular en las células COS-1 utilizando un anticuerpo específico anti-Flag.

La expresión del RNAm de LTA-T y LTA-C se evaluó mediante *reacción en cadena de la polimerasa con transcriptasa inversa* (RT-PCR) a las 7h, 24h y 48h después de la transfección, analizando el nivel de expresión del RNAm de LTα entre los dos alelos y de los niveles de expresión de proteínas de LTA-T y LTA-C por Western blot.

Aislamiento del RNA y RT-PCR:

El RNA total procedente de las neuroretinas de 3 donantes del banco de tejidos de Castilla y León y de dos muestras de sangre periférica de sujetos sanos procedentes de la Unidad de Medicina Molecular de la Universidad de Salamanca, fue aislado mediante el reactivo Trizol (Invitrogen™, Carlsbad, CA, USA) de acuerdo con las especificaciones del fabricante. Las concentración finales de RNA en las muestras fue medida en una nanogota mediante un espectrofotómetro (Thermo Scientific, Waltham, MA, USA).

El *ADN complementario* (cDNA) fue sintetizado con el sistema Improm-II™ Reverse Transcription (Promega, Madison, WI, USA) de acuerdo con las especificaciones del fabricante con 1µg total de ARN por cada reacción. El cDNA de la LTA se amplifico de estas muestras mediante la reacción de la polimerasa (GoTaq Hot Start Polymerase. Promega, Madison, WI, USA) utilizando los siguientes primers:

-Forward: 5'-ATGACACCACCTGAACGTCTC-3'

-Reverse: 5'-CTACAGAGCGAAGGCTCCAA-3'

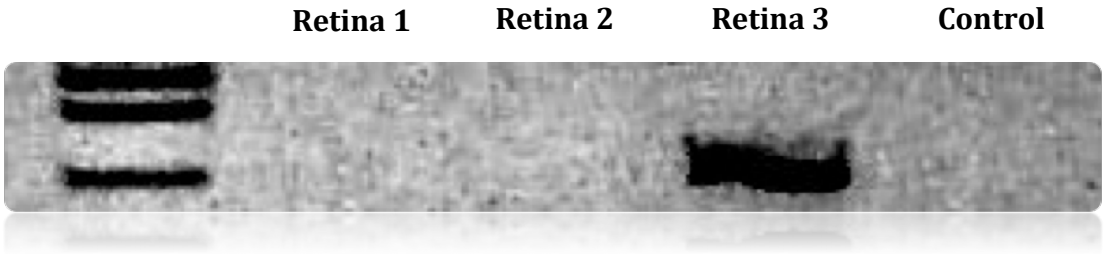
El ADN se amplificó mediante el siguiente protocolo: 5 min de desnaturalización a 94°C, seguido por 35 ciclos de 30s a 94°C, 58°C durante 30s, y a 72°C durante 1min.

La *Gliceraldehído-3-fosfato deshidrogenasa* (GAPDH) se utilizó como control interno en cada muestra, utilizando los siguientes primers:

-Forward: 5'-CCACCCATGGCAAATTCCATGGCA-3'

-Reverse: 5'-TCTAGACGGCAGGTCAGGTCCACC-3'

Los fragmentos resultantes se separaron en gel de agarosa 3,5%. Los fragmentos teñidos con Bromuro de Etidio fueron analizados bajo una fuente de *luz ultravioleta* (UV), utilizando el sistema de análisis de imagen: Kodak Digital Science ID.



Los productos de la LTA RT-PCR fueron secuenciados para identificar los diferentes transcriptos obtenidos, así como el genotipo del SNP rs2229094 de tamaño completo mediante el analizador genético ABI Prism 3100 (Applied Biosystems, Foster City, CA, USA).

Clonación, secuenciación y transformación:

Generación de los vectores de expresión de la LTA

El cDNA completo, portando el alelo C del SNP LTA rs2229094, fue amplificado de las muestras de sangre periférica utilizando los siguientes primers con los lugares de restricción EcoRI and NotI y siguiendo el protocolo de PCR ya descrito (los sitios de restricción aparecen subrayados).

-Forward: 5'-GAATTCACACCACCTGAACG-3'

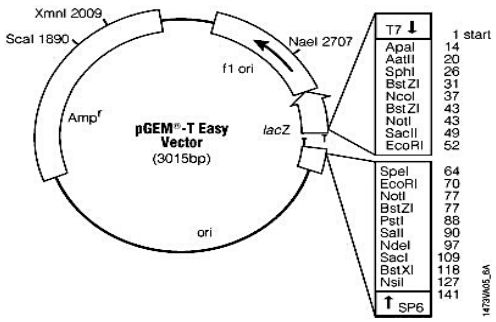
-Reverse: 5'-GCGGCCGCCTACAGAGCGAAGG-3'

Los productos de la PCR fueron clonados en el vector pCEFL-Flag en los sitios EcoRI-NotI, para generar los vectores pCEFL-Flag-LTA-C y pCEFL-Flag-LTA-T. La sustitución del SNP LTA rs2229094 (T>C) fue introducida en el vector pCEFL-Flag-LTA-C usando el kit de mutagenesis: QuikChange™ Site-Directed Mutagenesis (Stratagene, San Diego, CA,USA) y los siguientes primers:

-Forward: 5'-CTCCCAAGGGTGTGTGGCACCACCC-3'

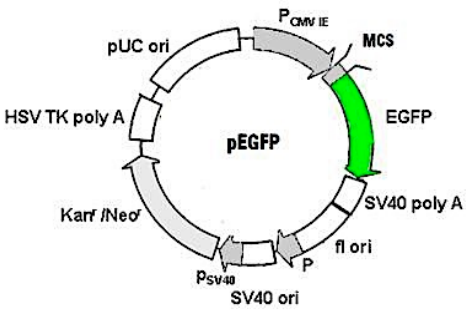
-Reverse: 5'-GGGTGGTGCCACACACCCTTGGGAG-3'

Clonación en vectores de expresión



pGEM-T Vector

Enzimas: **ApaI, SacI**

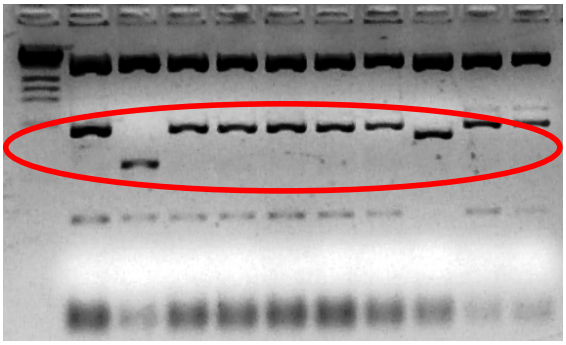


pEGPF Vector

Enzimas: **ApaI, EcoI**

Todos los vectores fueron transformados en bacterias E.Coli DH5α y el DNA plásmidico fue purificado usando el siguiente kit: Rapid DNA plasmid miniprep kit (Genedan, S.L, Barcelona, Spain). Todos los constructos fueron verificados mediante secuenciación automática.

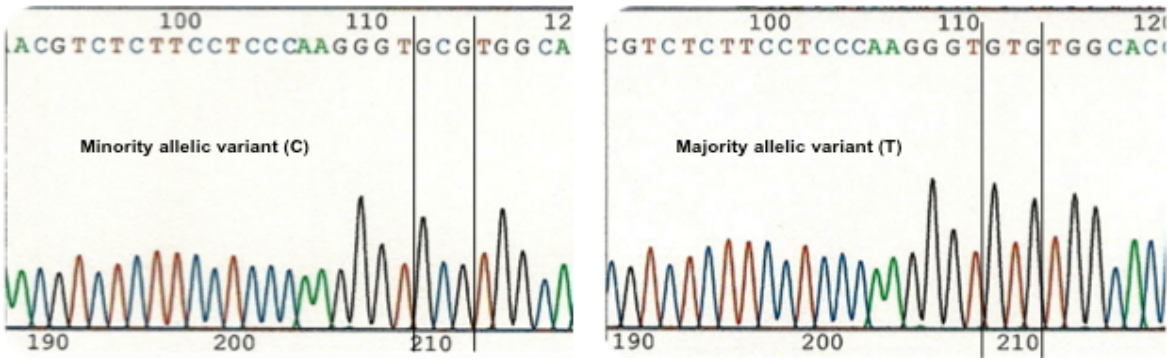
Transformación en bacterias E.Coli



Transformación en DH5 alpha competent E. Coli mediante un shock térmico a 42ºC

Gel de Agarosa1.5% (w/v) mostrando los TBE Vector con los insertos de LTA-T and LTA-C

Secuenciación de las variantes T y C



<u>Cultivo celular y transfección de DNA y expresión de proteínas</u>:

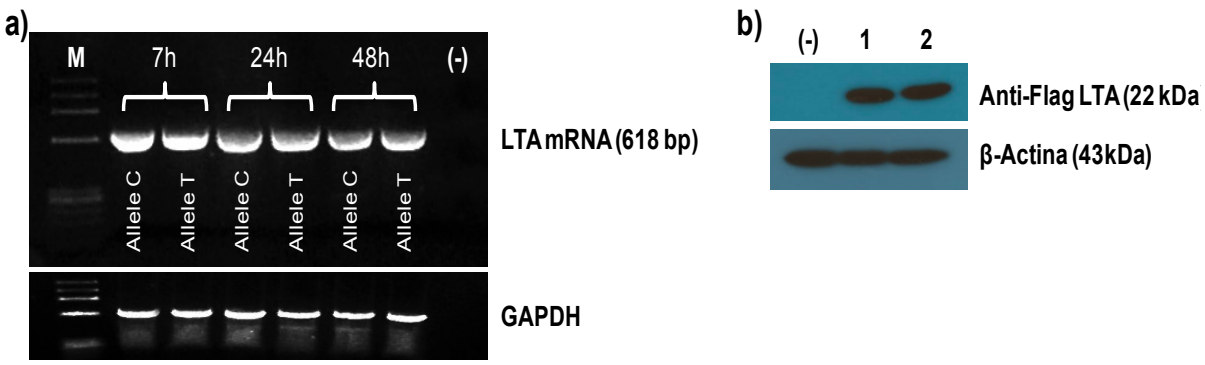
Con el fin de averiguar si la sustitución rs2229094 T por C en el péptido señal de LTA pudiera afectar a la correspondiente localización de la proteína, se realizó un ensayo de inmunofluorescencia para identificar la localización sub-celular de los alelos T y C (pCEFL-Flag-LTA-T y pCEFL-Flag-LTA-C) en la línea celular COS1 a las 48 horas después de la transfección.

Las células COS-1 fueron cultivadas en el medio Dulbecco's modified Eagle suplementado con suero fetal bovino al 10%, 1% de L-glutamina y 1% de penicilina + estreptomicina a 37ºC, con el 5% de CO_2 atmosférico. Las células con un crecimiento exponencial fueron transfectadas con 1µg de ADN total plasmidico (pCEFL-Flag-LTA-C y pCEFL-Flag-LTA-T) usando el reactivo de transfección X-tremeGENE HP DNA Transfection Reagent (Roche, Switzerland) con un ratio de 3:1. Después de 24-48 h de la transfección se analizo la expresión de ARN y el nivel de proteínas de los vectores pCEFL-Flag-LTA-C y pCEFL-Flag-LTA-T mediante RT-PCR y Western blot.

Western Blot

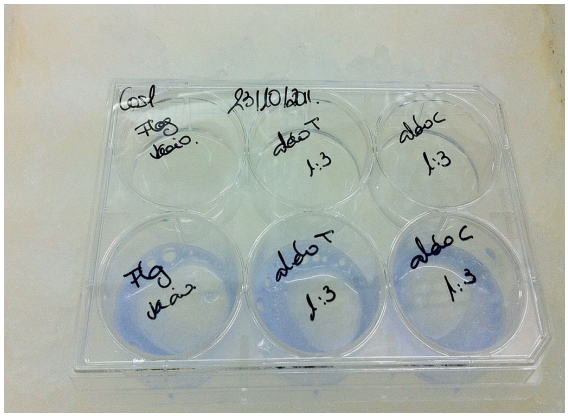
Las células COS-1 tras 24-48 h después de la transfección fueron re-suspendidas en el siguiente buffer (50mM Tris pH7.5, 150mM NaCl, 5mM EDTA y 1% NP40) y suplementadas con Complete Mini (Roche, Switzerland) para la extracción de proteínas, mediante la utilización del kit BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Los extractos de proteína (200 µg) fueron fraccionados mediante el SDS-PAGE, transferidos a una membrana de inmovilización de Millipore (Millipore, Billerica, MA, USA) e incubadas con anticuerpos primarios específicos anti-Flag M2 y anti-β-actin (Sigma-Aldrich Co, St. Louis, MO, USA). Para la detección se utilizo el sistema ECL Plus detection System (GE Healthcare, Buckinghamshire, UK) con anticuerpos secundarios antiratón conjugados HRP241 (Sigma-Aldrich Co, St. Louis, MO,USA).

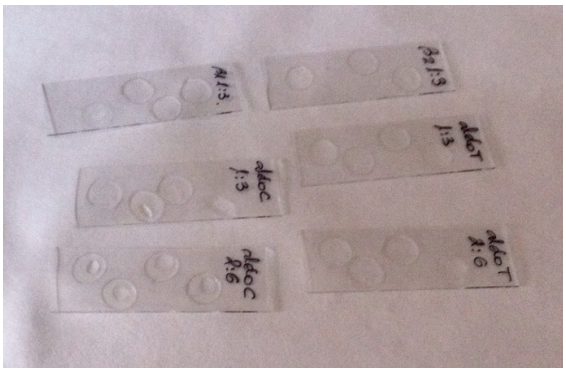
Análisis de expresión de los alelos T y C del SNP LTA rs2229094



Análisis con immunofluorescencia confocal:

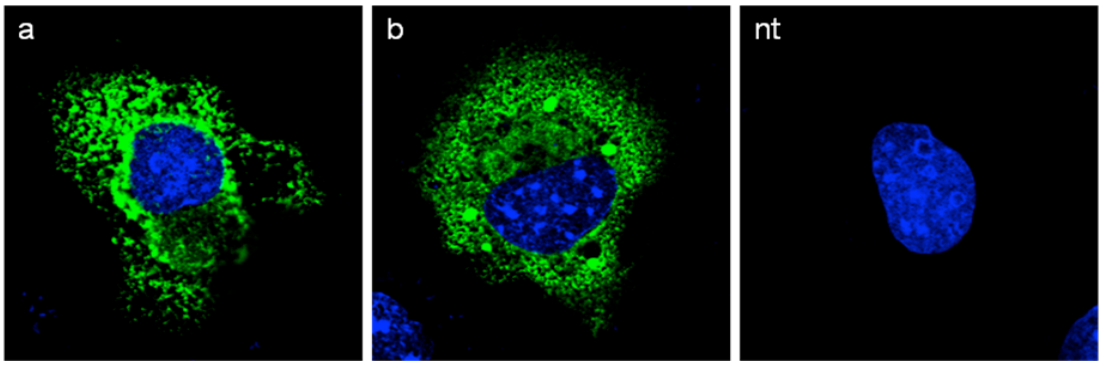
Las células COS-1 transfectadas fueron fijadas en formaldehido al 3.7% en PBS y hielo y permeabilizadas con 0.5% Triton X-100 in PBS. Posteriormente fueron incubadas con anticuerpos primarios específicos anti-Flag M2 (Sigma-Aldrich Co, St. Louis, MO, USA) y anticuerpos secundarios Alexa Fluor 488 Donkey Anti-cabra IgG *conjugados con isotiocianato de fluoresceína*(FITC) (Invitrogen, Carlsbad, CA,USA). Finalmente las células fueron teñidas con DAPI (4',6-diamidino-2-phenylindole) y montadas en portas en presencia del medio Mowiol.





Las imágenes de inmunofluorescencia fueron capturadas usando un microscopio confocal LEICA TCS SP5 DMI-6000B (Leica Microsystems Inc, Wetzlar, Germany) con una longitud de onda de excitacion de 488nm (para el FITC), y fueron analizadas mediante el sistema LEICA LAS AF (Leica Microsystems Inc, Wetzlar, Germany).

El procesamiento final de las imágenes se realizo con el programa Final Adobe Photoshop (Adobe Systems).



Inmunofluorescencia con Cofocal de los alelos T y C del SNP LTA rs2229094

Expresión y localización de la LTA en muestras de retina humana:

-La expresión de LTA se evaluó tanto a nivel en el RNAm como de los niveles de proteína, utilizando muestras de retina humana procedentes del Banco de tejidos Regional de Castilla y León y muestras de sangre periférica de sujetos sanos.

El RNAm fue analizado mediante RT-PCR en muestras de RNA total procedente de 3 neuro-retinas de donantes sanos obtenidas entre 30 y 60 minutos post-mortem y de 2 muestras de sangre periférica de sujetos sanos.

La localización de LTA se investigó mediante análisis inmunohistoquímico utilizando muestras de retina humana con diagnostico previo de DR crónico durante el periodo 1990-2013, procedentes del archivo de muestras de patología del laboratorio de Patología Ocular del I.O.B.A, y una muestra de retina sana procedente del banco de tejidos de Castilla y León. Además, se utilizaron secciones de tejido cerebral (área hipocampal) procedentes de la Fundación *Centro de Investigación en Enfermedades Neurológicas* (CIEN) (Madrid), como controles positivos.

Para la tinción inmunohistoquímica se utilizó un anticuerpo específico frente a LTA a una dilución 1:10 (HPA007729 Sigma-Aldrich®, St. Louis, MO) y el kit Envision™ de sistema de detección de FLEX para visualizar la reacción (Agilent's

Dako products Glostrup, Denmark). Las imágenes fueron tomadas con un microscopio óptico Leica DM4000 B equipado con una cámara digital Leica DFC490. El procesamiento final de las imágenes se realizó con el programa Adobe Photoshop.

La expresión de la LTA fue evaluada de manera semicuantitativa: (-: negativo; +: débilmente positiva; ++: moderadamente positiva; +++: fuertemente positiva).
Las muestras fueron evaluadas por tres observadores, de los cuales dos hicieron las evaluaciones de forma enmascarada, en el laboratorio de Patología Ocular del I.O.B.A. y en el servicio de Histología de la Facultad de Medicina de la Universidad de Valladolid.

Trabajo 2:

En el **trabajo 2,** se llevo acabo un estudio de asociación genética de casos y controles analizando la distribución del SNP rs1042522 del gen TP53 en una muestra de 555 muestras de ADN, procedente de la primera etapa proyecto *Retina-4* (Rojas J et al 2010, Rojas J et al 2013). El genotipado del SNP rs1042522 del gen TP53 se realizó en la Unidad de Medicina Molecular y en el IBSAL de la Universidad de Salamanca desconociendo el estado clínico de los pacientes, y utilizando la técnica *Polymerase Chain Reaction-Restriction Fragment Length Polymorphism* (PCR-RFLP) (Matlashewski GJ et al 1987, Lazar V et al 1993).

La PCR-RFLP es una técnica frecuentemente utilizada en los estudios moleculares, que consiste en la combinación de dos métodos: Amplificación por PCR del gen a estudio, y posterior digestión (o corte en fragmentos) del producto amplificado con enzimas de restricción, para ver los fragmentos resultantes o fragmentos de restricción de ese gen. Este método es útil para detectar pequeñas inserciones o deleciones en determinados fragmentos de restricción de un gen, ya que el tamaño de los mismos se verá aumentado o disminuido, respectivamente. En otros casos, ciertas mutaciones puntuales en un gen, que alteran la secuencia de nucleótidos del mismo, pueden crear nuevos lugares de restricción o hacer desaparecer

aquellos presentes en el gen normal, lo que alterará el patrón de los fragmentos de restricción observables por electroforesis (Zhang R et al 2005).

Criterios de inclusión-exclusión proyecto Retina-4 :

Todos los participantes fueron pacientes con un DRR primario sometidos a cirugía de vitrectomia vía pars plana (VPP) como cirugía reparadora.

Los criterios de exclusión fueron los siguientes: edad menor de 16 años, DR traccional, DR exudativo, DR traumático o iatrogénico. DR secundario a un agujero macular o a un desgarro gigante (más de 3 husos horarios) y presencia de VRP mayor de grado B (de acuerdo con la clasificación de Machemer (Machemer R et al. 1991)) en el momento de la cirugía.

Aquellos pacientes que no desarrollaron VRP tras 3 meses de seguimiento desde el momento de la cirugía de VPP, fueron clasificados como *grupo control*. Aquellos que desarrollaron VRP grado C1 o grado superior de acuerdo con la clasificación de Machemer (Machemer R et al. 1991) despúes de la cirugía VPP fueron clasificados como *grupo casos*. En total se analizaron 134 casos y 416 controles: 203 procedentes de España (36.9%), 68 procedentes de Portugal (12.4%), 121 de Holanda (22%), y 158 del Reino Unido (28.7%).

Genotipado:

El SNP rs1042522 del gen TP53 fue analizado y amplificado mediante la utilización de la reacción en cadena de la polimerasa y un alelo específico, usando como primers:
-Forward 5´-TCTACAGTCCCCCTTGCCGT-3´
-Reverse 5´-CTGACCGTGCAAGTCACAGA-3´

La enzima BstU1 (Bsh1236I Fermentas, Thermo Scientific, Germany) se utilizó como enzima de restricción para discriminar los alelos. Los fragmentos resultantes se separaron en gel de agarosa 3,5%, se tiñeron con Bromuro de Etidio y fueron

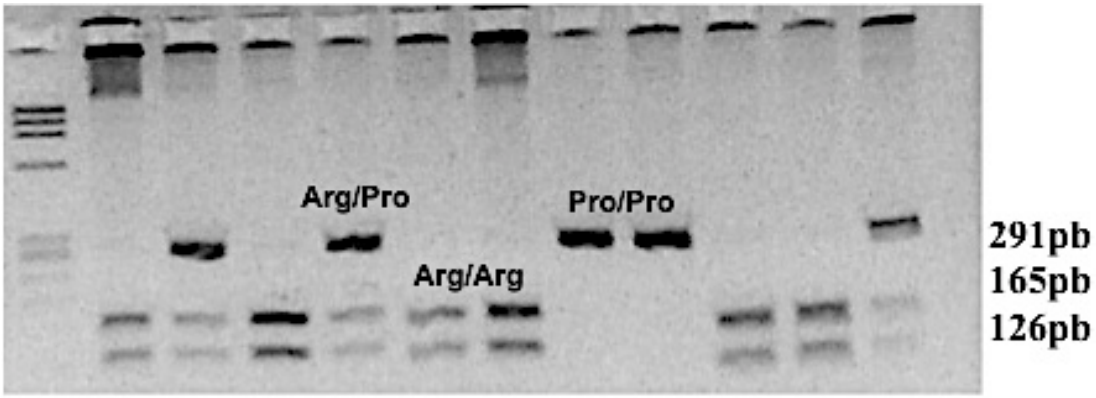
analizados bajo una fuente de luz ultravioleta (UV), utilizando el sistema de análisis de imagen: Kodak Digital Science ID.

Los fragmentos generados tras la tras la digestión enzimática migraron de la siguiente forma:

-1 fragmento de de 291 pb para los homocigotos Pro/Pro.

-2 fragmentos de 165 y 126 pb para los homocigotos Arg/Arg.

-3 fragmentos de 126, 165 y 291 pb para los heterocigotos Pro/Arg.



PCR products of Arg and Pro after digestion

Las reacciones de PCR fueron realizaron mediante una mezcla de reacción de 50 ml, que contiene 100-200 ng de ADN diana, 20 pmol de cada cebador, 2.5 mM $MgCl_2$, 50 mM de cada dNTP y 1,25 U de HotMaster Taq ADN polimerasa (5 Primer GmbH, Hamburgo, Alemania). El ADN se amplificó mediante los siguientes pasos: 5 min de desnaturalización a 94°C, seguido por 35 ciclos de 30s a 94°C, 58°C durante 30s, y a 72°C durante 30s, con una elongación final a 72°C durante 10 min.

Análisis estadístico:

El análisis estadístico se realizó utilizando el software SPSS versión 16.0 para Macintosh (SPSS, Inc., Chicago, IL) y el software R (R Foundation for Statistical Computing, Viena, Austria). La calidad de los datos fue evaluada en los grupos control mediante el *equilibrio Hardy-Weinberg* usando el test Chi-cuadrado. Las frecuencias genotípicas fueron estimadas en cada sub-grupo.

Las proporciones de los diferentes genotipos, así como la proporción de homocigotos Pro/Pro procedentes de las muestras de diferentes países se analizaron en 2 fases con el fin de llevar a cabo una replicación del estudio. Primero, se analizaron las sub-muestras de España y Portugal y posteriormente tras resultados significativos se hicieron las muestras procedentes de Holanda y el Reino Unido. Además se analizaron las posibles diferencias geográficas considerando a las muestras de España y Portugal como poblaciones del hemisferio sur y a Holanda y el Reino Unido como poblaciones del hemisferio norte. Además también se compararon las frecuencias genotípicas y alélicas entre los casos y controles en la muestra global (España + Portugal + Holanda + Reino Unido).

Las asociaciones se analizaron mediante el Chi-cuadrado y el test exacto de Fisher. La fuerza de asociación fue medida mediante la odds-ratio (OR) y el intervalo de confianza al 95% (CI 95%). Se consideraron dos modelos de herencia: *el modelo co-dominante*, en el que cada genotipo proporciona un riesgo de enfermedad diferente y no aditivo y el *modelo recesivo*, en el que son necesarios 2 copias del alelo Pro para cambia el riesgo. El criterio de información de Akaike (AIC) se utilizó para elegir el modelo de herencia que mejor se ajusta a los datos (Akaike H. 1974).

Trabajo 3:

En el **trabajo 3**, se llevo a cabo un estudio de asociación genética de casos y controles analizando la distribución del SNP rs2279744 del gen MDM2 en una muestra de 555 muestras de ADN, procedente de la primera etapa proyecto *Retina-4* (Rojas J et al 2010, Rojas J et al 2013). Además se evaluó la posible asociación entre los SNPs rs1042522 del gen TP53 y el rs2279744 del gen MDM2. El genotipado del SNP rs2279744 se realizó en la Unidad de Medicina Molecular y en el IBSAL de la Universidad de Salamanca, utilizando la técnica PCR-RFLP (Alhopuro P et al 2005, Di Vuolo V et al 2011).

Criterios de inclusión-exclusión proyecto Retina-4 :

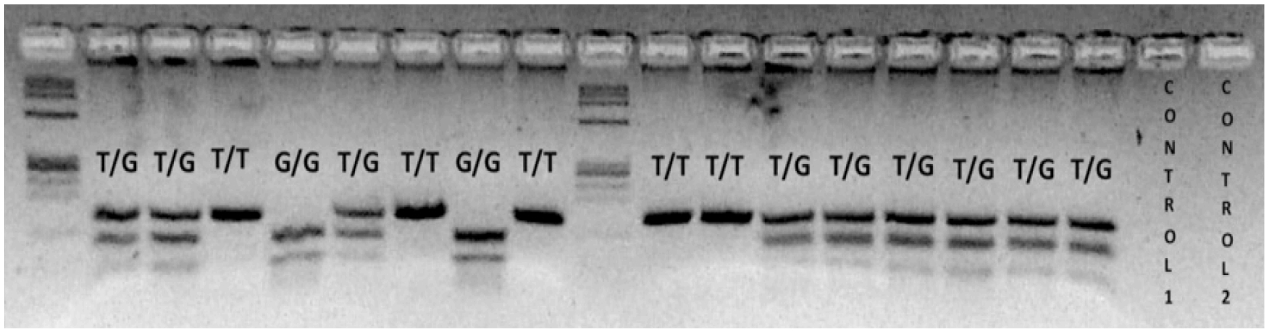
Los criterios de inclusión y exclusión para los casos y controles fueron los mismos que se ya se han detallado (Rojas J et al 2010, Rojas J et al 2013, Pastor-Idoate S et al 2013).

Genotipado:

El SNP rs2279744 del gen MDM2 se analizó y se amplificó mediante la utilización de la reacción en cadena de la polimerasa y un alelo específico, usando como primers:
-Forward: 5'-GAGGTCTCCGCGGGAGTTC-3'
-Reverse: 5'-CTGCCCACTGAACCGGC-3'

La enzima MspA1I (New England Biolabs, Inc, Ipswich, MA, USA) fue utilizada como enzima de restricción para discriminar los diferentes alelos. Los fragmentos resultantes se separaron en gel de agarosa 3,5% tras la incubación con la enzima y el gel fue teñido con Bromuro de Etidio para su posterior visualización bajo una fuente de luz U, utilizando el sistema de análisis de imagen: Kodak Digital Science ID.

Los fragmentos de la PCR generados tras la digestión enzimática migraron de la siguiente forma:
-1 fragmento de 155 pb para los homocigotos T (T/T).
-2 fragmentos de 101y 54 pb para los homocigotos G (G/G).
-3 fragmentos de 157, 101 y 54 pb para los heterocigotos TG (T/G).



PCR products of G and T after digestion

Las reacciones de PCR se realizaron mediante una mezcla de reacción de 50 ml, que contiene 100-200 ng de ADN diana, 20 pMol de cada cebador, 2.5 mM $MgCl_2$, 50 mM de cada dNTP y 1,25 U de HotMaster Taq ADN polimerasa (5 Primer GmbH, Hamburgo, Alemania). El ADN se amplificó mediante los siguientes pasos: 5 min de desnaturalización a 94°C, seguido por 35 ciclos de 30 s a 94°C, 61°C durante 30 s, y a 72°C durante 30 s, con una elongación final a 72°C durante 10 min.

Análisis estadístico:

El análisis estadístico utilizado fue el mismo los mismos que el ya se ha detallado en el trabajo 2 (Pastor-Idoate S et al 2013).

La posible interacción genética entre p53 y MDM2 se analizó entre los pacientes portadores de la variante Pro del SNP rs1042522 y los de la variante G del SNP rs2279744 en la muestra global y en las sub-muestras de los diferentes países.

Se consideraron dos modelos de herencia: *el modelo co-dominante* y el *modelo recesivo*. El criterio AIC fue utilizado para elegir el mejor modelo de herencia (Akaike H. 1974).

Además para ajustar los valores de significación estadística (p-valores) para múltiples comparaciones se realizo test de permutación o pruebas de re-aleatoriedad (resampling). Se barajaron 1000 etiquetas al azar de la serie de casos y controles para obtener la correcta distribución de la prueba estadística en la

hipótesis de no asociación. El ranking de la prueba estadística real entre los test estadísticos barajados proporciona los p-valores ajustados.

Trabajo 4:

En el trabajo 4, se llevo acabo un estudio de asociación genética de casos y controles analizando la distribución de los SNPs rs4645878 del gen BAX y rs2279115 del gen BCL-2 en una muestra de 555 ADNs procedentes de pacientes incluidos en la primera etapa proyecto Retina-4 (Rojas J et al 2010, Rojas J et al 2013). El genotipado de los SNPs se realizó en la Universidad de Salamanca, utilizando la técnica PCR cuantitativa ó PCR en tiempo real en un equipo *Step-One Plus Real-time PCR* (Applied Biosystems), variante de la PCR utilizada para amplificar y simultáneamente cuantificar de forma absoluta el producto de la amplificación del ADN (Heid CA et al 1996, Watson JD et al 2004).

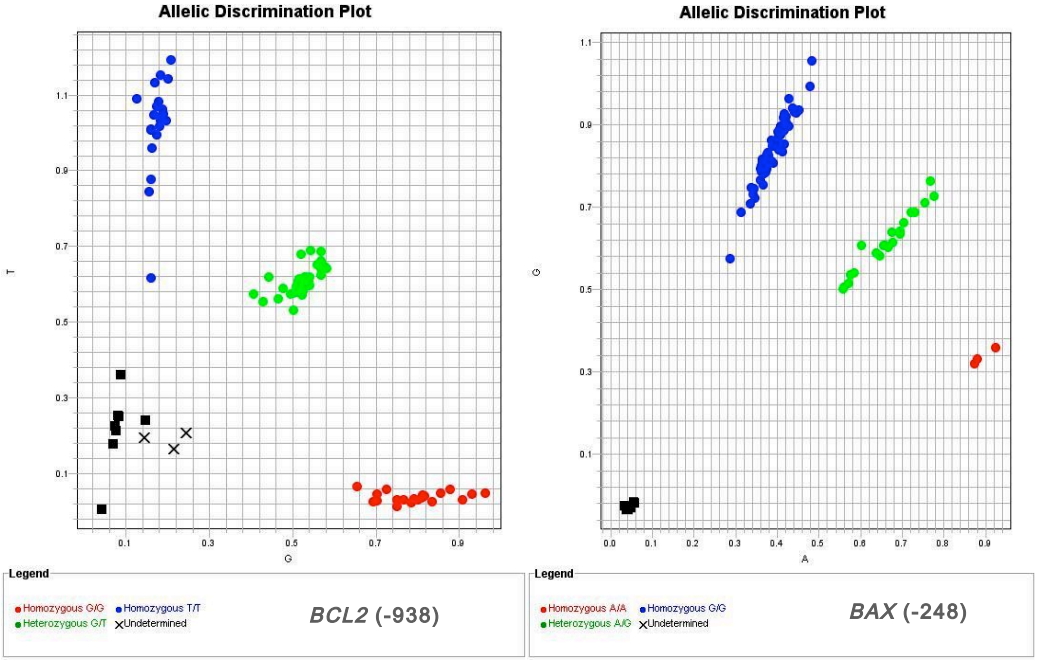
Criterios de inclusión-exclusión proyecto Retina-4 :

Los criterios de inclusión y exclusión para los casos y controles fueron los mismos que ya se han detallado (Rojas J et al 2010, Rojas J et al 2013, Pastor-Idoate S et al 2013a, Pastor-Idoate S et al 2013b).

Genotipado:

Los SNPs rs4645878 del gen BAX y rs2279115 del gen BCL-2 fueron analizados y amplificados mediante la utilización de PCR cuantitativa y de sondas específicas TaqMan® (*TaqMan 50-exonuclease allelic discrimination assays*. Applied Biosystems, Foster City, CA, USA) acuerdo con las especificaciones del fabricante (Applied Biosystems). Esta técnica permite medir la producción de productos de PCR mediante un sistema de sondas marcadas mediante dos fluorocromos. La fluorescencia que producen es específica de la amplificación que estemos estudiando, además de permitir que se usen varios fluorocromos en la misma reacción y detectar varios ADN/RNA al mismo tiempo (Heid CA et al 1996).

Para la PCR se utilizó la siguiente mezcla: 15ng de DNA genómico, 5µl de TaqMan® SNP genotyping Mastermix (Applied Biosystems) y 0.25µl de TaqMan® SNP Genotyping Assay (SNP ID C_3044428 para el SNP rs2279115 del gen BCL-2 y SNP ID C_27848291 para el SNP rs4645878 del gen BAX, Applied Biosystems) en un volumen final de 10µl. Las condiciones de la PCR fueron: 95°C durante 10min, seguidos por 40 ciclos a 95°C durante 15s, 60°C durante 1min y finalmente 60°C durante 1min. Para mantener una calidad en las amplificaciones cada muestra fue procesada por duplicado para cada SNP.



Gráficos de amplificación, mostrando la separación de los tres tipos de genotipos, así como los controles negativos (esquina inferior izquierda), en el análisis de PCR en tiempo real, para los SNPs BCL-2 (-938)(derecha) y BAX (−248)(izquierda).

Análisis estadístico:

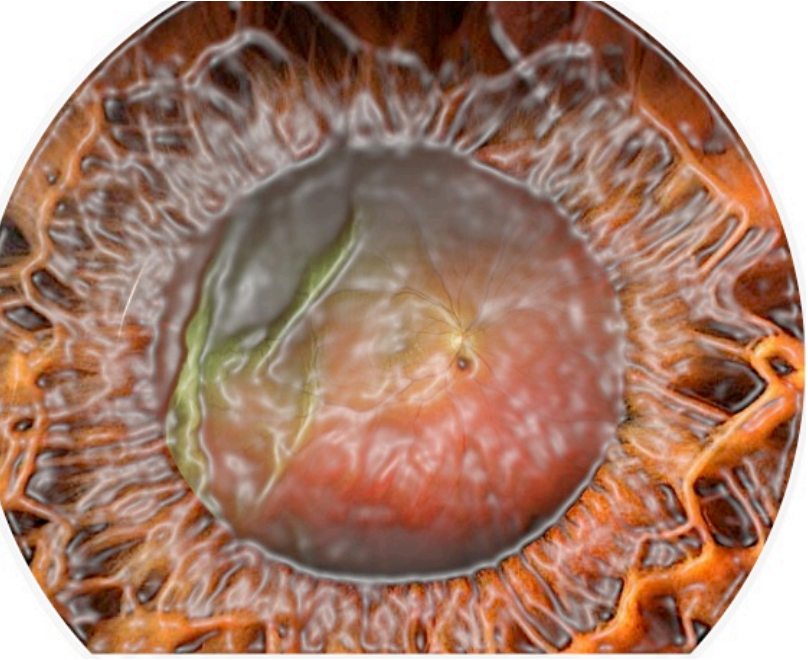
El análisis estadístico utilizado fue el mismo los mismos que el ya se ha detallado en el trabajo 2 y 3 (Pastor-Idoate S et al 2013a, Pastor-Idoate S et al 2013b).

Se consideraron dos modelos de herencia para el análisis de BAX: el *modelo dominante*, en el que una sola copia del alelo A es suficiente para modificar el riesgo y el ser portador de dos copias lo modifica en igual magnitud, es decir, que

tanto los portadores del genotipo AG como los del AA tienen el mismo riesgo. Por tanto, la combinación AG + AA se compara con el genotipo GG. y el *modelo aditivo,* en el que cada copia de A modifica el riesgo en una cantidad aditiva, por lo tanto los homocigotos AA tienen el doble de riesgo que los heterocigotos AG. Para el para el análisis de BCL-2 se consideraron el *modelo dominante*, y el *modelo sobredominante*, en el que los individuos homocigotos para el genotipo más frecuente (GG) y el genotipo variante (AA) tienen el mismo riesgo. El criterio de selección AIC fue utilizado para elegir el mejor modelo de herencia (Akaike H. 1974).

Trabajo 5:

En este último trabajo, se han recogido los conocimientos y las ideas del grupo de Retina del I.O.B.A en los últimos 20 años sobre la patogénesis de la VRP, procedentes de las líneas de investigación proyecto *Retina-1* y *Retina-4.*



Resultados del trabajo 1:

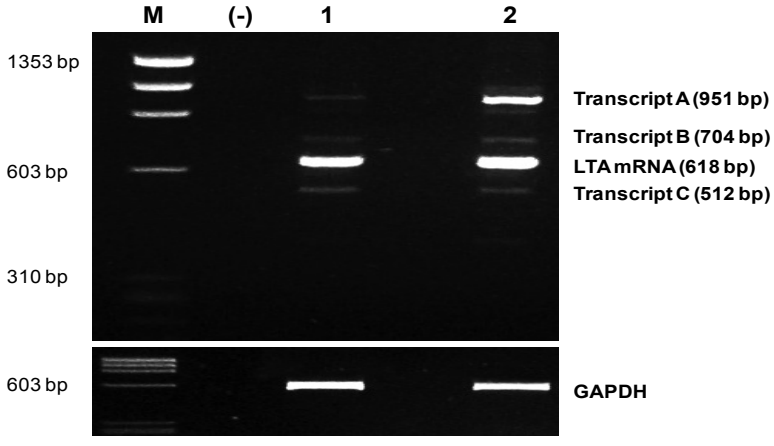
En el **trabajo 1**, se estudió la funcionalidad del polimorfismo rs2229094 (T>C) del gen LTA y se determinó la expresión y localización de la LTA en muestras de retina humana.

Funcionalidad del polimorfismo rs2229094 (T>C) del gen LTA:

Expresión del ARNm del LTA:

Los niveles de expresión obtenidos del ARNm del la LTA procedente de las 3 neuro-retinas fueron inferiores a los niveles de detección de la prueba utilizada. Sin embargo, en las muestras de sangre periférica, no sólo se obtuvieron niveles significativos de ARNm de la LTA, sino que además el análisis de RT-PCR, reveló transcriptos alternativos (Figura 1).

Figura 1: Productos de RT-PCR del ARNm de la LTA detectados por electroforesis en gel de agarosa al 3% teñido con Bromuro de Etidio.

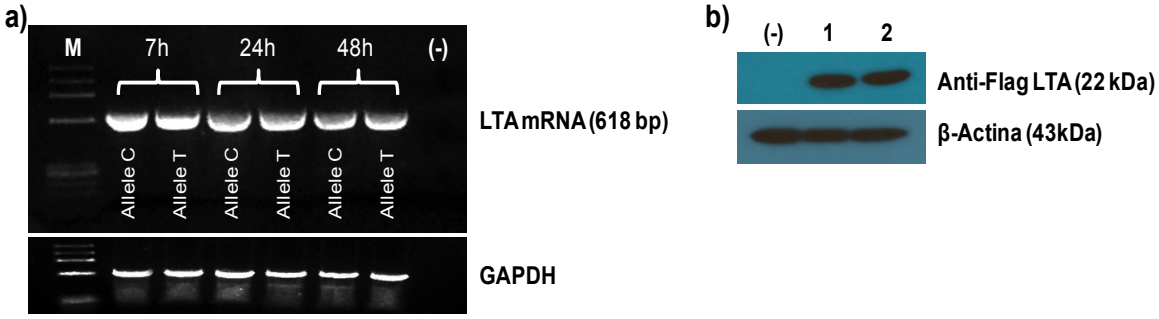


Se detectó la expresión del ARNm de LTA en las dos muestras de sangre periférica de sujetos sanos (carril 1 y 2). El análisis de RT-PCR reveló diferentes transcritos alternativos de la LTA (A, B y C). La secuenciación automática reveló que la transcripto A mantiene los intrones 2 y 3 de gen LTA, el transcripto B mantiene el intrón 2, mientras que el transcripto C carecía del exón 3 de gen LTA. Además, todos estos transcritos incluyen codones de parada prematuros, lo que lleva a isoformas truncadas de la proteína LTA que no poseen ninguna función.

La expresión de GAPDH se utilizó como control interno. El carril M fue el marcador de peso molecular ΦX174 ADN / HaeIII (Promega, Madison, WI, EE.UU.). El carril (-) fue la reacción control que carece de la transcriptasa inversa.

Caracterización funcional de los alelos T y C del SNP LTA rs2229094:

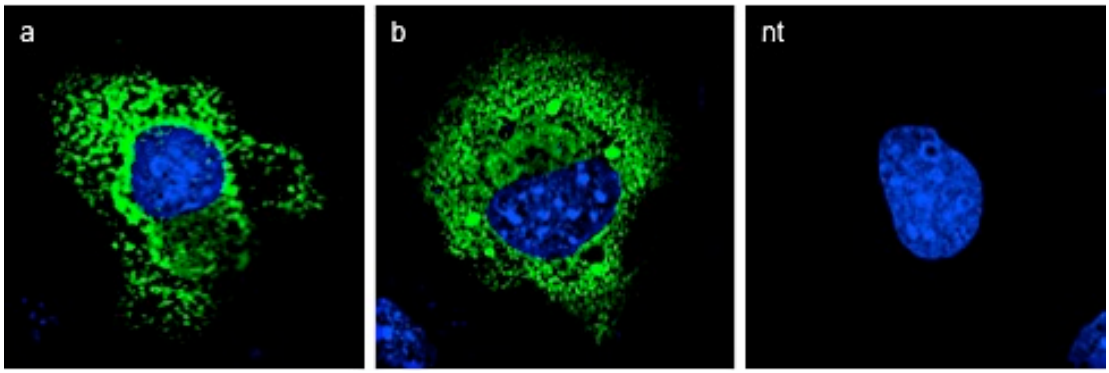
No se encontraron diferencias en la expresión de ARNm entre los alelos LTA-T y los LTA-C en las RT-PCR a las 7h, 24 y 48h después de la transfección (Figure. 2). Tampoco se encontraron diferencias a nivel de expresión proteica entre los alelos LTA-T y los LTA-C con la técnica de Western-blot (Figura 2).



(a) Expresión de RNAm de ambos alelos de la LTA mediante RT-PCR a 7, 24 y 48 horas después de la transfección. La expresión de GAPDH se utilizó como control interno. El carril M fue el marcador de peso molecular ΦX174 ADN / HaeIII (Promega, Madison, WI, EE.UU). El carril (-) fue la reacción control que carece de la transcriptasa inversa. (b) La expresión de proteína de ambos alelos LTA se analizó mediante transferencia Western a las 48 horas después de la transfección usando anticuerpo anti-Flag. El carril 1 muestra el alelo LTA-C, el carril 2 muestra el alelo

LTA-T y el carril (-) muestra el control negativo (células COS-1 no transfectadas). La expresión de β-actina se utilizó como control interno.

Con la finalidad de averiguar si la sustitución de T por C en el péptido señal del SNP rs2229094 pudiese afectar a la localización final proteica se llevo a cabo un estudio con inmunofluorescencia sin obtener tampoco diferencias entre los dos alelos (Figura 3).

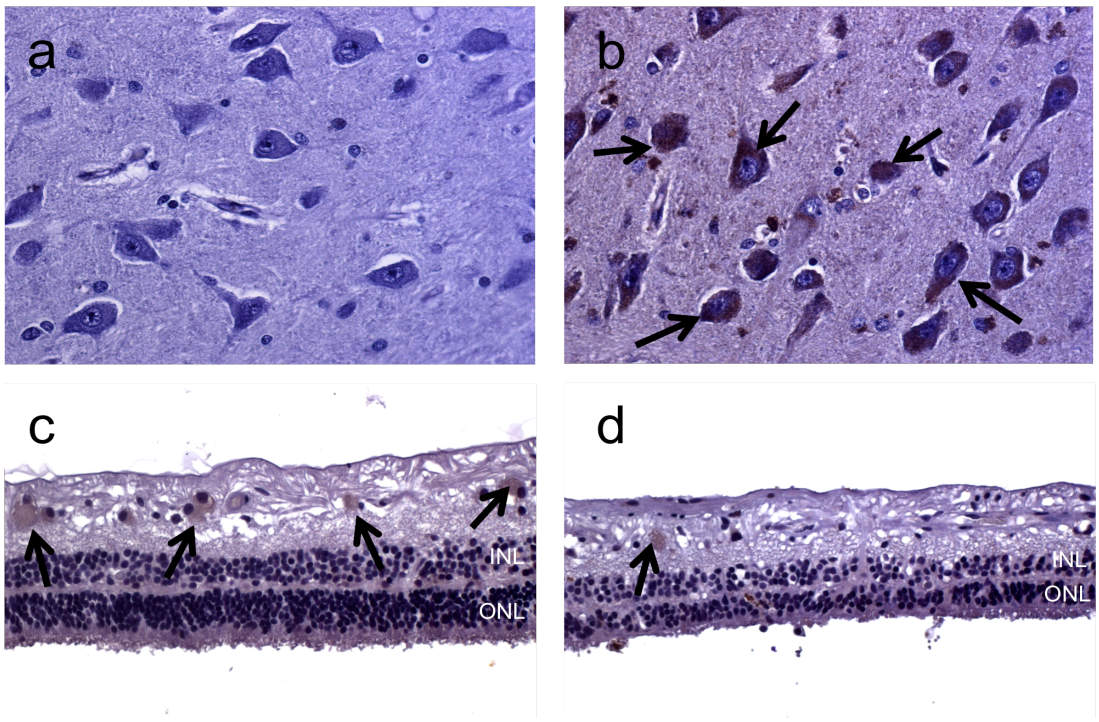


48 horas después de la transfección, se realizó la inmunofluorescencia con microscopia confocal, utilizando anticuerpos anti-Flag (que tiñen de color verde). Los núcleos celulares se tiñeron con DAPI (que tiñe en azul). Tanto el alelo LTA-C (a) como el alelo LTA-T (b) se localizaron principalmente en el citoplasma celular, sin encontrar diferencias en su localización. La imagen "nt" corresponde a las células COS-1 no transfectadas.

Aunque los resultados de este estudio sugieren una falta de funcionalidad del SNP LTA rs2229094, este SNP podría seguir siendo válido como un biomarcador para identificar pacientes de alto riesgo de desarrollar VRP tras un DR, debido a su fuerte asociación con esta complicación retiniana, demostrada anteriormente en trabajos del grupo (Rojas J et al 2009, Rojas J et al 2010). Además el gen LTA se encuentra dentro de locus TNF, que está estrechamente vinculado con los genes del TNFA y LTβ, y el gen TNFA también esta fuertemente asociado al desarrollo de la VRP (Rojas J et al 2010, Rojas J et al 2013).

Expresión y localización de la LTA en muestras de retina humana:

Se detectó una tinción positiva para LTA en secciones de cerebro que sirvieron de control, confinada a las células neuronales del hipocampo y sus citoplasmas (Figura 4. B). La intensidad de la inmunotinción fue moderada para las secciones de la retina sanas controles (Figura 4. C). mientras que la intensidad de la tinción y el número de células teñidas fueron notablemente menor (débilmente positivo) en las secciones de la retina de ojos con RD crónicas (Figura. 4D). En ambos casos (retinas sanas y con DR crónicos) la inmunotinción se limitó a las células neuronales ganglionares, a sus membranas celulares y a sus citoplasmas (Figura. 4C, 4D).



Inmunohistoquímica: (a) hipocampo humano adulto con ningún anticuerpo primario (control negativo), magnificación original 400X. (b) hipocampo humano adulto con tinción anti-LTA, mostrando positividad intensa (+++) en el citoplasma de las neuronas del hipocampo (flechas negras) 400X. (c) retina humana saludable con tinción anti-LTA (control). Mostrando moderada positividad citoplásmica (++) en las células ganglionares (flechas negras), 200X. (d) tinción anti-LTA (caso) en retina humana con DR crónico mostrando débil positividad citoplasmática (+) en las células ganglionares (flecha negro), 200X. Además se objetivo un

adelgazamiento de la retina y una degeneración de los fotorreceptores segmentos externos en la retinas con DR crónico. INL: capa nuclear interna.
ONL: capa nuclear externa.

La evaluación morfológica con microscopía óptica mostró que la inmunotinción para LTA tanto para las retina control como para las retinas con DR crónico se encuentra principalmente en la capa de fibra nerviosas y en la capa de células ganglionares de la retinas (Figura 4C, 4D). También se detectó inmunotinción débil y apenas visible en otras neuronas retinianas (amacrinas y / o bipolares), situadas en la capa nuclear interna.

Los resultados del presente estudio muestran una menor tinción inmunohistoquimica en las secciones procedentes de retinas con un DR crónico. Es posible que al tratarse de DR crónicos las muestras no presenten concentraciones elevadas de citoquinas pro-inflamatorias y esa sea la razón de su negatividad.

Los mediadores pro-inflamatorios, tales como TNFA y LTA, relacionadas con el proceso isquémico alcanzan concentraciones máximas a las 72h (Berger S et al 2008), como ocurre tras un DR agudo. Además, el aumento de la muerte células neuronales que se produce en un DR crónico podría ser otra de las posibles causas de una menor cantidad de inmunotinción en las secciones con DR crónico.

Resultados del trabajo 2:

En el **trabajo 2**, se llevo acabo un estudio de asociación genética de casos y controles analizando la distribución del SNP rs1042522 en una muestra de 555 muestras de ADN, procedente de la primera etapa proyecto Retina-4 (Rojas J et al 2010, Rojas J et al 2013).

La distribución de las frecuencias genotípicas obtenidas en las muestras procedentes de los 4 países están representadas en la siguiente tabla (Pastor-Idoate S et al 2013a).

Tabla1. Distribución de las frecuencias genotípicas del SNP rs1042522

Países	Arg/Arg *Casos/Controles*	Pro/Pro *Casos/Controles*	Arg/Pro *Casos/Controles*	p valor[1] *test de Fisher*	p<0.05 95% CI[2] *Casos/Controles*	OR
España	27.1%/45.1%	**35.6%**/6.25% [2]	37.3%/48.61%	***<0.05***[1]	*[29.01-42.18]/[2.29-10.20]*[2]	8.5
Portugal	33.3%/43.39%	**20%**/3.77% [2]	46.7%/52.8%	***<0.05***[1]	*[10.49-29.50]/[1.35-8.89]*[2]	6.5
UK	35.5%/36.2%	**12.9%**/9.4%	51.6%/54.3%	>0.05[1]	-	
Holanda	31%/33.7%	**24.1%**/9.8% [2]	44.8%/56.5%	>0.05[1]	*[16.49-31.70]/[4.51-15.09]*[2]	3.3

[1]Comparación de proporciones genotípicas entre las muestras. Se hallaron diferencias significativas entre casos y controles en España y Portugal.
[2]El análisis entre los homocigotos Pro, mostro diferencias en España, Portugal y Holanda.

Los resultados de este estudio mostraron que los portadores españoles y portugueses de la variante homocigota Pro/Pro tienen un riesgo de hasta 4 veces más de desarrollar VRP tras un DR en comparación con los que portaban la variante Arg en homocigosis (Tabla 2) (Pastor-Idoate S et al 2013a).

Tabla 2. Modelos de herencia utilizados en la muestra global y resultados de OR usando el modelo recesivo en España + Portugal y en Holanda + Reino Unido (UK).

Modelos	Genotipo	Controles		Casos		OR	CI OR 95%		p-valor	AIC*
Co-dominante	Arg/Arg	165	39.7	41	30.6	1.00				
	Arg/Pro	219	52.6	58	43.3	1.07	[0.68	1.67]	<0.001	588.6
	Pro/Pro	32	7.7	35	26.1	***4.40***	***[2.44***	***7.93]***		
Dominante	Arg/Arg	165	39.7	41	30.6	1.00				
	Arg/Pro-Pro/Pro	251	60.3	93	69.4	1.49	[0.98	2.26]	<0.001	611.1
Recesivo	Arg/Arg-Arg/Pro	384	92.3	99	73.9	1.00			***<0.001***	586.7
	Pro/Pro	32	7.7	35	26.1	***4.24***	***[2.50***	***7.19]***		
Sobre-dominante	Arg/Arg-Pro/Pro	197	47.4	76	56.7	1.00			<0.001	611.2
	Arg/Pro	219	52.6	58	43.3	0.69	[0.46	1.02]		
España+Portugal		186	94.4	50	67.6	1.00	***[3.72***	***17.69]***	***<0.001***	291.3
		11	5.6	24	32.4	***8.12***				
Holanda+UK		198	90.4	49	81.7	1.00	[0.96	4.68]	0.07	291.3
		21	9.6	11	18.3	2.12				

Esta observación fue confirmada posteriormente en la población de pacientes holandeses y en el análisis de la muestra global con un riesgo de 4.24 veces más para los homocigotos Pro/Pro (Tabla 3) (Pastor-Idoate S et al 2013a).

Tabla 3. Distribución del SNP rs1042522 en la muestra global. (test de Fisher).

Genotipos	Arg/Arg	Arg/Pro	Pro/Pro	Total	P-valor	IC 95%
Casos	41 (30.59%)	58 (43.28%)	35 **(26.1%)***	**134** (100%)	<0.05	[18.67-33.52]
Controles	165 (39.66%)	219 (52.64%)	32 (7.69%)	**416** (100%)		[5.1-10.2]
Total	**206**	**277**	**67**	**550**		

No se encontraron diferencias en el análisis de la población británica, aunque se observó una tendencia similar a la población de Holanda. La ausencia de correlación en la población británica dentro del presente estudio podría deberse al hecho de que la frecuencia de los alelos del codón 72 del p53 difiere con la latitud, existiendo un aumento de las variantes Pro dentro de las poblaciones cercanas a la línea ecuatorial, mientras que existe un predominio de la variente Arg en latitudes más al norte (Sjalander A et al 1995, Frank AK et al 2011). Sin embargo, debido a que los perfiles genéticos de los pacientes holandeses fueron similares a los de españoles y portugueses, deberían buscarse otros posibles factores implicados en esta diferencia en futuros estudios. En este sentido, otra de las variables que podrían haber influido en la diferencia de resultados es la diversidad étnica en el grupo de pacientes analizados procedentes del Reino Unido superior a la de las otras poblaciones al tratarse de pacientes atendidos en Londres.

Los resultados de este trabajo señalan que los portadores del alelo Pro del polimorfismo en el gen p53, asociado con una disminución en la función apoptótica de p53, tienen un mayor riesgo de desarrollar PVR después de un DR. Es posible especular en el sentido de que el proceso de apoptosis mediado por p53 podría desempeñar un papel clave en la generación de VRP después de la cirugía DR.

Este estudio, además pone de relieve el papel de la genética como una herramienta útil en la identificación de pacientes de alto riesgo susceptibles de desarrollar VRP e indica que el alelo Pro podría tener un riesgo significativo de desarrollo de VRP tras un DR, pudiendo por lo tanto ser utilizado como un posible marcador de riesgo.

Resultados del trabajo 3:

En el **trabajo 3**, se llevo a cabo un estudio de asociación genética de casos y controles analizando la distribución del SNP rs2279744 en una muestra de 555 muestras de ADN, procedente del proyecto *Retina-4* (Rojas J et al 2010, Rojas J et al 2013)

La distribución de las frecuencias genotípicas obtenidas en las muestras procedentes de los 4 países están representadas en la siguiente tabla (Pastor-Idoate S et al 2013b).

Tabla 1. Distribución de los genotipos y frecuencias alélicas entre casos y controles

Países	Genotipo	Controles	Casos	P-valor Test de Fisher	P-valor corregido	Alelos	Controles	Casos	Controles (95% IC Alelos)	Casos (95% IC Alelos)	P-valor Chi Cuadrado test	OR*	IC OR 95%
España	T/T T/G G/G	72 (50.0%) 64 (44.4%) 8 (5.6%)	18 (30.5%) 30 (50.9%) 11 (18.6%)	0.0037[1]	0,0039[2]	G T	80 (27.8%) 208 (72.2%)	52 (44.0%) 66 (56.0%)	[22.6-32.9] [67.0-77.4]	[35.1-53.0] [46.9-64.9]	0.0012[3]	2.0	[1.31-3.19]
Portugal	T/T T/G G/G	27 (51.0%) 20 (37.7%) 6 (11.3%)	3 (20.0%) 7 (46.7%) 5 (33.3%)	0.0387[1]	0,0449[2]	G T	32 (30.2%) 74 (69.8%)	17 (56.7%) 13 (43.3%)	[21.4-38.9] [61.7-78.5]	[39.0-74.4] [61.0-25.6]	0.0156[3]	3.0	[1.31-6.95]
UK	T/T T/G G/G	66 (50.0%) 56 (42.4%) 10 (7.6%)	7 (22.6%) 17 (54.8%) 7 (22.6%)	0.0047[1]	0,0059[2]	G T	76 (28.8%) 188 (71.2%)	31 (50.0%) 31 (50.0%)	[23.3-34.2] [65.7-76.6]	[37.5-62.5] [37.5-62.5]	0.0015[3]	2.4	[1.40-4.35]
Holanda	T/T T/G G/G	40 (43.5%) 45 (48.9%) 7 (7.6%)	4 (13.8%) 19 (65.5%) 6 (20.7%)	0.0037[1]	0,0049[2]	G T	59 (32.0%) 125 (68.0%)	31 (53.5%) 27 (46.5%)	[25.3-38.8] [61.1-74.8]	[40.6-66.2] [46.5-59.3]	0.0023[3]	2.4	[1.27-464]

[1] Test de Fisher (H_o. Independencia entre genotipos entre casos y controles). Se hallaron diferencias significativas entre casos y controles en el genotipo G/G en España y Portugal y en Holanda y Reino Unido.

[2]Test de permutación para comparaciones múltiples

[3]El análisis entre los homocigotos G/G, mostro diferencia en España, Portugal, Holanda y Reino Unido.

Los resultados del presente estudio mostraron que los portadores españoles y portugueses de la variante homocigota G/G tienen un riesgo de hasta 5.4 veces más de desarrollar VRP tras un DR en comparación con los que portaban la variante T en homocigosis (tabla 2) (Pastor-Idoate S et al 2013b).

Tabla 2. Modelos de herencia utilizados en la muestra global y resultados de OR usando el modelo codominante en España + Portugal y en Holanda + Reino Unido.

Modelos	Genotipo	Controles	Casos	OR*	IC 95%	P-valor	AIC*	P-valor corregido
Co-dominante	T/T T/G G/G	205 (48.7%) 185 (43.9%) 31 (7.4%)	32 (24.0%) 73 (54.4%) 29 (21.6%)	1.00 2.53 5.99	 [1.59-4.01] [3.20-11.24]	2.0374e-08	584.1	0,0009[1]
Dominante	T/T T/G-G/G	205 (48.7%) 216 (51.3%)	32 (24.0%) 102 (76.0%)	1.00 3.03	- [1.95-4.70]	2.1790e-07	590.7	0,0009[1]
Recesivo	T/T-T/G G/G	390 (92.6%) 31 (7.4%)	105 (78.4%) 29 (21.6%)	1.00 3.47	- [2.00-6,02]	1.4218e-05	598.7	0,0009[1]
Sobre-dominante	T/T-G/G T/G	236 (56.1%) 185 (43.9%)	61 (45.6%) 73 (54.4%)	1.00 1.53	- [1.03-2.26]	3.3371e-02	613.0	0,0319[1]
España+Portugal	T/T T/G G/G	99 (50.3%) 84 (42.6%) 14 (7.1%)	21 (28.4%) 37 (50.0%) 16 (21.6%)	1.00 2.08 5.44	- [1.13-3.82] [2.30-12.7]	0.0003	307.7	0,0019[1]
Holanda+UK	T/T T/G G/G	106 (47.3%) 101 (45.1%) 17 (7.6%)	11 (18.3%) 36 (60.0%) 13 (21.7%)	1.00 3.43 7.30	- [1.66-7.11] [2.80-19.01]	2.6566e-05	277.8	0,0009[1]

[1]Test de permutación para comparaciones multiples

[2]Resultados de OR usando el modelo co-dominate en España + Portugal y en Holanda + Reino Unido (UK).

Esta observación fue también confirmada en la población de pacientes holandeses y del Reino Unido, y en el análisis de la muestra global con un riesgo de hasta 5.9 veces mayor en los homocigotos G/G (tabla 2)(Pastor-Idoate S et al 2013b).
La distribución de las frecuencias genotípicas en la muestra global están representadas en la siguiente tabla (Pastor-Idoate S et al 2013b).

Tabla 3. Distribución de los genotipos y frecuencias alélicas entre casos y controles en la muestra global.

Genotipos	**T/T**	**T/G**	**G/G**	**Total**	**IC 95%***	**P-valor Test de Fisher**	**P-valor Corregido**	**OR[2]**	**IC OR 95%[2]**
Casos	32 (23.9%)	73 (54.5%)	29 (21.6%) *	134 (100%)	[42.954.8] *	1.6738e08	0.0009[1]	2.3	[1.733.05][2]
Controles	205 (48.7%)	185 (43.9%)	31 (7.4%)	421 (100%)	[26.232.4] *			-	-
Total	237	258	60	555					

[1]Test de permutación para comparaciones múltiples.
[2]Resultados de OR. Análisis de los homocigotos G/G entre casos y controles.

Interacción genética entre el SNP rs1042522 y el SNP rs2279744:

Cuando se analizaron los pacientes que portaban la variante Pro/Pro del gen p53 y la variante G/G del gen MDM2 se encontró un efecto adicional con un riesgo de desarrollo de la VRP tras un DR de hasta 10,19 veces mayor. Sin embargo ambos SNPs sólo se encontraron presentes en 16 de los 555 pacientes de la muestra global. Aunque los resultados fueron significativos se considera que la muestra es demasiado pequeña para obtener conclusiones robustas acerca de la posible asociación o interacción entre los dos SNPs y el desarrollo de VRP tras un DR, por lo que se hacen necesarios nuevos estudios para confirmar estos resultados (tabla 4)(Pastor-Idoate S et al 2013b).

Table 4. Interacción genética entre el SNP rs1042522 y el SNP rs2279744 en la muestra global y en las muestras procedentes de los diferentes países

Genotipo	Muestra	Controles	Casos	P-valor	P-valor Corregido	OR*	95% IC OR
Pro/Pro + G/G	Global	4	12	2.1139e-05	0.0009 [1]	10.19	[3.2-31.9]
	España+Portugal	1	7	0.0005	0,0009[1]	20.4	[2.4-169.5]
	Holanda+UK	3	5	0.0130	0,0169[1]	6.5	[1.5-28.2]

[1]Test de permutación para comparaciones múltiples.

[2]Resultados de OR de los pacientes que portan ambos genotipos (Pro/Pro + G/G).

Los resultados de este trabajo señalan que los portadores del alelo G de el gen MDM2, asociado con una disminución en la función apoptótica de p53, presentan un mayor riesgo de desarrollar PVR. Se podría especular de nuevo que el proceso de apoptosis mediado por p53 podría desempeñar un papel clave en la generación de VRP después de la cirugía DR. Además, se ha publicado que la supresión de la expresión del gen p53 podría ser un evento necesario en el desarrollo del DR y la VRP, y que agentes tales como la Nutlina-3, que impiden la interacción entre el p53 y MDM2, manteniendo los niveles de p53, podrían ser eficaces en la profilaxis del DR y en la VRP (Lei H et al 2012).

Este estudio, vuelve a poner en relieve el importante papel de la genética como una herramienta útil en la identificación de pacientes de alto riesgo, indicando que el alelo G del SNP del gen MDM2 al igual que el alelo Pro del SNP del gen p53 podrían ser utilizados como posibles marcadores de riesgo o como futuras dianas terapéuticas.

Resultados del trabajo 4:

En el trabajo 4, se llevo acabo un estudio de asociación genética de casos y controles analizando la distribución de los SNPs rs4645878 y rs2279115 localizados en los genes BAX y BCL-2, en la misma muestra de 555 del proyecto Retina-4 (Rojas J et al 2010, Rojas J et al 2013).

SNPs rs4645878:

Para el SNP G(-248)A-BAX (rs4645878), los resultados de este estudio mostraron que los portadores españoles homocigotos A/A del SNP BAX (rs4645878) tenían mayor riesgo de desarrollar VRP tras un DR en comparación con los que portaban la variante G en homocigosis (Tabla.1)(Pastor-Idoate S et al 2015). Esta observación fue confirmada en el análisis de la población general (Tabla 2)(Pastor-Idoate S et al 2015). Sin embargo, no se observaron diferencias en el análisis del resto de grupos por separado (Portugal, Holanda y Reino Unido).

Tabla 1. Distribución de genotipos y frecuencias alelicas de BAX en las muestras procedentes de diferentes países, así como en las muestras del Sur (España + Portugal) y del hemisferio norte (UK + Holanda).

Países	Genotipo	Casos		Controles		p-valor Test de Fisher	Alelos	Casos		Controles		95% IC Alelos		p-valor Chi Cuadrado test	OR *	IC OR 95%
												Casos	Controles			
España	A/A G/A G/G	3 20 36	5.08% 33.9% 61.0%	5 29 110	3.47% 20.1% 76.3%	0.0659[1]	AA GG	26 92	22.0% 78.0%	39 249	13.5% 86.5%	[15.4-30.8] [69.2-84.8]	[10.0-18.1] [81.8-90.1]	0.0479[2]	1.8	[1.04.-3.13]
Portugal	A/A G/A G/G	1 4 10	6.67% 26.6% 66.7%	0 13 40	0.00% 24.5% 75.5%	0.2343[1]	AA GG	6 24	20.0% 80.0%	13 93	12.2% 87.8%	[8.4-39.1] [60.8-91.6]	[6.9-20.4] [79.6-93.0]	0.3622[2]	1.7	[0.61-5.19]
UK	A/A G/A G/G	1 9 21	3.23% 29.0% 67.7%	2 28 102	1.52% 21.2% 77.2%	0.3459[1]	AA GG	11 51	17.7% 82.2%	32 232	12.1% 87.8%	[9.6-29.9] [70.0-90.4]	[8.5-16.8] [83.1-91.4]	0.2989[2]	1.5	[0.73-3.30]
Holanda	A/A G/A G/G	2 9 18	6.9% 31.0% 62.0%	2 22 68	2.1% 23.9% 73.9%	0.2411[1]	AA GG	13 45	22.4% 77.6%	26 158	14.1% 85.9%	[12.9-35.6] [64.4-87.0]	[9.6-20.2] [79.8-90.4]	0.1645[2]	1.7	[0.83-3.69]
España + Portugal	A/A G/A G/G	4 24 46	5.41% 32.4% 62.2%	5 42 150	2.54% 21.3% 76.2%	0.0492[1]	AA GG	32 116	21.6% 78.4%	52 342	13.2% 86.8%	[15.4-29.3] [70.7-84.5]	[10.1-17.0] [82.9-89.9]	0.0212[2]	1.8	[1.11-2.95]
UK + Holanda	A/A G/A G/G	3 18 39	5.0% 30.0% 65.0%	4 50 170	1.79% 22.2% 75.9%	0.1414	AA GG	47 73	39.2% 60.8%	172 276	38.4% 61.6%	[15.4-29.3] [70.7-84.5]	[10.1-17.0] [82.9-89.9]	0.9232[2]	1.6	[0.99-2.84]

[1]Test de Fisher. ($H_{o.}$ Independencia entre genotipos entre casos y controles). Se hallaron diferencias significativas entre casos y controles en el genotipo A/A en los países del hemisferio Sur (España + Portugal).

[2]El análisis entre los homocigotos A/A, mostro diferencias en los países del hemisferio Sur (España + Portugal).

Tabla 2. Distribución de genotipos y frecuencias alelicas de BAX en la muestra global.

Muestra Global	Genotipo	Casos		Controles		p-valor Test de Fisher	Alelos	Casos		Controles		95% IC Alelos		p-valor Chi Cuadrado test	OR*	IC OR 95%
												Casos	Controles			
BCL-2	A/A G/A G/G	26 46 62	19.4% 34.3% 37.7%	84 180 157	19.9% 42.7% 37.3%	0.1455[1]	AA GG	98 170	36.6% 63.4%	348 494	41.3% 58.7%	[30.8-42.6] [57.3-69.1]	[37.9-44.7] [55.2-62.0]	0.2064[2]	0.8	[0.61-1.08]
BAX	A/A G/A G/G	7 42 85	5.2% 31.3% 63.4%	9 92 320	2.1% 21.8% 76.0%	0.0084[1]	AA GG	56 212	20.9% 79.1%	110 732	13.0% 86.9%	[16.2-26.3] [73.6-83.7]	[10.9-15.5] [84.4-89.1]	0.0029[2]	1.7	[1.23-2.51]

[1] Test de Fisher. (H_o. Independencia entre genotipos entre casos y controles). Se hallaron diferencias significativas entre casos y controles en el genotipo A/A para BAX en la muestra global.

[2] Test del Chi cuadrado. El análisis entre los homocigotos A/A, mostro diferencias en BAX.

Cuando se realizo el análisis geográfico, solamente se observaron diferencias significativas en los países del hemisferio Sur, (España + Portugal), con un riesgo de desarrollar VRP tras un DR de hasta 1.8 veces mayor para los portadores homocigotos A/A del SNP G(-248)A-BAX (rs4645878) (Tabla 3)(Pastor-Idoate S et al 2015).

Tabla 3. Modelos de herencia utilizados en la muestra global y resultados de OR usando el modelo dominante para BCL-2 y modelo aditivo para BAX en España + Portugal y en Holanda + Reino Unido (UK).

Muestra Global		*BCL-2*					*BAX*				
Modelos	Genotipo	OR*	95% IC OR		p-valor	AIC*	OR*	95% IC OR		p-valor	AIC*
Co-dominante	C/C C/A A/A	1.00 0.65 0.78	- [0.42 [0.46	- 1.00] 1.33]	0.1452	615.7	1.00 1.72 2.93	- [1.11 [1.06	- 2.66] 8.09]	0.0124	610.8
Dominante	C/C C/A-A/A	1.00 0.69	- [0.47	- 1.02]	0.0655	614.1	1.00 1.83	- [1.20	- 2.77]	0.0052	609.7
Recesivo	C/C-C/A A/A	1.00 0.97	- [0.59	- 1.58]	0.8893	617.5	1.00 2.52	- [0.92	- 6.91]	0.0812	614.5
Sobre-dominante	C/C-A/A C/A	1.00 0.70	- [0.47	- 1.05]	0.0817	614.5	1.00 1.63	- [1.06	- 2.51]	0.0283	612.7
Aditivo	-	0.84	[0.64	1.10]	0.1951	6.15.9	1.72	[1.21	2.44]	0.0030	6.08.8
España+Portugal		1.00 0.50	- [0.29	- 0.86]	0.0119	315.4	1.75	[1.09	2.83]	0.0229	316.6
Holanda+UK		1.00 0.95	- [0.53	- 1.68]	0.8489	296.8	1.66	[0.98	2.80]	0.0626	293.4

Las proteínas pro-apoptóticas como bax son necesarias y esenciales para la disfunción mitocondrial que ocurre en respuesta a una señal apoptotica o en respuesta a los agonistas necroptoticos (Irrinki KM et al 2011). Sin embargo, la reducción en la expresión de BAX o incluso su total deficiencia, como en los modelos knock-out de BAX, solo parecen proteger contra las respuestas apoptóticas (Janssen K et al 2009), sin comprometer la inducción de necrosis o la activación de otras vías de muerte celular no apoptóticas tales como la desencadenada por el retículo endoplásmico inducida por el estrés celular o la autofagia aumentada también en situaciones de estrés oxidativo y de hipoxia (Janssen K et al 2009).

Los resultados de esta primera parte del **trabajo 4** señalan que los portadores del alelo A del gen BAX, asociado con una disminución en la función apoptótica de p53, tienen un mayor riesgo de desarrollar PVR después de un DR. Este nuevo hallazgo refuerza la hipótesis de que el proceso de apoptosis mediado por p53 podría desempeñar un papel clave en la generación de VRP después de la cirugía DR.

SNPs rs2279115:

Para el SNP C (-938) A- BCL-2 (rs2279115), los resultados de este estudio, no mostraron diferencias significativas en al análisis de los portadores homocigotos A/A en las muestras por países (tabla 4)(Pastor-Idoate S et al 2015) observación que fue confirmada en el análisis de la muestra global (tabla 2)(Pastor-Idoate S et al 2015).

Tabla 4. Distribución de genotipos y frecuencias alelicas de BCL-2 en las muestras procedentes de diferentes países, así como en las muestras del Sur (España + Portugal) y del hemisferio norte (UK + Holanda).

Países	Genotipo	Casos		Controles		p-valor Test de Fisher	Alelos	Casos		Controles		95% IC Alelos		p-valor Chi Cuadrado test	OR*	IC OR 95%
												Casos	Controles			
España	A/A C/A C/C	9 20 30	15.2% 33.9% 50.8%	24 69 51	16.6% 47.9% 35.4%	0.1126[1]	AA CC	38 80	32.2% 67.8%	117 171	40.6% 59.4%	[24.0-41.5] [69.2-84.8]	[34.9-46.5] [81.8-90.1]	0.1305[2]	0.7	[0.44.-1.09]
Portugal	A/A C/A C/C	4 5 6	26.6% 33.3% 40.0%	18 23 12	33.9% 43.4% 22.6%	0.4376[1]	AA CC	13 17	43.3% 56.7%	59 47	55.6% 44.4%	[25.9-62.3] [37.6 -74.0]	[45.7-65.2] [34.8-54.2]	0.3438[2]	0.6	[0.26-1.37]
UK	A/A C/A C/C	7 10 14	22.5% 32.3% 45.2%	27 54 51	20.4% 41.0% 38.6%	0.6670[1]	AA CC	24 38	38.7% 61.3%	108 156	40.9% 59.1%	[26.8-51.9] [48.0-73.1]	[34.9-47.1] [52.8-65.0]	0.7943[2]	0.9	[0.51-1.60]
Holanda	A/A C/A C/C	6 11 12	20.7% 38.0% 41.3%	15 34 43	16.3% 37.0% 46.7%	0.7822[1]	AA CC	23 35	39.6% 60.4%	64 120	34.7% 65.3%	[27.3-53.3] [46.6-72.6]	[28.0-42.1] [57.8-71.9]	0.5689[2]	1.2	[0.67-2.26]
España + Portugal	A/A C/A C/C	13 25 36	17.6% 33.8% 48.6%	42 92 63	21.3% 46.7% 31.9%	0.0409[1]	AA CC	51 97	34.4% 65.6%	176 218	44.7% 55.3%	[26.9-42.7] [57.2-73.0]	[39.7-49.7] [50.2-60.2]	0.0405[2]	0.6	[0.43-0.96]
UK + Holanda	A/A C/A C/C	13 21 26	21.6% 35.0% 43.4%	42 88 94	18.7% 39.3% 41.2%	0.7834[1]	AA CC	47 73	39.1% 60.9%	172 276	38.4% 61.6%	[30.5-48.5] [51.4-69.4]	[33.9-43.0] [56.9-66.1]	0.9232[2]	1.0	[0.68-1.56]

[1] Test de Fisher. (H_0. Independencia entre genotipos entre casos y controles). Se encontraron diferencias significativas entre casos y controles en el genotipo A/A en los países del hemisferio Sur (España + Portugal).

[2]El análisis entre los homocigotos A/A, mostro diferencias en los países del hemisferio Sur (España + Portugal).

Cuando se realizo el análisis geográfico, se observaron diferencias significativas en los países del hemisferio Sur, (España+Portugal), mostrando un efecto protector frente al desarrollo de VRP tras un DR en los portadores homocigotos A/A del SNP C (-938) A- BCL-2 (rs2279115) (Tabla 3)(Pastor-Idoate S et al 2015).
Este efecto protector de BCL-2 parece ser contradictorio y no estar en consonancia con los resultados anteriores. Sin embargo, aunque una sobreexpresión de BCL-2 como ocurriría con los portadores del alelo A en homocigosis, ocasionaría una disminución en la respuesta apoptótica, a diferencia del SNP G(-248)A (rs4645878) del gen BAX que también daría lugar a una disminución en la respuesta apoptótica, la sobreexpresión de BCL-2 es capaz de inducir una efecto inhibitorio en otras vías de muerte celular como la necrosis programada mediada por TNFA (Irrinki KM et al 2011) u otras vías no apoptoticas como la autofagia. De hecho, algunos estudios han sugerido la uso de inhibidores de BCL-2 como una nueva posible diana en la terapia contra el cáncer (Kang MH et al 2009). Además, se ha publicado que la sobre-expresión de BCL-2 podría tener un efecto protector el desarrollo de la VRP, ya que podía interactuar en otras vías de muerte celular que se encuentran también activadas en situaciones de estrés oxidativo e hipoxia (Irrinki KM et al 2011).

Este estudio, además de indicar una posible diana terapéutica en la profilaxis de la VRP, pone de nuevo en relieve el importante papel de la genética en la identificación de pacientes de alto riesgo, indicando que tanto el alelo A del SNP rs4645878 del gen BAX como del SNP rs2279115 del gen BCL-2 junto con el alelo G del SNP rs2279744 del gen MDM2 y el alelo Pro del SNP rs1042522 del gen p53 podrían ser utilizados como posibles biomarcadores o como elementos para identificar futuras dianas terapéuticas.

Trabajo 5:

Aunque en realidad no forma parte de los trabajos exigidos para la tesis doctoral, esta revisión realizada por parte de los miembros del grupo de Retina, sintetiza los conocimientos y las ideas del grupo de Retina del IOBA en los últimos 20 años

sobre la patogénesis de la VRP, procedentes de muchos proyectos, y entre ellos de los denominados *Retina-1* y *Retina-4.*

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LIMITACIONES DE LOS TRABAJOS

Limitaciones del trabajo 1:

Respecto al trabajo de la LTA existen básicamente dos limitaciones importantes: por una lado la escasez de la muestra y por otro el tiempo de evolución de las DR.

La obtención de material humano siempre es un problema cuando no se trata de patología tumoral o cuando se trata de material procedente de biopsias. Ya que este no es el caso de los DR ha sido complejo encontrar material adecuado en el archivo del laboratorio de Patología Ocular del I.O.B.A. Se intentó obtener mas casos de otros laboratorios pero no fue posible, ya que se trata de muestras difíciles de encontrar.

Por otro lado, los globos estudiados han sido enucleados por diversas complicaciones ajenas al propio DR, por lo que todos los desprendimientos son crónicos, y este hecho ha podido influir en los resultados del análisis inmunohistoquímico. Es evidente que hubiese sido preferible obtener desprendimientos de menor tiempo de evolución pero no ha sido factible.

Estas muestras con DR crónico, pueden haber influenciado en los resultados presentando una menor tinción inmunohistoquimica. Es posible que al tratarse de DR crónicos las muestras no presentasen concentraciones elevadas de citoquinas pro-inflamatorias y esa fuese la razón de su negatividad, con respecto al control de

retina sana, y tampoco se realizó un test previo de Western blot, para cuantificar los niveles de LTA.

Los mediadores pro-inflamatorios, tales como TNFA y LTA, relacionadas con el proceso isquémico alcanzan concentraciones máximas a las 72h (Berger et al 2008), como ocurre tras un DR agudo. Además, el aumento de la muerte células neuronales que se produce en un DR crónico podría ser otra de las posibles causas de una menor cantidad de inmunotinción en las secciones con DR crónico.

Por último, otra de las grandes limitaciones de este estudio, es la falta de estudios previos con los que poder comparar los resultados. Ya que, hasta donde sabemos, es la primera vez que se miden los niveles de LTA en retina humana.

Limitaciones de los trabajos 2, 3 y 4:

Se acepta que los estudios de asociación permiten establecer las diferentes frecuencias de ciertos SNPs en poblaciones afectas y en las controles con el fin de identificar aquellos marcadores genéticos que se asocian al desarrollo de una determinada patología. Sin embargo este tipo de estudios tienen algunas limitaciones.

Una de las principales limitaciones en cualquier estudio de asociación, y desde luego en el presente trabajo, es el tamaño muestral (Dempfle et al 2008). Comparando con otros estudios de asociación, la muestra constaba de 555 muestras de ADN, procedentes de la primera etapa proyecto *Retina-4* (Rojas J et al 2010, Rojas J et al 2013)), lo que podría considerarse como relativamente escasa. Uno de los inconvenientes de una muestra pequeña, es el efecto que puede tener sobre la potencia del estudio, pudiendo no ser adecuada como para obtener conclusiones. Sin embargo, la recogida de más muestras no es una tarea fácil, ya que la prevalencia de la VRP es de un 5% a un 10% de los casos operados de DRR y la prevalencia del DR en la población general es de 1.5 casos nuevos por 10.000 habitantes y año (Mitry D et al 2010). Por eso no es de extrañar que dentro de los no muy numerosos estudios que existen en la literatura, el presente aporte el

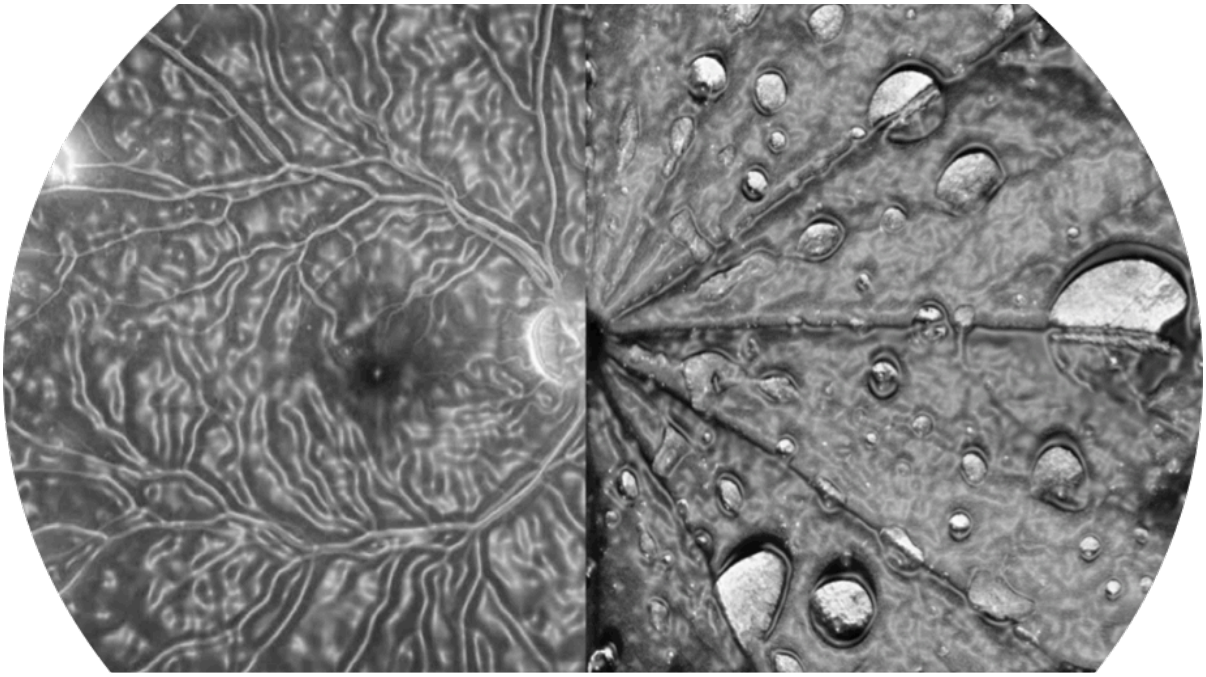
mayor tamaño muestral si se compara con otros estudios de asociación enfocados a la VRP (Yu H et al 2013). Afortunadamente, los resultados encontrados en los trabajos 2, 3 y 4, son coherentes con los anteriores hallazgos reportados por nuestro grupo (Rojas J et al 2010, Rojas J et al 2013).

También es importante señalar que las muestras procedentes de la primera etapa *Proyecto Retina-4,* provenían de diferentes países, y en el caso del Reino Unido, es difícil mantener la homogeneidad en la composición genética, debido a la superior diversidad étnica que experimenta en este caso el entorno asistencial de Londres sobre el resto de las muestras de otros países europeos. Además, a pesar de que existe un cierto grado de parentesco genético entre los países de Europa Occidental en mayor o menor medida, existen diferencias entre el eje Norte-Sur (Genographic Project by National Geographic), lo que podría explicar, por ejemplo, la ausencia de correlación en los resultados obtenidos en el trabajo 2 en la población británica en cuanto a las frecuencias del SNP rs1042522 en el gen p53 (Frank et al 2011, Sjalander et al 1995). Razones que han motivado a considerar en nuestros análisis a Holanda y Reino Unido como países de la zona Norte y a España y Portugal como países de la zona Sur.

Aunque los análisis de las muestras se han llevado a cabo en dos fases, primero analizando las muestas de la zona Sur y tras obtener resultados positivos, analizando las muestras de la zona Norte, con la finalidad de replicar los resultados, estos han de ser interpretados con precaución hasta que sean confirmados nuevos estudios de replicación, con muestras diferentes tanto temporalmente como geográficamente.

Por último otra de las principales limitaciones de los estudios de asociación son los falsos positivos (Crawford et al 2005). Hay numerosas razones que pueden originar falsas asociaciones. Unas de ellas, es la que resulta de una clasificación inadecuada de los sujetos en estudio (alteraciones en el fenotipado). Otra, la derivada del propio tratamiento estadístico de los datos. Además ciertos marcadores pueden tener un efecto en ciertas poblaciones y no en otras. Por todo eso, ocurre en ocasiones que las asociaciones encontradas en algunas poblaciones

no son definitivas (Ioannidis et al 2001) y muchos investigadores consideran que la replicación de estos estudios es fundamental (Patterson et al 2005).



CONCLUSIONES

De los resultados de los diferentes trabajos que conforman esta tesis, pueden extraerse las siguientes conclusiones generales:

1. Los SNPs funcionales han permitido una mejor comprensión de las bases moleculares de la VRP, y además ponen de relieve el importante papel de la genética como una herramienta útil en la identificación de pacientes de alto riesgo y de nuevas dianas terapéuticas.

2. A pesar de que los mecanismos responsable de la VRP no son completamente entendidos, se acepta que la inflamación y la muerte celular juegan un papel crucial en su patogénesis y los resultados de estos trabajos parecen confirmarlo.

3. La apoptosis juega un papel importante en las alteraciones secundarias del DR, y parece jugar también un papel decisivo en la génesis de la VRP, lo que debería tenerse en cuenta al proponer nuevos tratamientos.

Estas conclusiones generales están basada en las siguientes sub-conclusiones derivadas de los hallazgos del grupo de trabajo:

CONCLUSIONES

Conclusiones del trabajo 1:

Funcionalidad del polimorfismo rs2229094 (T>C) del gen LTA:

Los resultados de este estudio sugieren una falta de funcionalidad del SNP LTA rs2229094. Sin embargo, este SNP podría seguir siendo válido como un biomarcador para identificar pacientes de alto riesgo de desarrollar VRP tras un DR, debido a su fuerte asociación con esta complicación retiniana, demostrada en trabajos del grupo.

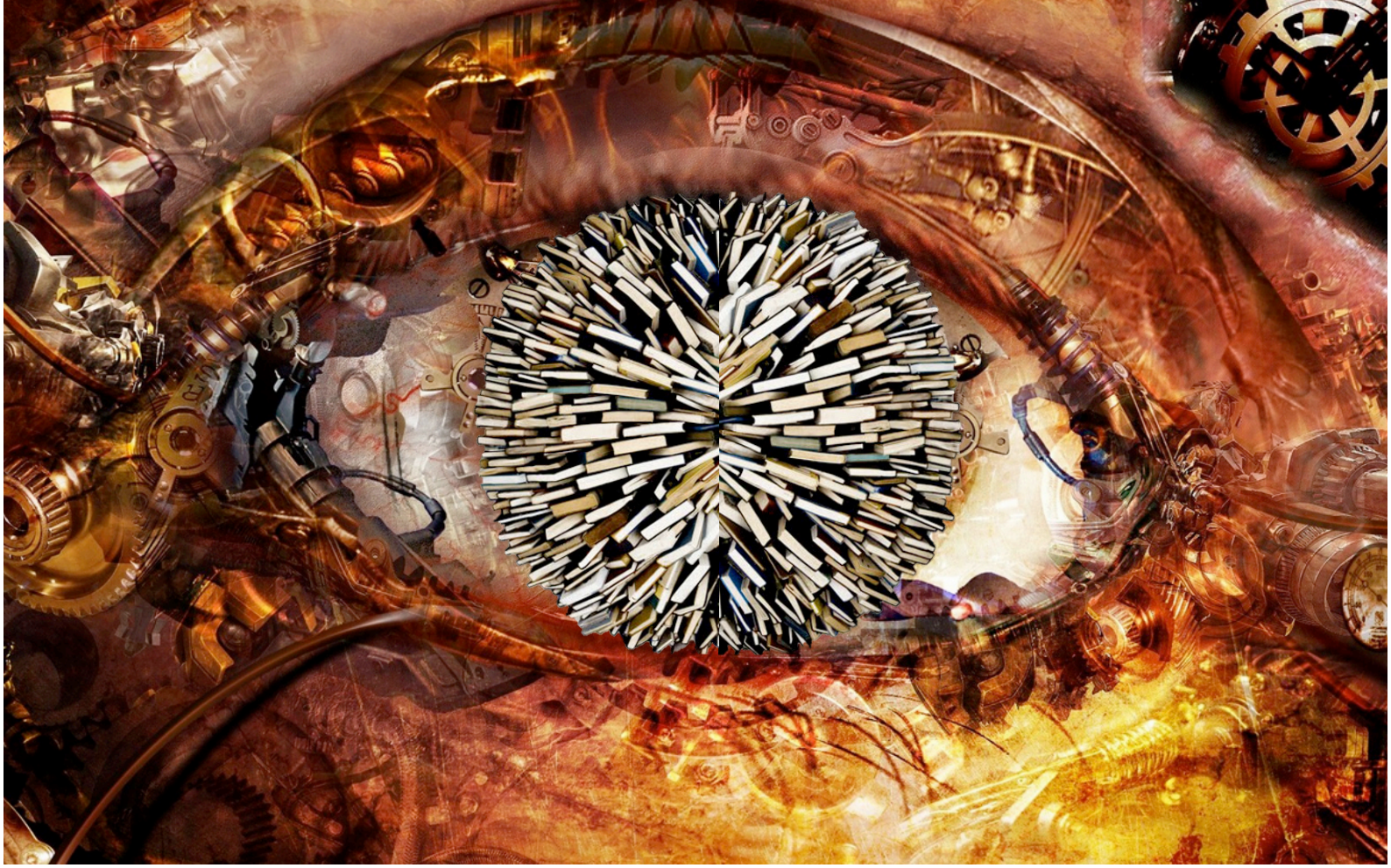
Expresión y localización de la LTA en muestras de retina humana:

Los resultados de este estudio muestran una menor tinción inmunohistoquimica en las secciones procedentes de retinas con un DR crónico con respecto a la retina sana utilizada como control. Sin embargo, debido a las limitaciones del estudio no se pueden obtener conclusiones sobre que papel tiene la LTA en la retina afectas de DR. Por lo que son necesarios más estudios en este sentido.

Conclusiones de los trabajos 2, 3 y 4:

Los estudios de asociación permiten señalar en los trabajos realizados que los pacientes portadores del alelo Pro del SNP rs1042522 del gen p53, del alelo G del SNP rs2279744 del gen MDM2 y del alelo A del SNP rs4645878 del gen BAX presentan un mayor riesgo de desarrollar VRP después de un DR, por lo que podrían ser utilizados como posibles biomarcadores en la identificación de pacientes de alto riesgo o como elementos para identificar futuras dianas terapéuticas.

Además los pacientes portadores del alelo A del SNP rs2279115 del gen BCL-2 podrían tener un efecto protector frente al desarrollo de la VRP, ya que la sobre-expresión de esta proteína no solamente esta asociada a una disminución en la función apoptótica, sino que además tiene induce un efecto inhibitorio en otras vías de muerte celular ó la autofagia.



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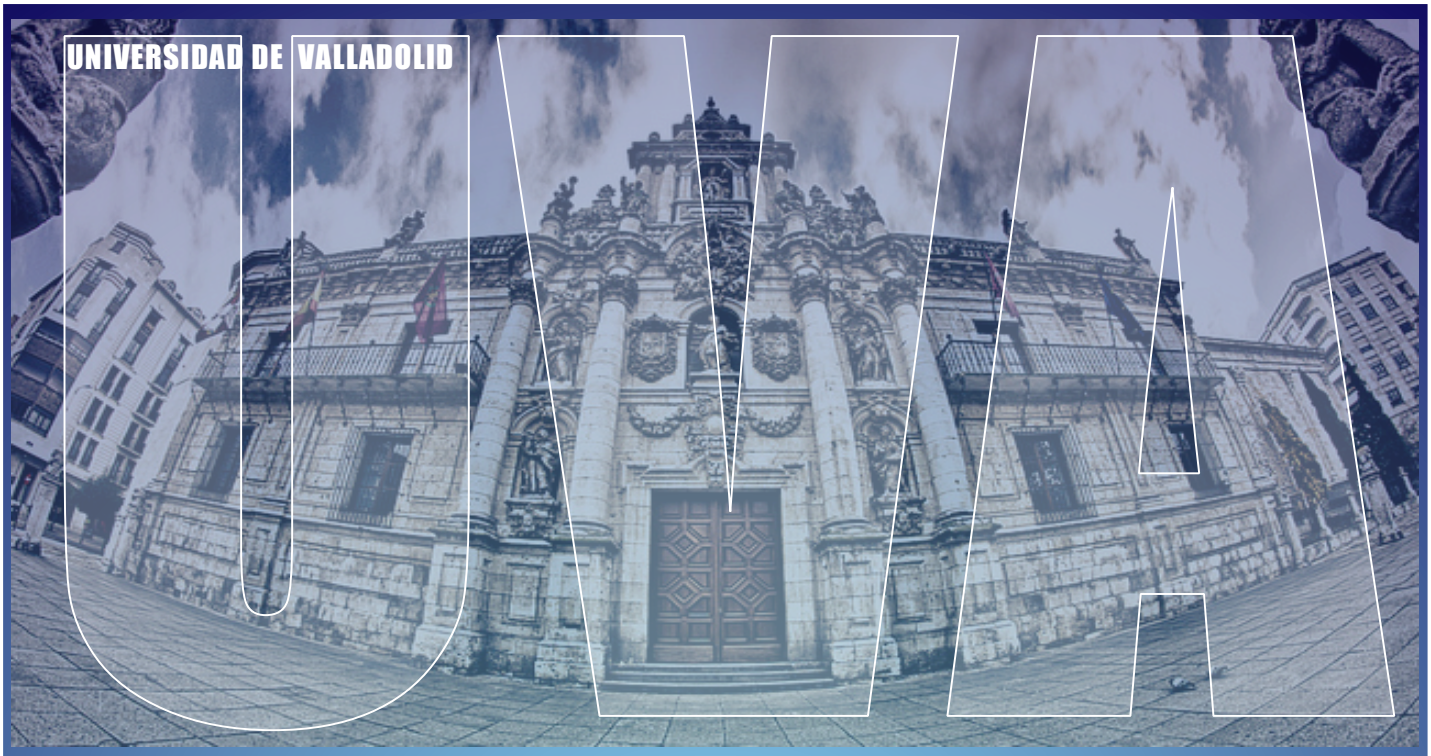

Genetics of Inflammation, Apoptosis Pathway and Other Cell Death Mechanisms in Retinal Detachment and Proliferative Vitreoretinopathy

The Retina 4 Project

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2015

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"The fact is, I felt completely hepless when I looked at you. I was so frightened by your eyes that I lost whatever fighting spirit I had" said the engraver

Matsumura's voice became soft. "Perharps, " the master said. "But I know this: you were determined to win and I was just as determined to die if I lost. That was the difference between us"

Karate-do. My way of life. Gichin Funakoshi

Abstract

Proliferative vitreoretinopathy (PVR) is still the major cause of failure in retinal detachment (RD). Currently, there is no effective medical treatment, nor prophylaxis for this disease, and surgery has shown unsatisfactory anatomic and functional results, despite great advances in vitreoretinal surgery during the last decades.

Most research has attempted to identify clinical risk factors for PVR developing; however, these variables do not completely explain the probability of its onset. PVR is currently considered a complex disease in which there is an interaction between environmental factors (clinical variables) and the genetic profile of each subject.

Our group have focused in the last decade on some other mechanisms involved in RD and PVR with the aim of finding new markers of PVR risk or targets for its prophylaxis.

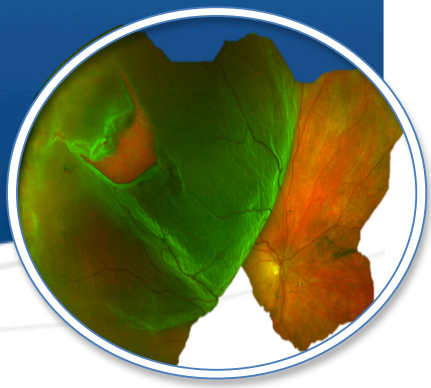
Single nucleotide polymorphisms have important implications for human genetic diseases. They may help to identify a genetic predisposition for certain diseases, either as a causative factor or protective risk factor. And they may help to increase our knowledge of the molecular causes of some conditions.

This PhD project emphasizes the genetic component of this disease and its possible usefulness for developing a new generation of predictive biomarkers. Also this project has incorporated a major review which summarizes the our ideas on the mechanisms and pathogenesis of PVR in the last 20 years.

Contents

Doctoral Degree Program in Visual Sciences
I.O.B.A-University of Valladolid

General Introduction



As mentioned before, 4 of the 5 papers presented in this thesis are part of the scientific output of the second stage of the *Retina 4 project*. This second stage was conducted at IBSAL (University of Salamanca-Spain) in collaboration with the Ocular Pathology Lab at I.O.B.A (University of Valladolid-Spain). Also, an additional work, recently published, which collects all the results from the two main lines of research of the retina group (*Retina-1 and Retina-4 project*) is presented as chapter 5.

All of the studies were approved by the institutional research committee of each centre and followed the tenets of the Declaration of Helsinki. All patients gave a written informed consent before entering in the study. All tissue samples were used in accordance with applicable laws for research involving human tissues and samples (Law 14/2007, of 3 July, on Biomedical Research) and in accordance with the Valladolid Universitary Clinic Hospital Steering Committee (22-April-2014).

All of the studies presented have in common the attempt to provide more information about the pathogenesis of PVR, and the ability to identify potential biomarkers for the diagnosis of high risk patients and potential therapeutic targets. The efforts of this thesis have been directed to investigate two major events in the PVR development: inflammation and the genes involved in the cell death.

Chapter 1:
Assessment of inflammation in PVR

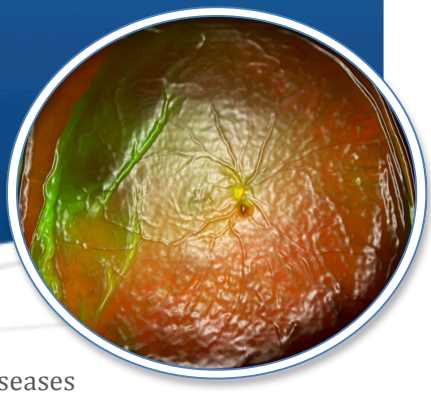
- **Pastor-Idoate S,** Rodríguez-Hernandez I, Rojas J, Gonzalez-Buendia L, Delgado-Tirado S, Lopez JC, González-Sarmiento R, Pastor JC. Functional Characterization of rs2229094 (T>C) polymorphism and LTA Expression in Human Retina. The Retina 4 project. Paper has already submitted to ACTA Ophthalmol Journal. 2015.

PVR is a cell-based inflammatory response, and, as in other inflammatory responses, genetic susceptibility may play an important role. The severity of inflammatory conditions and the circulating levels of some cytokines could be partially determined by related polymorphisms (Rojas J et al 2010).

The purpose of this study was to determine the expression and localization of lymphotoxin alpha (LTA) in human retinas, and investigate the functionality of the LTA rs2229094 (C13R) (T>C) polymorphism, previously associated to PVR development.

Within the LTA gene, located in the TNF locus (6p21.3), the LTA rs2229094 polymorphism consists of a T to C substitution that encodes a cysteine to arginine change at codon 13 (Cys13Arg). Our group found a strong association between this SNPs and patients who had suffered PVR after RD (Rojas J et al 2009, Rojas J et al 2010). In addition, in three predictive models of PVR developed by our group base don the analysis of genetic variables, LTA rs 2229094 polymorphism was the highest individual predictor for patients at risk for developing PVR (Rojas J et al 2009).

General Introduction



LTA is a member of the TNF family of cytokines with inflammatory and immunologic activities (Aggarwal BB.2003). It plays a key role in communication between lymphocytes and stromal cells, eliciting cytotoxic effects on cells. It also induces the expression of vascular cell-adhesion molecule 1 (VCAM-1) on vascular endothelial cells and recruits natural killer (NK) cells to parenchymal organs or lesion areas (Fogler WE et al 1996).

LTA, with a 40% of homology to TNFA, shares the same membrane receptors as TNFA (Naoum JJ et al 2006). Previous studies reported high vitreous levels of TNFA and its receptors (TNFR 1 and 2) in eyes with PVR (Limb GA et al 2001) and local production of TNFA has been suggested to occur in these eyes (El-Ghrably IA et al 1999). However, retinal expression and localization of LTA have not been previously described in either normal or RD eyes.

LTA is able to act through LTA–TNFR1 complex as TNFA, but it seems to be differences in their signal strength or capabilities (Etemadi N et al 2013). In particular, it has been reported that LTA is less able to promote TNFR1-induced cell death and nuclear factor kB (NF-kB) activation (Chaturvedi MM et al 1994), expression of cell surface markers (Andrews JS et al 1990) and cytokine production (Oster W et al 1987, Broudy VC et al 1987) than TNFA. In addition, TNFA and LTA have shown protective effects in neuronal cells when acting via TNFR2, especially in ischemic conditions both in brain and in retina (Fontaine V et al 2002, Shohami E et al 1999). Thus, despite LTA is able to induce signals via TNFR1, and could have a crucial role in the initiation and development of some inflammatory diseases (Calmon-Hamaty F et al 2011, Buch MH et al 2004), due to its diverse bioactivities, it is presently not clear under which conditions LTA promotes beneficial or deleterious effects on neuronal tissues.

Therefore further studies will be critical to further understanding the role of LTA in retinal neuronal cells.

Chapter 2, 3 and 4: Assessment of cell death in PVR

- **Pastor-Idoate S**, Rodriguez-Hernández I, Rojas J, Fernández I, García-Gutierrez MT, Ruiz-Moreno JM, Rocha-Sousa A, Ramkissoon Y, Harsum S, Maclaren RE, Charteris D, van Meurs J, González-Sarmiento R, Pastor JC; Genetics on PVR Study Group. (2013). The p53 codon 72 polymorphism (rs1042522) is associated with proliferative vitreoretinopathy: The Retina 4 Project. Ophthalmology. 120(3):623-628.

- **Pastor-Idoate S**, Rodríguez-Hernández I, Rojas J, Fernández I, García-Gutiérrez MT, Ruiz-Moreno JM, Rocha-Sousa A, Ramkissoon Y, Harsum S, MacLaren RE, Charteris D, VanMeurs JC, González-Sarmiento R, Pastor JC; Genetics on PVR Study Group. (2013). The T309G MDM2 gene polymorphism is a novel risk factor for proliferative vitreoretinopathy. The Retina 4 Project. PLoS One. 9;8(12):e82283.

- **Pastor-Idoate S**, Rodríguez-Hernandez I, Rojas J, Fernandez I, Garcia-Gutierrez MT, Ruiz-Moreno JM, Rocha-Sousa A, Ramkisson Y, Harsum S, MacLaren RE, Charteris DG, VanMeurs JC, González-Sarmiento R, Pastor J.C on behalf of the Genetics on PVR Study Group. (2015). BAX and BCL-2 polymorphisms, as predictors of proliferative vitreoretinopathy development in patients suffering retinal detachment: The Retina 4 Project. Acta Ophthal Dec 2014 (accepted for publication 18/02/2015.

General Introduction

In these three studies, the role in the development of PVR of rs1042522 in the p53 gene, the rs2279744 in the MDM2 gene, and rs4645878 and rs2279115 polymorphisms in the BAX and BCL-2 genes respectively, were evaluated.

Current studies have highlighted the involvement of extrinsic and intrinsic pathways of apoptosis in retinal cells after RD, and also, the existence of other death pathways, such as programmed necrosis (which are more inflammatory pathways) and is enhanced when apoptosis is inhibited (Lo AC et al 2011). Furthermore, it has been reported that suppression of p53 expression might be a necessary event in the development of RD and PVR, and maintaining levels of p53 with agents such as Nutlin-3, which prevents the interaction between p53 and MDM2, might be effective in the prophylaxis of RD and also PVR vitreous-induced contraction (Lei H et al 2012).

Apoptosis is critically important during various developmental processes, it is necessary to rid the body of pathogen-invaded cells and also is involved in the removal of inflammatory cells and the evolution of granulation tissue into scar tissue (Elmore S. 2007). Moreover, inappropriate apoptosis is an important factor in many human pathologic conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer. In addition, it has been reported that a deregulation of apoptosis during wound healing can lead to pathologic forms of healing such as excessive scarring and fibrosis (Elmore S. 2007).

These SNPs were analyzed not only because they are related to each other, but also with the p53 gene and the TNFA in the activation of secondary cell death pathways as necroptosis. Also they are associated with decreased or increased levels of apoptosis, and with cell proliferative responses.

Thus, deregulation in the apoptosis during wound healing and the activation of other cell death pathways could lead to pathologic forms of healing, such as excessive scarring and fibrosis in some patients after RD.

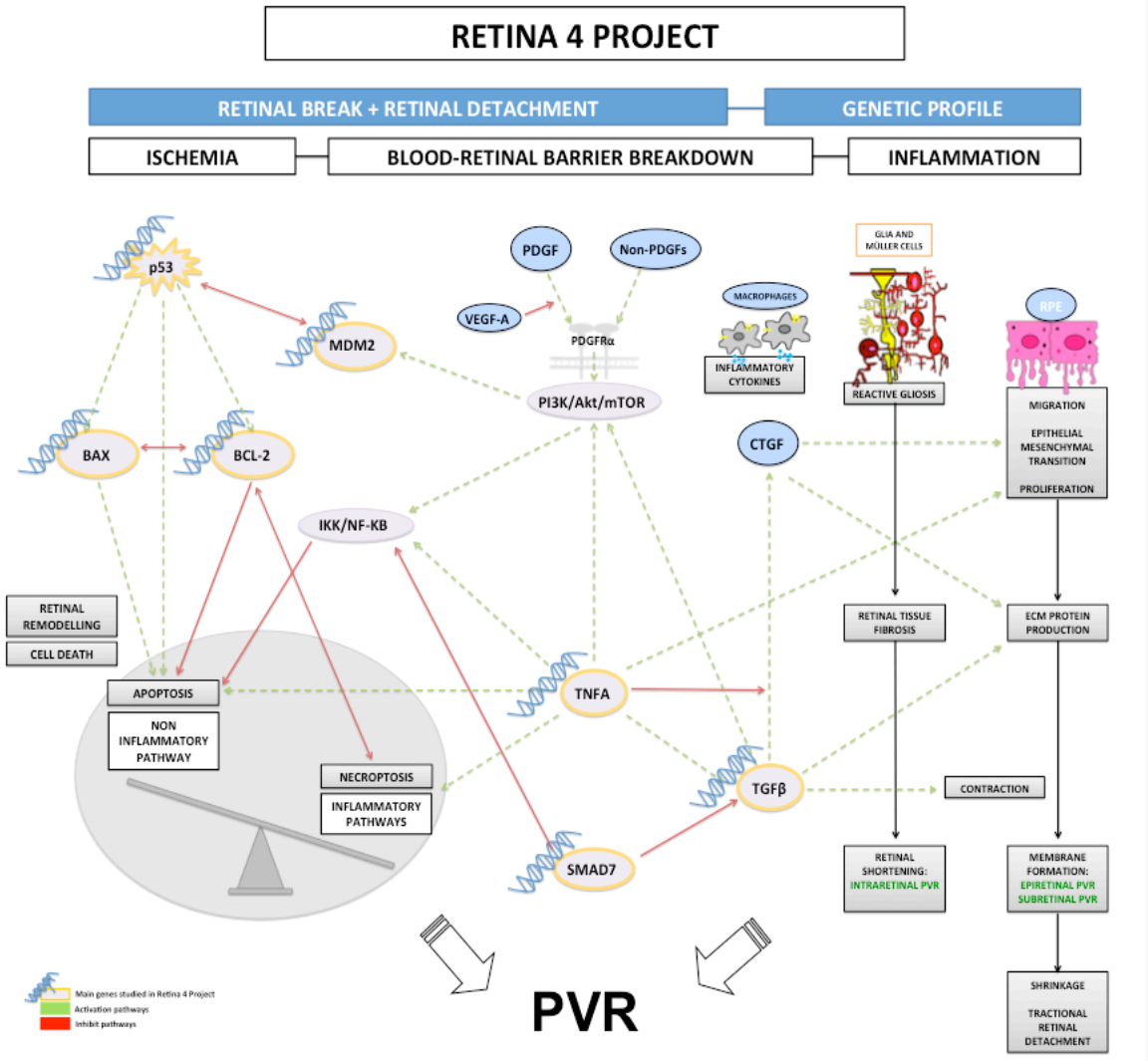
Although, all of these SNPs are widely associated with tumors and inflammatory processes, their role in the RD and in the development of PVR has not yet been studied.

Chapter 5: Major review on PVR

- Pastor JC, Rojas J, **Pastor-Idoate S**, Di Lauro S, Gonzalez-Buendia L, Delgado-Tirado S. Proliferative vitreoretinopathy: A new concept of disease pathogenesis and practical consequences. Prog Retin Eye Res. 2015 Jul 21. EPUB ahead of print].

This latest work collects the knowledge and the ideas of the retina group on the pathogenesis in PVR.(*Retina 1 project and Retina 4 project*). Although this manuscript does not comply with the requirements for the authorship for being part of the compendium of works presented in this PhD project, this final work summarizes and combines the findings of the previous works, extending the information of the investigations in this complication during the past 20 years.

General Introduction



(Scheme modified from Pastor JC et al 2015)

and Objectives

The general hypothesis of this PhD is that alterations and modifications in both, the inflammatory cascade and the main cell death pathway (apoptosis), induced by genetic variations, might be associated with the development of the PVR after a RD.

This general assumption is based on the following sub-hypotheses derived from the findings achieved in the different Works.

Chapter 1: Assessment of inflammation in PVR

The SNP rs2229094, which is located in the LTA signal peptide gene may be functional and modify the production or location of the LTA protein. Furthermore, the LTA protein levels, could be altered (increased or decreased) in retinas of patients who have suffered a RD.

Chapter 2, 3 and 4: Assessment of cell death in PVR

The SNPs rs1042522, rs2279744, rs4645878 and rs2279115, located in p53, MDM2, Bax and Bcl-2 genes are associated with decreased or increased levels of apoptosis, and cellular proliferative responses, and may be associated with increased risk in the PVR development.

Objectives

The overall objective of this PhD was to relate the presence of these SNPs in DNA samples from patients who have suffered PVR after a RRD and establish whether there are statistically significant associations with the development of this complication. In addition, the functional characterization of one SNP of the LTA gen was performed.

The DNA samples used for these purposes come from the biological collection obtained for the project called *Retina-4.*

The principal objective can be subdivided into the following secondary objectives:

Chapter 1: Assessment of inflammation in PVR

- Functional characterization of rs2229094 (T>C) polymorphism and LTA expression and localization in human retina.

Chapter 2, 3 and 4: Assessment of cell death in PVR

- To compare the distribution of a p53 gene polymorphism among European subjects undergoing primary RD surgery in relation to the development of PVR.
- To evaluate the impact of T309G MDM2 polymorphism (rs2279744) in PVR and the possible association between rs2279744 and rs1042522 in patient who have suffered PVR after RD.
- To compare the distribution of BCL-2 -938C>A (rs2279115) and BAX -248G>A (rs4645878) genotypes among European subjects undergoing RRD surgery in relation to the further development of PVR.

Chapter 1

Functional Characterization of rs2229094 (T>C) polymorphism and LTα Expression in Human Retina The Retina 4 project

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Meeting Presentation: ARVO Annual Meeting, May, 2010 and 2014

Functional Characterization of rs2229094 (T>C) polymorphism and LTA Expression in Human Retina. The Retina 4 project

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WORD COUNT: 3.182

ABSTRACT

Purpose: To determine the expression and localization of lymphotoxin alpha (LTA) in human retinas, and investigate the functionality of the LTA rs2229094 (C13R) (T>C) polymorphism, previously associated to proliferative vitreoretinopathy (PVR) development**.**

Methods: Total RNA from 3 healthy human retinas were extracted and subjected to reverse transcription polymerase chain reaction (RT-PCR) analysis, using flanking primers of LTA cDNA. In addition, 3 human eyes with retinal detachment (RD) and 1 healthy control eye were subjected to immunohistochemistry (IHC) with specific antibodies against LTA.
The Functionality of the T and C alleles was assessed by using pCEFL-Flag expression vector and transient transfection assays in the COS-1 cell line. In addition, expression analysis by RT-PCR, Western Blot and subcellular localization of both alleles, by immunofluorescence assay were performed.

Results: RT-PCR analysis revealed no significant levels of mRNA LTA in healthy human retinas. Sequential IHC staining showed differences between healthy human and RD retinas. No differences in mRNA and protein expression levels and in subcellular localization between both alleles were found. Both alleles were located in the cytoplasm.

Conclusions: Although results suggest lack of functionality, and it appears to be no differences in the IHC studies, this polymorphism could remain as a valid

biomarker to identify high-risk patients to develop PVR after RD, due to the strong association between PVR and LTA C13R polymorphism previously shown by our group.

INTRODUCTION:

Proliferative vitreoretinopathy (PVR) is still the major cause of failure in retinal detachment (RD) surgery,[1] affecting 5% to 10% of RD and accounting for approximately 75% of all primary failures after RD surgery. [1, 2]

PVR is a multifactorial disease,[3-7] considered as an abnormal wound-healing process induced by a retinal break, whereupon the levels of certain inflammatory cytokines (e.g., tumor necrosis factor-alpha (TNFA)) and growth factors (e.g. transforming growth factor beta (TGFB)) are increased and play an important role in its pathogenesis.[3, 4, 8, 9]

PVR is a cell-based inflammatory response, and, as in other inflammatory responses, genetic susceptibility may play an important role.[6] Single nucleotide polymorphisms have an important implication in human genetic diseases. They may help to know the genetic predisposition for certain diseases, either as a causative factor or as a signal of protective risk factor. The severity of inflammatory conditions and the circulating levels of some cytokines could be partially determined by related polymorphisms.[10]

That is one of the reasons why the search for predictive molecular risk factors (biomarkers) of PVR susceptibility has been intensified in recent years.

Lymphotoxin alpha (LTA), a member of the TNF family of cytokines, was initially isolated on the basis of an anti-tumor activity. Later, this cytokine was shown to have inflammatory and immunologic activities.[11] LTA plays a key role in communication between lymphocytes and stromal cells, eliciting cytotoxic effects on cells. It also induces the expression of vascular cell-adhesion molecule 1 (VCAM-1) on vascular endothelial cells and recruits natural killer (NK) cells to parenchymal organs or lesion areas.[12]

Within the LTA gene, located in the TNF locus (6p21.3), the LTA rs2229094 polymorphism consists of a T to C substitution that encodes a cysteine to arginine change at codon 13 (Cys13Arg). Previously, our group examined the relationship between this polymorphism and PVR development, finding a strong genetic association in patients who had suffered PVR after a rhegmatogenous RD.[4] In addition, in three predictive models of PVR developed by our group based on the analysis of genetic variables, LTA rs2229094 polymorphism was the highest individual predictor for patients at risk for developing PVR.[13] However, despite its shown association, the polymorphism functionality and the role of LTA in human retinas and in the pathogenesis of PVR remains to be determined.

Thus, the purpose of this study was to characterize the functionality of rs2229094 polymorphism and analyze the role of LTA in healthy human retinas and retinas with previous RD, as a part of the project named Retina 4.

MATERIAL AND METHODS:

All tissue samples were used in accordance with applicable laws for research involving human tissues and samples (14/2007 of Biomedical Research) and the Declaration of Helsinki. The study was approved by the Research Committee of the IOBA, Eye Institute (University of Valladolid, Valladolid, Spain) and by the Ethics Committee of the Valladolid Clinic University Hospital (Valladolid, Spain).

Human samples

Three post-mortem neuroretinas from Regional tissue bank (Castilla y León, Spain) from subjects with no reported past history of eye disease within the range of 40-60 years old, and two peripheral blood samples from healthy donors were obtained for RNA extraction. Eyes were enucleated within 30 to 60 minutes after death and were immersed in ice-cold transport medium composed of Dulbecco's modified Eagle's medium CO_2-independent medium without L-glutamine supplemented with 1% antibiotic-antimycotic mixture (penicillin, streptomycin and amphotericin B) (Gibco, Paisley, UK). Eyes were transported on dry ice to the laboratory where, under aseptic conditions, each eyeball was bisected with scissors, dividing the ocular globe into anterior and posterior eye-cups. The vitreous was removed, and the posterior eyecup was placed into a dish with clean transport medium. A paintbrush was used to mechanically detach the neuroretina from the retinal pigmented epithelium layer (RPE).

In addition, three human eye globe specimens diagnosed of RD during the period 1990-2013 selected from the archives of the IOBA Ocular Pathology Laboratory (University of Valladolid, Spain) and one healthy eye from a Regional eye tissue bank were fixed in formalin and embedded in paraffin for immunohistochemical (IHC) analysis.

Normal adult human brain sections from the hippocampal area, provided by the Fundación Centro de Investigación en Enfermedades Neurológicas (CIEN) tissue bank (Madrid, Spain), were used as a positive control for the IHC study.

Immunohistochemical Study

The aforementioned samples were used for the IHC study. 3-µm sections were mounted on FLEX IHC Microscope slides (Dako, Glostrup, Denmark) and

subjected to deparaffinization and hydration followed by heat-induced epitope retrieval in citrate buffer (pH 6.0). IHC staining was performed on these sections using a specific antibody against LTA (dilution 1:10; following manufacturer's instructions; reference HPA007729, Sigma-Aldrich Co, St. Louis, MO, USA) and the reaction was visualized by the EnVision™ FLEX detection system kit (Dako, Glostrup, Denmark). Normal adult human brain sections from the hippocampal area were used as a positive control. Images were taken using a Leica DM4000 B light microscope equipped with a Leica DFC490 digital camera (Leica Microsystems Inc, Wetzlar, Germany), and final processing of images was done with the Adobe Photoshop (Adobe Systems) program.

The slides were evaluated semi-quantitatively in a blind fashion by a pathologist using the following scoring system: (-: negative; +: weakly positive; ++: moderately positive; +++: strongly positive).

RNA isolation and Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from 3 post-mortem neuroretinas and 2 peripheral blood samples of human subjects was isolated using Trizol reagent (Invitrogen™, Carlsbad, CA, USA) according to manufacturer protocol. RNA concentration was determined by spectrophotometry on a Nanodrop (Thermo Scientific, Waltham, MA, USA). cDNA was synthesized with the Improm-II™ Reverse Transcription System (Promega, Madison, WI, USA) according to manufacturer specifications, with 1 µg total RNA per reaction. LTA cDNA was amplified from these samples with GoTaq Hot Start Polymerase (Promega, Madison, WI, USA) using flanking primers 5'-ATGACACCACCTGAACGTCTC-3' (forward) and 5'-

CTACAGAGCGAAGGCTCCAA-3' (reverse) following the protocol: denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and polymerization at 72°C for 1 minute. The GAPDH expression levels in each sample were used as internal control using 5'-CCACCCATGGCAAATTCCATGGCA-3' (forward) and 5'-TCTAGACGGCAGGTCAGGTCCACC-3' (reverse) primers. PCR products were separated by electrophoresis in TBE agarose gels and LTA RT-PCR products were sequenced to identify the different LTA transcripts obtained and the LTA rs2229094 genotype of the LTA full-length of each sample in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Generation of LTα expressing vectors

Complete cDNA carrying LTA rs2229094 allele C was amplified from a peripheral blood sample with primers containing EcoRI and NotI sites: 5'-GAATTCACACCACCTGAACG-3' (forward) and 5'-GCGGCCGCCTACAGAGCGAAGG-3' (reverse) (restriction sites are underlined) following the program described above. The product was cloned in frame with an amino-terminal Flag epitope into the EcoRI-NotI sites of mammalian vector pCEFL-Flag to generate pCEFL-Flag-LTA-C. The polymorphic substitution rs2229094 was introduced in pCEFL-Flag-LTA-C with the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA, USA) using the following primers: 5'-CTCCCAAGGGTGTGTGGCACCACCC-3' (forward) and 5'-GGGTGGTGCCACACACCCTTGGGAG-3' (reverse) to generate pCEFL-Flag-LTA-T. All vectors were transformed in E. coli DH5α and plasmid DNA was purified using the Rapid DNA plasmid miniprep kit (Genedan,

S.L., Barcelona, Spain) All constructs were verified by automated sequencing.

Cell culture and DNA transfections

COS-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin-streptomycin at 37ºC in a humidified 5% CO_2 atmosphere. Exponentially growing COS-1 cells were transiently co-transfected with 1 µg total plasmid DNA (pCEFL-Flag-LTA-C and pCEFL-Flag-LTA-T) using X-tremeGENE HP DNA Transfection Reagent (Roche, Switzerland) at ratio 3:1. After 24-48 h cells were harvested to assess expression at RNA and protein level of pCEFL-Flag-LTA-C and pCEFL-Flag-LTA-T vectors by RT-PCR and Western blot. RT-PCR was performed as described above.

Western Blot

COS1 cells were harvested 24-48 h after transfection and resuspended in lysis buffer (50mM Tris pH7.5, 150mM NaCl, 5mM EDTA and 1% NP40) supplemented with Complete Mini (Roche, Switzerland) for protein extraction. Protein concentrations were determined using the BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Protein extracts (200 µg) were fractionated by SDS-PAGE, transferred to an Immobilon-P membrane (Millipore, Billerica, MA, USA) and incubated with primary specific antibodies anti-Flag M2 and anti-β-actin (Sigma-Aldrich Co, St. Louis, MO, USA). The ECL Plus detection System (GE Healthcare, Buckinghamshire, UK) with HRP-conjugated anti-mouse secondary antibodies (Sigma-Aldrich Co, St. Louis, MO, USA) was used for detection.

Confocal immunofluorescence assay

COS1 cells were grown on glass cover-slips and transfected as indicated above. At 48 h after transfection, cells were fixed with ice-cold 3.7% formaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS and incubated with the primary antibody anti-Flag M2 (Sigma-Aldrich Co, St. Louis, MO, USA) and the secondary antibody Alexa Fluor 488 Donkey Anti-Goat IgG conjugated to fluorescein isothiocyanate (FITC) (Invitrogen, Carlsbad, CA, USA). Finally, cells were stained with DAPI (4',6-diamidino-2-phenylindole) (Sigma-Aldrich Co, St. Louis, MO, USA) and cover-slips were mounted onto microscope slides in the presence of Mowiol mounting medium. Fluorescence images were captured with a LEICA TCS SP5 DMI-6000B confocal microscope (Leica Microsystems Inc, Wetzlar, Germany) using excitation wavelength of 488nm (for FITC) and were analyzed with LEICA LAS AF (Leica Microsystems Inc, Wetzlar, Germany). Final processing of images was done with the Adobe Photoshop (Adobe Systems) program.

RESULTS:

LTA mRNA expression

LTA expression was evaluated both at mRNA and protein levels. LTA mRNA expression was analyzed by RT-PCR in total RNA samples from 3 healthy neuroretinas obtained within 30 to 60 minutes post-mortem. mRNA LTA expression was below the significance level of the used method and none of the 3 healthy human neuroretinas showed expression of LTA mRNA.

In addition, in order to determine whether the LTA mRNA is expressed in

peripheral blood, RT-PCR analysis was carried out using the total RNA extracted from two peripheral blood samples from healthy subjects. We detected the mRNA expression of LTA in both DNA blood samples. Interestingly, the RT-PCR analysis also revealed different alternative LTA transcripts (Figure. 1). However, the analysis by sequencing of these new transcripts revealed premature stop codons, and consequently, LTA isoforms without any function.

LTA protein expression and localization

Constitutive LTA protein expression was evaluated by immunohistochemistry in a healthy human eye, chronic RD donor eyes and in adult human brain sections from the hippocampal area (used as a positive control) (Figure. 2A). Strong positive staining for LTA was detected in control brain sections, which was confined to hippocampal neuronal cells and their cytoplasms (Figure. 2B). The intensity of the immunostaining was moderate in retinal sections from control eye (Figure. 2C), whereas the intensity of the immunostaining and the number of stained cells were noticeably lesser (weakly positive) in retinal sections from chronic RD eyes (Figure. 2D). In both cases (normal and chronic RD eyes) the immunostaining was confined to ganglion neuron cells and their membranes and cytoplasms (Figure. 2C, 2D).

Light microscopy morphologic assessment showed that immunostaining for LTA in control donor and chronic RD eyes was mostly found in ganglion cells located in the nerve fiber and retinal ganglion cell layers (Figure.2C, 2D). At the level of light microscopy, some faint and barely visible immunostaining was also observed into other neurons retinal cells (amacrine and/or bipolar), located in

the inner nuclear.

Functional characterization of the T and C alleles of LTA rs2229094 polymorphism

The functionality of the T and C alleles of LTA rs2229094 polymorphism was investigated by cloning these alleles in the pCEFL-Flag expressing vector and by analyzing their expression (pCEFL-Flag-LTA-T and pCEFL-Flag-LTA-C) both at RNA and protein level as well as their subcellular localization in COS1 cells using a specific anti-Flag antibody.

mRNA expression of LTA-T and LTA-C was evaluated by RT-PCR at 7h, 24h and 48h after transfection (Figure. 3A). No differences in the level of mRNA expression of LTA between the two alleles were detected. In addition, no differences were observed in the protein expression levels of LTA-T and LTA-C by Western Blot analysis (Figure. 3B).

In order to investigate whether the rs2229094 T to C substitution in the signal peptide of LTA may affect the corresponding protein localization, we performed an immunofluorescence assay to study the subcellular location of the T and C LTA alleles (pCEFL-Flag-LTA-T and pCEFL-Flag-LTA-C) in the Cos1 cell line at 48 hours after transfection. We observed that both LTA alleles were located mainly in the cellular cytoplasm; however, no differences in the subcellular location were observed between the two alleles (Figure. 4 A, 4B).

DISCUSSION:

As mentioned, PVR is a complex process, involving not only ischemic tissue damage, but also inflammation and proliferation of several types of intraretinal

local cells.

Currently, PVR is considered a multifactorial disease and may be the result of interaction between genetic and environmental factors.[3-7] Despite the fact that the exact mechanisms responsible of PVR are not completely understood, it is widely accepted that inflammation plays an important role in its pathogenesis.[14]

LTA Expression and Localization

TNF superfamily of cytokines regulates many physiological processes, including inflammation, proliferation, differentiation, and cell death.[15] TNFA and LTA are two molecules that play an important role in inflammation. Previous studies demonstrate that LTA, with a 40% of homology to TNFA, shares the same membrane receptors as TNFA, exerting its biological effect largely via TNF receptor 1 (TNFR1) and TNFR2 activation.[16]

The implication of pro-inflammatory cytokines, especially TNFA, in PVR pathogenesis has been demonstrated. Previous studies reported high vitreous levels of TNFA and its receptors (TNFR 1 and 2) in eyes with PVR[17] and local production of TNFA has been suggested to occur in these eyes.[18] However, retinal expression and localization of LTA have not been previously described in either normal or RD eyes.

We observed a positive immunostaining for LTA predominantly localized in the cytoplasm of ganglion cells, as well as on the cell surface of the retina in control and chronic RD eyes. This particular localization could be due probably because LTA is internalized after its receptor binding as TNFA.[19-22] Surprisingly, despite RNA LTA expression in the 3 neuroretinas (enucleated within 30 to 60 minutes after death and immersed in ice-cold transport medium) from donors

343 was undetectable by RT-PCR, we found positive immunostaining for LTA in the
344 control eye. Moreover, the immunostaining was stronger in the control eye
345 (fixed in formalin and embedded in paraffin) than in chronic RD eyes. A possible
346 explanation for this might be that donor eyes in which RT-PCR was performed,
347 were enucleated within 30 to 60 minutes after death and were immersed
348 immediately in ice-cold transport medium, preventing a long period of time
349 under ischemia and subsequent initiation of inflammatory responses. On the
350 other hand, the time-limited response of TNFA and LTA after its initial high
351 peaks around 72h after ischemia[23] suggesting that there are immediate pro-
352 inflammatory responses after RD. Our RD samples presented chronic RD, in
353 which immediate pro-inflammatory mediators may not be released as it occurs
354 in acute RD. Also, the increase of neuronal cell death could be one of the
355 possible causes leading to a smaller number of positive cells and weaker
356 immunostaining in chronic RD eyes.
357 Moreover, it has been previously described that the addition of exogenous
358 TNFA to organotypic culture of porcine neuroretinas results in greater
359 hypertrophy of glial cells and a higher level of retinal disorganization, with the
360 processes of Müller cells crossing the OLM and forming gliotic membranes in
361 the subretinal space.[24] Similar observations also occur in PVR[8, 24, 25] and have
362 been described by our group when neuroretinas were cultured with
363 macrophages.[26]
364 LTA is able to act through LTA–TNFR1 complex as TNFA, but it seems to be
365 differences in their signal strength or capabilities.[27] In particular, it has been
366 reported that LTA is less able to promote TNFR1-induced cell death and
367 nuclear factor-kB (NF-kB) activation,[28] expression of cell surface markers[29] and

cytokine production[30, 31] than TNFA. In addition, TNFA and LTA have shown protective effects in neuronal cells when acting via TNFR2, especially in ischemic conditions both in brain and in retina.[32, 33] Thus, despite LTA is able to induce signals via TNFR1, and could have a crucial role in the initiation and development of some inflammatory diseases,[34, 35] due to its diverse bioactivities, it is presently not clear under which conditions LTA promotes beneficial or deleterious effects on neuronal tissues. Therefore further studies will be critical to further understanding the role of LTA in retinal neuronal cells after a RD.

Functionality of the T and C alleles of LTA rs2229094 polymorphism

LTA gene is located on chromosome 6, between the HLA class II and class I loci. LTA gene polymorphisms are also shown to be associated with the inflammatory and immunomodulatory diseases including cancer.[36-41] In addition, the contribution of TNF genes to PVR susceptibility has been previously discussed. A positive association of TNFA and LTA polymorphisms with PVR has been reported.[4,6] However, the functionality of most of these polymorphisms remains currently unknown.

LTA rs2229094 polymorphism is a non-synonymous polymorphism located in the signal peptide of LTA gene, which results in a change from cysteine to arginine in codon 13 (Cys13Arg). While cysteine is a neutral hydrophobic amino acid, arginine is a hydrophilic, positively charged amino acid. Regarding the results of bioinformatic tool analysis,[4] the hydropathy profile changed for each allelic variant could affect the function of LTA protein or modify its production or its localization, due to the hydrophobicity of the signal peptide and the

asymmetric distribution of the hydrophobic and hydrophilic potentials which determine the translocation specificity of proteins.[42-44]

However, the functional analysis performed in the present study did not show any differences in the expression levels between the LTA-T and C alleles (cysteine and arginina alleles, respectively). Furthermore, the subcellular location of C allele did not vary with respect to the T allele, both being located in the cytoplasm, suggesting that the C allele has no significant effect in the LTA gene transcriptional regulation and subcellular location. The cytoplasmic location of LTA could be due to, as explained above, LTA internalization after its receptor binding as TNFA.[19-22]

Although the results suggest lack of functionality of the LTA rs2229094 polymorphism, this polymorphism could be remain as a valid biomarker to identify high-risk patients to develop PVR after RD, due to strong association between PVR and LTA rs2229094 polymorphism previously shown by our group. And because of LTA gene is located within TNF locus, and it is closely linked to TNFA and LTB genes. Therefore, modifications derived from functional polymorphisms in any of these could lead to collateral alterations in the neighboring genes.[42] Additionally, LTA could somehow control the expression of TNFA, and the absence of LTA could interfere with the production of this cytokine, which would allow us suggest an alternative therapeutic approach by blocking this cytokine and reinforce the potential role that cytokines from the TNF-family and their signaling pathways may play in PVR pathogenesis.

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FIGURES

Figure 1: RT-PCR products of LTA mRNA detected by 3% agarose gel electrophoresis stained by Ethidium bromide.
mRNA expression of LTA was detected in the two peripheral blood samples from healthy subjects (lane 1 and 2). Interestingly, RT-PCR analysis revealed different alternative LTA transcripts (A, B and C). Automatic sequencing revealed that transcript A retained introns 2 and 3 of LTA, transcript B retained intron 2, while transcript C lacked exon 3 of LTA. However, all of these three transcripts had altered reading frames and included premature stop codons, leading to truncated LTA isoforms that may have no function.
GAPDH expression was used as internal control. Lane M was ΦX174 DNA/HaeIII molecular weight marker (Promega, Madison, WI, USA). Lane (-) was a control reaction lacking reverse transcriptase.

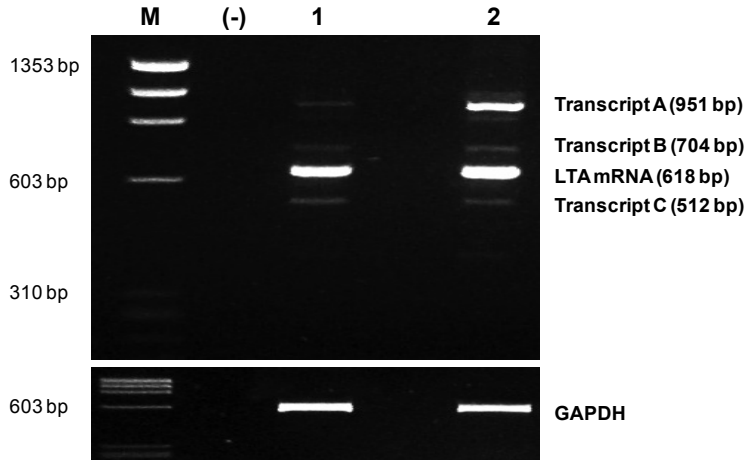


Figure 2: Immunohistochemical images of **(a)** Adult human hippocampus with no primary antibody (negative control), original magnification 400X. **(b)** Adult human hippocampus anti-LTA staining, showing intense positivity (+++) within the cytoplasm of hippocampal neurons (black arrows) 400X. **(c)** Healthy human retina anti-LTA staining (control). Moderate LTA cytoplasmic positivity (++) is observed in ganglion cells (black arrows), 200X. **(d)** Anti-LTA staining (case) in human retina with chronic RD showing weak cytoplasmic positivity (+) in ganglion cells (black arrow), 200X.
Note retinal thinning and photoreceptor outer segments degeneration after RD. INL: inner nuclear layer. ONL: Outer nuclear layer.

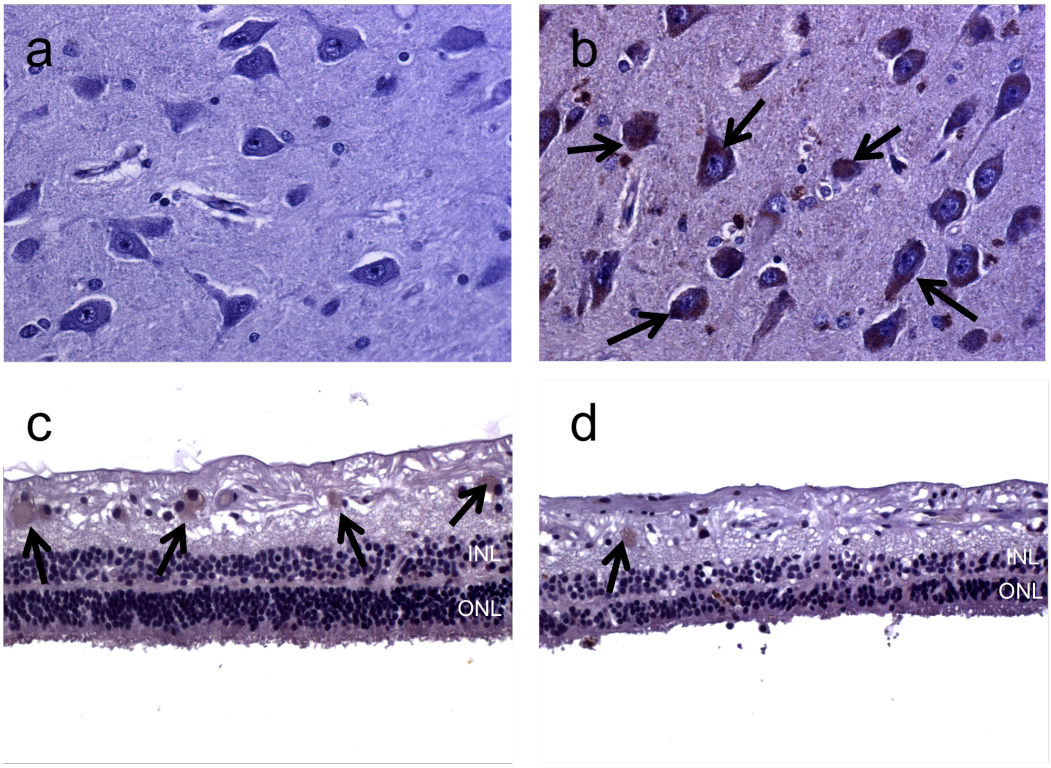


Figure 3. Expression analysis of T and C alleles of LTA rs2229094 polymorphism.
COS1 cells were transfected with plasmids encoding LTA-C allele (pCEFL-Flag-LTA-C) and LTA-T allele (pCEFL-Flag-LTA-T). **(a)** mRNA expression of both LTA alleles was assessed by RT-PCR at 7, 24 and 48 hours after transfection. GAPDH expression was used as internal control. Lane M was ΦX174 DNA/HaeIII molecular weight marker (Promega, Madison, WI, USA). Lane (-) was a control reaction lacking reverse transcriptase. **(b)** Protein expression of both LTA alleles was evaluated by western blot at 48 hours after transfection using anti-Flag antibody. Lane 1 was LTA-C allele, lane 2 was LTA-T allele and lane (-) was a negative control (not transfected COS1 cells). β-actin expression was used as internal control.
No differences in RNA or protein expression levels were observed between the LTA-C and T alleles.

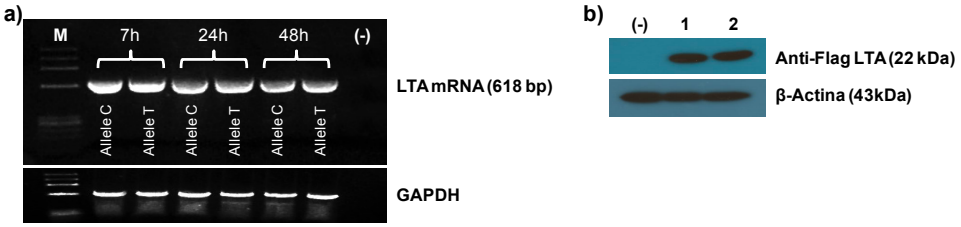
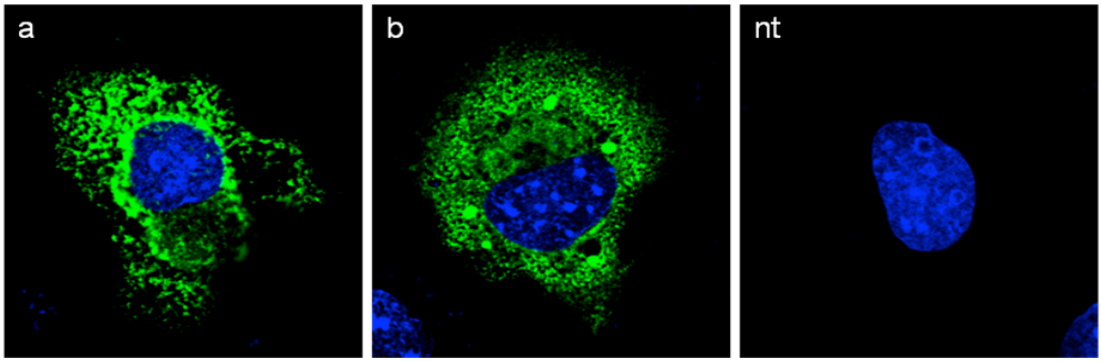


Figure 4: Subcellular localization of T and C alleles of LTA rs2229094 polymorphism.
COS1 cells were transfected with plasmids encoding LTAC allele (pCEFL-Flag-LTAC) and LTAT allele (pCEFL-Flag-LTAT). 48 hours after transfection,

confocal immunofluorescence assay was performed using anti-Flag antibody (green) and cell nuclei were stained with DAPI (blue). Both LTA C allele **(a)** and T allele **(b)** were mainly located in the cytoplasm, without finding any differences in their cellular localization. nt: not transfected COS1 cells.



Chapter 2

The p53 codon 72 polymorphism (rs1042522) is associated with proliferative vitreoretinopathy The Retina 4 Project

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The *p53* Codon 72 Polymorphism (rs1042522) Is Associated with Proliferative Vitreoretinopathy

The Retina 4 Project

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Purpose: To compare the distribution of a *p53* gene polymorphism among European subjects undergoing primary retinal detachment (RD) surgery in relation to the development of proliferative vitreoretinopathy (PVR).

Design: Case-controlled gene association study conducted as a component of the Retina 4 Project (a European multicenter study).

Participants and Controls: Five hundred fifty DNA samples, 134 with PVR secondary to primary RD and 416 with RD without PVR.

Methods: The *p53* codon 72 polymorphism (rs1042522) was analyzed using allele-specific primer polymerase chain reaction. Proportions of genotypes and the proline (Pro-P) homozygote groups between subsamples from different countries were analyzed in 2 phases. In the first, subsamples from Spain and Portugal were analyzed. After significant results were found, samples from the United Kingdom (UK) and The Netherlands were analyzed (second phase). Genotypic and allelic frequencies were compared between cases and controls in the global sample.

Main Outcome Measures: Single significant associations with PVR.

Results: A significant difference ($P<0.05$, Fisher exact test) was observed regarding the p53 genotype frequencies at codon 72 between the PVR cases and the non-PVR controls in Spain and Portugal (phase I), but not in the UK or The Netherlands (phase II). Analysis of Pro homozygote carriers between cases and controls revealed differences in Spain (29.01–42.18 and 2.29–10.20, respectively), Portugal (10.49–29.50 and 1.35–8.89, respectively), and The Netherlands (16.49–31.70 and 4.51–15.09, respectively), but no differences in the UK (7.68–18.1 and 4.85–13.94, respectively). The odds ratio of Pro carriers from Spain and Portugal together was 8.12 (95% confidence interval [CI], 3.72–17.69; $P<0.05$), whereas the odds ratio of Pro carriers from the UK and The Netherlands was 2.12 (95% CI, 0.96–4.68; $P = 0.07$). All control samples were in Hardy-Weinberg equilibrium. Considering the entire sample, significant differences were found in genotype frequencies between cases (RR, 30.59%; RP, 43.28%; PP, 26.11% [R = Arg; P = Pro]) and controls (RR, 39.66%; RP, 52.64%; PP, 7.69%) and in Pro homozygote carriers between controls (Pro homozygote 95% CI, 18.67–33.52) and cases (Pro homozygote 95% CI, 5.1–10.2).

Conclusions: Results indicate that the Pro variant of *p53* codon 72 polymorphism is associated with a higher risk of PVR developing after a primary RD. Further studies are necessary to understand the role of this polymorphism in the development of PVR.

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Proliferative vitreoretinopathy (PVR) is one of the major causes of failure in retinal detachment (RD) surgery,[1] affecting 5% to 10% of RD cases and accounting for approximately 75% of all primary failures after RD surgery.[1,2] It is considered an abnormal wound-healing process induced by a retinal break and escape of retinal pigment epithelium cells into a proinflammatory vitreous enviroment.[3–7] Although PVR was identified in 1983 as an independent

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entity,[8] there are no currently available treatments for its prophylaxis. Thus, in most cases, its management implicates repeated procedures at a significant cost[9] and, above all, with very poor anatomic and functional results.[10–13]

Most research has attempted to identify clinical risk factors for PVR developing; however, these variables do not completely explain the probability of its onset.[14] Single nucleotide polymorphisms have important implications for human genetic diseases. They may help to identify a genetic predisposition for certain diseases, either as a causative factor or protective risk factor. Finally, they may help to increase our knowledge of the molecular causes of some conditions.

Previous studies described the contribution of the genetic component to PVR.[15,16] With this aim, the Retina 4 project, a case-control, European, multicenter study, was coordinated. As a result of the first part of this project, a strong association between PVR development and the rs2229094 (T→C) lymphotoxin α polymorphism was reported.[16] However, the genetics of PVR seem to be complex, and probably some other genes are involved in its development.

The tumor-suppressor gene *p53* is crucial for cell repair of genomic mutations that may give rise to many tumors. Also, it is well known to induce cell cycle arrest, apoptosis, senescence, or differentiation after cellular stress.[17] Numerous single nucleotide polymorphisms and other polymorphic variations have been described in the *p53* gene. The codon 72 polymorphism in *p53* regulates the interaction with nuclear factor-κB and transactivation of genes involved in apoptosis, immunity, and inflammation, and it has been associated with several cancers and inflammatory processes.[18–21] This polymorphism appears in humans under 2 variants, arginine (Arg-R) or proline (Pro-P). The Arg/Arg variant encodes a highly proapoptotic protein, whereas the Pro/Pro variant has the opposite effect.[19,22,23] This property correlates with a greater capacity to interact with the Murine double minute 2 protein, which facilitates nuclear export and mitochondrial localization.[22,24] Other differences between the *p53* variants have been reported: the ability to bind components of the transcriptional machinery, to activate transcription, to induce apoptosis, and to repress the transformation of primary cells.[25]

Besides its relationship to tumors and inflammatory processes, recent studies of the central nervous system have shown that the *p53* codon 72 polymorphism is related to a poor functional prognosis in patients who have had ischemic or hemorrhagic stroke.[26] Moreover, previous studies have shown apoptosis to be a major cause of neuronal loss after trauma, ischemia, and neurodegeneration in the central nervous system.[27] These findings are relevant because retinal tissue has a similar behavior to the brain tissue, including the scarring processes.[5,28] After RD, the outer retina is separated from the underlying retinal pigment epithelium, which provides the major metabolic and nutritional support, leading to relative ischemia and hypoxia of photoreceptors. These factors promote an increase in *p53* levels and the activation of various cell death mechanisms.[27,29] It has been reported that photoreceptor death after RD and subsequent visual decline could be caused by apoptosis and other pathways for RD-associated photoreceptor death.[27,30,31] Finally, a recent report suggested that increased expression of soluble apoptosis and adhesion molecules at the time of primary RD surgery is associated with the future development of PVR.[32] However, it has been reported that apoptotic bodies derived from retinal capillary endothelium induce the release of proangiogenic cytokines and chemokines as well as the expression of adhesion molecules facilitating endothelial progenitor cell recruitment, which could favor retinal healing.[32] Thus, the purpose of this study was to analyze the distribution of the codon 72 polymorphism in exon 4 of the *p53* gene in a large consecutive sample of patients with primary rhegmatogenous RD with and without PVR recruited from several European centers.

Patients and Methods

Design and Study Population

The association study was carried out among 550 patients from 7 centers: 3 in Spain, 2 in Portugal, 1 in the United Kingdom (UK), and 1 in The Netherlands. For analysis, the global sample was divided in subsamples according to country. The study was carried out in 2 phases. In the first phase, subsamples from Spain and Portugal were analyzed. After significant results were found in this first cohort, subsequent samples from the UK and The Netherlands were analyzed (second phase). To compare whether there were differences regarding geographic localization in the odds ratio (OR) analysis, Spain and Portugal were considered as southern countries and the UK and The Netherlands were considered as northern countries. The study was approved by the institutional research committee of each center and followed the tenets of the Declaration of Helsinki. All patients provided written informed consent before entering in the study.

DNA samples from cases and controls in the Retina 4 project were analyzed for this study. All participants were patients with a primary rhegmatogenous RD who underwent surgery. Exclusion criteria were age younger than 16 years; traumatic, tractional, exudative, or iatrogenic RD; RD secondary to macular hole or giant retinal tear (larger than 3 clock hours); and PVR grade higher than B (Machemer classification)[1] on admission for surgery. Those who did not demonstrate PVR after 3 months of follow-up were included in the control group. Those in whom PVR grade C1 or higher developed, according to Machemer classification, were included as cases.

Genotyping

Genotyping of codon 72 of the *p53* polymorphism was performed at the Molecular Medicine Unit, Department of Genetics, University of Salamanca, Salamanca, Spain. Those carrying out the genotyping were blinded to the clinical status of patients and used the polymerase chain reaction-restriction fragment length polymorphism technique.[33,34]

The Tp53 polymorphism was detected by amplifying genomic DNA with the forward primer 5′TCTACAGTCCCCCTTGCCGT-3′ and the reverse primer 5′- CTGACCGTGCAAGTCACAGA-3′.[33,34] The *p53* exon 4 was amplified within a 298-base pair (bp) DNA fragment that was digested with BstU1 (*Bsh1236I Fermentas fast digest restriction enzyme* [Thermo Scientific, Germany]), and the resulting fragments were separated on 3.5% agarose gel. The polymerase chain reaction fragments containing Arg and Pro alleles, after digestion, migrated as a 291-bp fragment for Pro homozygotes, as 2 fragments of 165 and 126 bp for Arg homozygotes, and as 3 fragments of 126, 165 and 291 bp for heterozygotes.

Table 1. Clinical Variables of Entire Sample

Characteristics	Controls No. with Retinal Detachment, n (%)	Cases No. with Proliferative Vitreoretinopathy, n (%)	Total	*P* Value
Race				
White	387 (71.5)	121 (22.36)	508	
Hispanic American	2 (50)	2 (50)	4	
Hindu	7 (46.6)	8 (53.3)	15	
Arabic North African	6 (100)	0 (0)	6	0.059
Sub-Saharan African	2 (66.6)	1 (33.3)	3	
Asian	3 (60)	2 (40)	5	
Unknown			9	
Total			550	
Phakic lens				
Yes	258 (77.24)	76 (22.75)	334	
No	137 (72.48)	52 (27.51)	189	0.224
Unknown			27	
Total			550	
Geographical location				
Northern countries (UK+Holland)	210 (77.7)	60 (22.2)	270	
Southern countries (Spain+Portugal)	197 (72.7)	74 (27.3)	271	0.171
Unknown			9	
Total			550	

UK = United Kingdom.

Statistical Analysis

The statistical analysis was conducted in both phases. The quality of data was evaluated in control subsamples by Hardy-Weinberg equilibrium using the chi-square test. Genotypic frequencies were estimated in each subsample. The proportions of genotypes and the Pro homozygote groups between subsamples were analyzed. Also, the genotypic and allelic frequencies were compared between cases and controls in the global sample.

Association was investigated using the chi-square and the Fisher exact tests. The strength of association was measured using ORs and 95% confidence intervals (CIs). Two inheritance models were considered: the codominant model, which allows every genotype to give a different and nonadditive risk, and the recessive model, in which 2 copies of the Pro allele are necessary to change the risk. The Akaike information criterion (AIC)[35] was used to choose the inheritance model that best fit the data. The statistical analyses were conducted using SPSS software version 16.0 for Macintosh (SPSS, Inc., Chicago, IL) and R software (R Foundation for Statistical Computing, Vienna, Austria).

Results

A total of 550 subjects including 134 cases and 416 controls were analyzed: 203 from Spain (36.9%), 68 from Portugal (12.4%), 121 from The Netherlands (22%), and 158 from the UK (28.7%). Some important clinical variables are shown in Table 1. A multiracial population with statistical differences in the British samples in comparison with the others groups was found. All control subsamples were in Hardy-Weinberg equilibrium. Status of the lens was determined because aphakia has been related to a higher incidence of PVR.[5,14]

Phase I: Genotypic Distribution of *p53* Codon 72 Polymorphism in Spain and Portugal

The frequencies of the genotypes in each country are shown in Table 2. The comparison of proportions of genotypes between subsamples showed a significant difference ($P<0.05$) between cases and controls. Also, a significant difference ($P<0.05$) in Pro homozygote carriers between subsamples in the control group (Pro homozygote 95% CI

Table 2. Distribution of Frequencies of the Genotypes

Countries	Arginine/Arginine (%)		Proline/Proline (%)		Arginine/Proline (%)		*P* Value (Fisher Exact Test)*	*P*<0.05 95% Confidence Interval†		Odds Ratio
	Cases	*Controls*	*Cases/Controls*	*Controls*	*Cases/Controls*	*Controls*		*Cases/Controls*	*Controls*	
Spain	27.1	45.1	35.6†	6.25†	37.3	48.61	<0.05*	29.01–42.18	2.29–10.20	8.5
Portugal	33.3	43.39	20†	3.77†	46.7	52.8	<0.05*	10.49–29.50	1.35–8.89	6.5
UK	35.5	36.2	12.9	9.4	51.6	54.3	>0.05*	—	—	
The Netherlands	31	33.7	24.1†	9.8†	44.8	56.5	>0.05*	16.49–31.70	4.51–15.09	3.3

— = confidence interval not statistically significant; UK = United Kingdom.
*Comparison of proportions of genotypes between subsamples. A significant difference was observed between cases and controls in Spain and Portugal but not in the UK and The Netherlands.
†Prohomozygote carrier analysis between different countries revealed differences in Spain, Portugal, and The Netherlands but no differences in the UK.

Table 3. Models of Inheritance in the Global Sample and Results of Odds Ratios Using a Recessive Model for Spain plus Portugal and The Netherlands plus the United Kingdom

Model	Genotype	Controls (*n*)	Controls (%)	Cases (*n*)	Cases (%)	Odds Ratio	95% Confidence Interval	*P* Value	Akaike Information Criterion*
Codominant	Arg/Arg	165	39.7	41	30.6	1.00		<0.001	588.6
	Arg/Pro	219	52.6	58	43.3	1.07	0.68–1.67		
	Pro/Pro	32	7.7	35	26.1	4.40	2.44–7.93		
Dominant	Arg/Arg	165	39.7	41	30.6	1.00		<0.001	611.1
	Arg/Pro-Pro/Pro	251	60.3	93	69.4	1.49	0.98–2.26		
Recessive	Arg/Arg-Arg/Pro	384	92.3	99	73.9	1.00		<0.001	586.7
	Pro/Pro	32	7.7	35	26.1	4.24	2.50–7.19		
Overdominant	Arg/Arg-Pro/Pro	197	47.4	76	56.7	1.00		<0.001	611.2
	Arg/Pro	219	52.6	58	43.3	0.69	0.46–1.02		
Spain+Portugal		186	94.4	50	67.6	1.00		<0.001	291.3
		11	5.6	24	32.4	8.12	3.72–17.69		
The Netherlands+UK		198	90.4	49	81.7	1.00		0.07	291.3
		21	9.6	11	18.3	2.12	0.96–4.68		

Arg = arginine; Pro = proline; UK = United Kingdom.
*A measure of the relative goodness of fit of a statistical model. It generally can be used for the identification of an optimum model in a class of competing models. Given a set of candidate models for the data, the preferred model is the 1 with the minimum Akaike Information Criterion value.

for Spain and Portugal, 29.01–42.18 and 10.49–29.50, respectively) and in the cases group (Pro homozygote 95% CI for Spain and Portugal, 2.29–10.20 and 1.35–8.89, respectively) was found. The OR of Pro carriers considering a recessive model (Arg/Arg plus Arg/Pro vs. Pro/Pro; AIC = 291.3 vs. 293.2 of codominant model) was 8.12 (95% CI, 3.72–17.69; $P<0.05$; Table 3).

Phase II: Genotypic Distribution of *p53* Codon 72 Polymorphism in the United Kingdom and The Netherlands

The frequencies of the genotypes in patients from the UK and The Netherlands are shown in Table 2. Distribution of genotypes between subjects from those countries did not show statistical differences. However, when the Pro homozygote carriers between cases and controls were analyzed, a significant difference in the group of Dutch patients was found (Pro homozygote 95% CI, 16.49–31.70 and 4.51–15.09, respectively; $P<0.05$). Although not statistically significant in patients from the UK, a similar trend was seen (Pro homozygote 95% CI, 7.68–18.1 and 4.85–13.94, respectively; $P>0.05$). No differences were found in the OR distribution of homozygous carriers of the Pro variant in patients from the UK and The Netherlands together considering a recessive model (AIC = 291.3 vs. 293.2 of codominant model; OR, 2.12; 95% CI, 0.96–4.68; $P = 0.07$; Table 3).

When all patients were grouped (Table 4), significant differences in the distribution of genotypes between the controls and cases ($P<0.05$) were found. Also, homozygous carriers of the Pro variant were more frequent in PVR cases than in controls ($P<0.05$; 95% CI, 18.67–33.52 and 5.1–10.2 for cases and controls, respectively). The OR of the Pro variant in the global sample using a recessive model (AIC = 586.7 vs. 588.6 of codominant model) was 4.24 (95% CI, 2.50–7.19; Table 3).

Discussion

Proliferative vitreoretinopathy is considered a multifactorial disease,[15,16] and it may result from interactions between genetic and environmental factors.[14–16] The lack of satisfactory results in the identification of patients at risk of developing PVR after RD by clinical characteristics[14] justifies the efforts to elucidate the genetic components[15,16] as a potential means of identifying high-risk patients before surgery and possibly to modify the treatment strategy in a more customized way.

In addition, recent research has highlighted the involvement of extrinsic and intrinsic pathways of apoptosis in retinal cells after RD and the existence of other mechanisms of cell death after RD when apoptotic pathways are inhibited.[27] The initiation of apoptosis and other death pathways, such as programmed necrosis, involves the activation of certain specific receptors on the cell surface. These death receptors mainly comprise the tumor necrosis factor (TNF) receptor family tumor necrosis factor receptor 1 (TNFR1) and TNF-related apoptosis-inducing ligand (TRAIL).[27]

Table 4. Distribution of p53 Codon 72 Polymorphism in the Entire Sample

Genotypes	Arginine/ Arginine	Arginine/ Proline	Proline/ Proline	Total	*P* Value*	95% Confidence Interval
Cases	41 (30.59%)	58 (43.28%)	35 (26.1%)†	134 (100%)	<0.05	18.67–33.52
Controls	165 (39.66%)	219 (52.64%)	32 (7.69%)	416 (100%)		5.1–10.2
Total	206	277	67	550		

*Fisher exact test.
†Analysis of pro-homozygote carriers between case and control group.

In recent years we have been exploring the genetic contribution to PVR. As a result of these studies, they have identified the potential contribution of tumor growth factor β[16] and lymphotoxin α[17] in PVR. Lymphotoxin α and TNF-α are proinflammatory cytokines that have a wide range of biologic functions involved in inflammation, apoptosis, and cell proliferation[36]; in addition, their intraocular levels are increased in eyes with PVR.[37–39]

p53 Is a protein involved in regulating apoptosis and has increased intracellular levels in response to DNA damage, uncontrolled cell proliferation, or telomere erosion.[40,41] The *p53* Arg72Pro polymorphism is located in exon 4 and consists of a change of guanine to cytosine at position 2 of codon 72, which is located in the Pro-rich region (at the N-terminal extreme) involved in the apoptotic functions of the p53 protein.[42] The Arg→Pro change affects the primary structure of the protein and generates functional differences because the Arg variant is associated with increased apoptosis.[22,43]

Several studies have reported the potential role of this polymorphism as a risk factor for several cancers and some inflammatory processes[18–21] in which apoptosis seems to have a crucial role. It recently was reported that carriers of the Arg/Arg genotype have a poorer functional prognosis after a stroke, probably associated with an increase of apoptotic death of neurons.[26] Furthermore, it has been associated with an increased risk of primary open-angle glaucoma compared with healthy subjects.[44]

Proliferative vitreoretinopathy remains the most common cause of recurrent RD after RD surgery. The development of PVR is a complex process involving humoral and cellular factors, and the distribution of genotypes of the *p53* codon 72 polymorphism in patients with PVR was considered a target for increasing the knowledge of this severe complication of RD.

The current results show that Spanish and Portuguese carriers of the homozygous Pro variant in homozygosis have a 4-fold increased risk to PVR after RD compared with those who carry the homozygous Arg variant (Table 2). This observation was confirmed in Dutch patients but not in a British population (although a similar trend was seen). The absence of correlation in the British group could be explained by the observation that frequency of the *p53* codon 72 alleles differs with latitude, increasing the Pro variants within populations close to the equator, whereas the Arg variant predominates in northern latitudes.[21,45]

However, because the Dutch patient genetic profiles were similar to those from Spain and Portugal, there must be some other factors implicated in this difference. In this sense, the possibility that differences could be the result of ethnic diversity in the group of patients from the UK cannot be ruled out because many patients undergoing RD treatment in London have ancestry from the Indian subcontinent.

Results of this work indicate that carriers of the Pro allele of the *p53* gene, associated with a decrease in apoptotic function of *p53*, have a higher risk of PVR developing after RD. It can be speculated that the reduction in the levels of apoptosis could energize migrating retinal pigment epithelium cells and inflammatory mediators directly, allowing a more aggressive cellular response. Alternatively, a greater resistance to apoptosis could sustain ischemic photoreceptors for longer periods, allowing these cells to release more cytokines and other growth factors to generate a more aggressive PVR response through secondary mechanisms and globally increase the intraocular inflammation after RD.

In summary, this study highlights the role of genetics as useful in the identification of high-risk patients who may be susceptible to PVR and indicates that the Pro allele could be a significant risk factor for PVR development after a primary RD and could be used as a possible marker of risk of PVR after RD. In conclusion, these results support a key role for *p53*-mediated apoptosis in the generation of PVR after RD surgery.

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Chapter 3

The T309G MDM2 gene polymorphism is a novel risk factor for proliferative vitreoretinopathy The Retina 4 Project

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The T309G MDM2 Gene Polymorphism Is a Novel Risk Factor for Proliferative Vitreoretinopathy

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Abstract

Proliferative vitreoretinopathy (PVR) is still the major cause of failure in retinal detachment (RD) surgery. It is believed that down-regulation in the p53 pathway could be an important key in PVR pathogenesis. The purpose was to evaluate the impact of T309G *MDM2* polymorphism (rs2279744) in PVR. Distribution of T309G *MDM2* genotypes among European subjects undergoing RD surgery was evaluated. Proportions of genotypes between subsamples from different countries were analyzed. Also, a genetic interaction between rs2279744 in *MDM2* and rs1042522 in *p53* gene was analyzed. Significant differences were observed comparing *MDM2* genotype frequencies at position 309 of intron 1 between cases (GG: 21.6%, TG: 54.5%, TT: 23.8%) and controls (GG: 7.3%, TG: 43.9%, TT: 48.7%). The proportions of genotypes between sub-samples from different countries showed a significant difference. Distribution of GG genotype revealed differences in Spain (35.1–53.0)/(22.6–32.9), Portugal (39.0–74.4)/(21.4–38.9), Netherlands (40.6–66.3)/(25.3–38.8) and UK (37.5–62.4)/(23.3–34.2). The OR of G carriers in the global sample was 5.9 (95% CI: 3.2 to 11.2). The OR of G carriers from Spain and Portugal was 5.4 (95% CI: 2.2–12.7), whereas in the UK and the Netherlands was 7.3 (95% CI: 2.8–19.1). Results indicate that the G allele of rs2279744 is associated with a higher risk of developing PVR in patients undergoing a RD surgery. Further studies are necessary to understand the role of this SNP in the development of PVR.

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Introduction

Proliferative vitreoretinopathy (PVR) is still the major cause of failure in retinal detachment (RD) surgery [1], affecting 5% to 10% of RD and accounting for approximately 75% of all primary failures after RD surgery [1,2]. It is considered an abnormal wound-healing process induced by a retinal break allowing the posterior escape of retinal pigment epithelium cells (RPE) into a pro-inflammatory vitreous environment [3–7]. In any RD, the blood-ocular barrier breaks down, possibly due to disruption of the photoreceptor-RPE cell interface, then inflammatory cells are recruited increasing the inflammatory mediators production into the vitreous cavity [8–10]. Growth factors and cytokines in the vitreous cavity seem to be responsible for RPE migration, metaplasia and proliferation [11–13], which can result in the development of epi and subretinal membranes which are some of the characteristic clinical features of PVR. Tangential contraction of the membranes leads to reduced internal diameter of the retina and subsequent tension which rapidly develops into RD once a break allows ingress of subretinal fluid [14–16]. Some of those growth factors are also responsible of the glial cell hypertrophy causing important changes inside of the retinal tissue and inducing a shortening of the neuroretina, the most severe form of PVR [17].

Although PVR was identified in 1983 as an independent entity [18], and many efforts have been made for treating and preventing it during these years, there is neither current available medical treatment nor prophylaxis. Nowadays, treatment for PVR consisted of surgery, with an anatomically successful of 60% to 80% in the less severe cases and below of 40% in the most severe cases [19,20]. Surgical procedures have a significant cost [21], involve the risk of recurrence [22], and above all have poor functional results [23–26]. Besides the efforts to identify nonsurgical approaches to treat PVR, they have not had success [27–29].

Most research has to date attempted to identify the clinical risk factors related to the development of PVR after RD; however,

these clinical factors do not completely explain the probability of its onset [30]. Since PVR is a cell-based inflammatory response, like other inflammatory responses, genetic susceptibility may have an important role. In previous studies, we have partially described the contribution of the genetic component to PVR [31–34]. Single nucleotide polymorphisms (SNPs) have important implications for human genetic diseases and they may help to identify the genetic predisposition of certain diseases, either as a causative or protective factor.

Besides the role of many inflammatory mediators in the development of PVR and RD [35], previous studies have shown increased level of p53 and the activation of various cell death mechanisms after RD [36,37]. Also it has been reported that photoreceptor death after RD and subsequent visual loss could be caused by apoptosis or other cell death pathways, such as programmed necrosis, when the pathways for apoptosis were inhibited by some drugs [38–40].

In normal unstressed cells, p53 is a very unstable protein with a half-life ranging from 5 to 30 min, which is present at very low levels owing to continuous degradation largely mediated by murine double min 2 protein (MDM2) [41,42]. Importantly, MDM2 itself is the product of a p53-inducible gene [42]. Thus, the two molecules are linked to each other through an autoregulatory negative feedback loop aimed at maintaining low cellular p53 levels in the absence of stress and limiting the duration and severity of various p53-mediated biological responses after a non-lethal stress response. Conversely, a hallmark of many cellular stress pathways such as DNA damage, hypoxia, ischemia, telomere shortening, and oncogene activation is the rapid stabilization of p53 via a block of its degradation [41].

Recently, it has been reported that the levels of p53 expression could be a checkpoint in the development of RD and PVR, and how its local increase in the vitreous by using inhibitors of MDM2, seem to be a promising approach as a prophylaxis in experimental RD and also in experimental PVR [43].

The *MDM2* gene is a key negative regulator of p53 and in humans seems to have two promoter-enhancer regions that regulate the levels of *MDM2* mRNA. The first promoter is 5′ to the first exon and likely regulates the basal level of MDM2 in a nonstressed cell. The second promoter region is in the first intron and this region increases the expression of MDM2 after a p53 response [41,42]. This intron is composed of 524 nucleotides with the T>G SNP at nucleotide 309. The G/G variant increases the binding affinity of the transcriptional activator Sp1 resulting in high levels of MDM2 protein; formation of transcriptionally inactive p53-MDM2 complexes and a consequent decreased activity of the p53 pathway [44,45].

Thus, the purpose of this study was to analyze the distribution of the *MDM2* T309G polymorphism in the first intron of *MDM2* gene, in a consecutive sample of patients undergoing primary rhegmatogenous RD surgery with and without PVR, recruited from several European clinical centres through the project named Retina 4.

Materials and Methods

Ethics Statement

The study was approved by the local Ethics Committees of Instituto de Oftalmobiología (IOBA-Retina Group) (Valladolid, Spain), Hospital San João (Porto, Portugal), Moorfields Eye Hospital (London, United Kingdom) and Rotterdam Eye Hospital (Rotterdam, Netherlands) and followed the tenets of the Declaration of Helsinki. All patients received written informed consent before entering in the study.

Design and study population

DNA samples from the Retina 4 project were analyzed. This work is made up in two steps: first, a candidate gene association study in the T309G polymorphism (rs2279744) located into the *MDM2* gene was carried out. Second, the interaction between this polymorphism and the *p53* codon 72 polymorphism (rs10425229) was investigated.

Candidate gene association study

The association study was carried out among 555 patients from 7 centers: 3 in Spain, 2 in Portugal, 1 in the United Kingdom (UK) and 1 in Netherlands. The global sample was divided in sub-samples according to the country for the analysis. This study was carried out in two phases. In the first one, sub-samples from Spain and Portugal were analyzed. After significant results were found in this first cohort, subsequent samples from the UK and the Netherlands were analyzed (second phase). To compare if there were differences regarding geographical localization in the odds ratio analysis, Spain and Portugal were considered as Southern countries and the UK and the Netherlands as Northern countries. Genotypic and allelic frequencies were also compared between cases and controls in the global series.

Detailed explanation of the exclusion and inclusion criteria for classification of patients has been provided in a previous publication [33]. In brief, all participants were patients with a primary rhegmatogenous RD who underwent surgery. Exclusion criteria were: age under 16 years old; traumatic, tractional, exudative or iatrogenic RD; RD secondary to macular hole or giant retinal tears (larger than 3 clock hours) and pre-operative PVR grade higher than B. Those who did not develop clinical signs of PVR after 3 months of follow-up were included in the control group. Those who developed PVR grade C1 or higher, according to Machemer classification, were included as cases.

Genetic interaction

Interaction between the *MDM2* polymorphism (rs2279744) and one SNP (rs1042522) located in the *p53* gene previously identified by our group as significantly associated to PVR [33] was investigated. Samples used for analyzing *p53* polymorphism were same samples than for the *MDM2* SNP. Carriers of Pro variant of rs1042522 and G variant of rs2279744, were analyzed in the global sample and in the sub-samples from different countries.

Genotyping

Genotyping of the *MDM2* T309G polymorphism was performed at the Molecular Medicine Unit at the University of Salamanca, (Salamanca, Spain) blinded to the clinical status of patients, using the PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) technique [46,47].

The *MDM2* T309G polymorphism was detected after amplification of genomic DNA with the forward primer 5'-GAGGTCTCCGCGGGAGTTC-3' and the reverse primer 5'-CTGCCCACTGAACCGGC-3'. *MDM2* intron 1 was amplified within a 155 pb DNA fragment that was digested with the restriction endonuclease MspA1I (New England Biolabs, Inc.).

PCR reactions were performed in a 50-µl reaction mixture containing 100–200 ng of target DNA, 20 pmol of each primer, 2.5 mM $MgCl_2$, 50 µM of each dNTP and 1.25 U of HotMaster *Taq* DNA polymerase (5 Prime GmbH, Hamburg, Germany). DNA was amplified with the following steps: an initial 5-min denaturation at 94°C, followed by 35 cycles of 94°C for 30 s, 61°C for 30 s, 72°C for 30 s, and a final elongation at 72°C for 10 min.

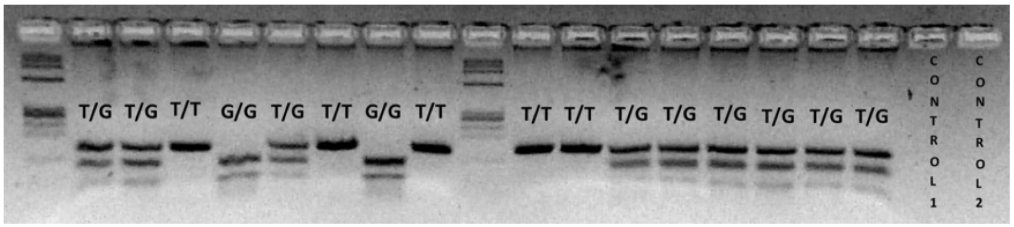

Figure 1. PCR-RFLP to determine *MDM2* SNP309 polymorphism. *MDM2* SNP309 T allele is not cleaved by MspA1I endonuclease and generates a single fragment of 155 bp. The *MDM2* SNP309 G allele is cleaved by MspA1I and generates two small fragments of 101 and 54 bp. The *MDM2* SNP309 heterozygote displays three fragments of 155, 101 and 54 bp.
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The resulting fragments were separated on 3.5% agarose gel (Figure 1) and the ethidium bromide–stained fragments were analyzed under a UV source, using the Kodak Digital Science ID image analysis system.

The polymerase chain reaction fragments, containing T and G alleles, after digestion migrated as 2 fragments of 101 and 54 bp for G homozygotes (GG), 1 fragments of 155 for T homozygotes (TT), and 3 fragments of 157, 101 and 54 bp for heterozygotes (TG).

Statistical analysis

The quality of data was evaluated in control sub-samples by Hardy-Weinberg equilibrium using the Chi-square test. Genotypic frequencies were estimated in each sub-sample. The proportions of genotypes and the G homozygote groups between sub-samples were analyzed. Also, the genotypic and allelic frequencies were compared between cases and control in the global sample and in the sub-samples from different countries. In the genetic interaction, patients carrying the Pro variant of rs1042522 and the G variant of rs2279744 were analyzed in the global sample and in the sub-samples from different countries.

Association was investigated using the Chi-Square and the Fisher's tests. The strength of association was measured using Odds Ratio (OR) and 95% confidence intervals (CI). Two inheritance models were considered: co-dominant model that allows every genotype to give a different and non-additive risk, and recessive model in which two copies of the G allele are necessary to change the risk. The Akaike Information Criterion (AIC) [48] was used in order to choose the inheritance model that best fitted the data. The statistical analyses were performed by using SPSS 16.0 for Macintosh and R software (Software Foundation's GNU project) [49].

In order to adjust p-values for multiple comparisons a permutation test was performed. We used 1000 random shuffles of the case/control labels to get the correct distribution of test statistics under the no-association hypothesis. The ranking of the real test statistic among the shuffled test statistics gives the adjusted p-values.

Results

Candidate gene association study

A total of 555 peripheral DNA blood samples including 134 cases and 421 controls were analyzed (203 from Spain (36.57%), 68 from Portugal (12.25%), 121 from Netherlands (21.80%) and 163 from the UK (29.36%). Regarding clinical information some significant associations were observed. The control group was significantly older than cases ($p<0.0001$) with the difference between median of 6 years (95% CI: 3.39-8.31). A significant association in patients with history of PVR in the fellow eye was found in the cases group. Also status of the lens was determined because aphakia has been related to a higher incidence of PVR [5,30] (Table 1). There were no significant associations with sex, race, affected eye or history of phakic status. There were no differences regarding the geographical localization or centre where the patients came from.

There were no failures for the genotyping process, with a global call rate of 96.21%. Additionally, in order to ensure accuracy of allele-specific results, a randomized selection of samples PCRs were assessed by an independent researcher unaware of the patients' status. All control sub-samples verified the Hardy-Weinberg equilibrium.

Phase I: genotypic distribution of *MDM2* T309G polymorphism in Spain and Portugal.

The frequencies of the genotypes in each country are shown in Table 2. The comparison of proportions of genotypes between sub-samples showed a significant difference ($p<0.05$) between cases and controls. Also a significant difference ($p<0.05$) in G homozygote carriers between sub-samples in the control group (CI G homozygote: Spain (22.6–32.9), Portugal (21.4–38.9)) and the cases group (CI G homozygote: Spain (35.1–53.0), Portugal (39.0–74.4)) was found.

The odds ratio of G carriers from Spain and Portugal together considering a co-dominant model (T/T, T/G and G/G) (AIC = 307.7 vs 311.4 of a recessive model) was 5.4 (95% CI: 2.3 to 12.7) (Table 3).

Phase II: genotypic distribution of *MDM2* T309G polymorphism in UK and the Netherlands.

The frequencies of the genotypes in patients from the UK and the Netherlands are shown in Table 1. Also, the distribution of genotypes between subjects from those countries showed statistical differences. When G homozygote carriers between cases and controls were analyzed a significant difference in both groups of patients was found in control (CI of G homozygote: Netherlands (25.3–38.8), UK (23.3–34.2)) and cases (CI of G homozygote: Netherlands (40.6–66.2), UK (25.3–38.8)). Also, differences were found in the odds ratio distribution of homozygous carriers of the G variant in patients from the UK and the Netherlands together considering a co-dominant model (AIC = 277.8 vs 288.4 of a recessive model) (odds ratio 7.3 (95% CI: 2.8 to 19.1) (Table 3).

When all samples were grouped, (Table 4) significant differences in the distribution of genotypes between the controls and cases ($p<0.05$) were found. Also homozygous carriers of the G variant were more frequent in PVR cases (CI: 42.9–54.8) than in controls (CI: 26.2–32.4). The odds ratio of the G variant in the global sample using a co-dominat model (AIC = 584.1 versus 598.7 of a recessive model) was 5.9 (CI: 3.2 to 11.2) (Table 3).

Table 1. Clinical characteristics of the whole sample.

Characteristics	Controls, n RD (%)	Cases, n PVR (%)	Total	P-value*
Race				0,064
-Caucasian	370 (73.56%)	133 (26.44%)	503	
-Hispano-American	6 (46.15%)	7 (53.85%)	13	
-Hindu	7 (58.33%)	5 (41.67%)	12	
-Arabic-North-African	6 (100%)	0	6	
-Sub-Saharan	2 (50%)	2 (50%)	4	
-Asian	3 (60%)	2 (40%)	5	
-Unknown			12	
Sex				0,362
-Male	248 (71.26%)	100 (25.37%)	348	
-Female	135 (75%)	45 (31.42%)	180	
-Unknown			27	
Status of the lens (Phakia)				0,233
-Yes	250 (74.63%)	85 (25.37%)	335	
-No	131 (68.58%)	59 (31.42%)	190	
-Unknown			30	
RD in fellow eye				0,528
-Yes	32 (76.19%)	10 (23.81%)	42	
-No	355 (68.58%)	136 (31.42%)	491	
-Unknown			22	
PVR in fellow eye				0,005(*)
-Yes	0	5 (100%)	5	
-No	141 (26.70%)	387 (73.30%)	528	
-Unknown			22	
Geographical location				0,171
-Northern countries (UK+Netherlands)	224 (78.87%)	60 (22.2%)	284	
-Southern countries (Spain+Portugal)	197 (72.7%)	74 (27.3%)	271	

RD: Retinal detachment; PVR: Proliferative vitreoretinopathy.
*p-value: Chi-squared or Fishers exact test in the statistical analysis of clinical characteristics of the whole sample of the global sample. **(*)** A significant association in patients with history of PVR in the fellow eye was found in the cases group.
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Allelic frequencies comparison

Significant differences in the analysis of the allelic frequencies were found between cases and controls in Spain and Portugal and between cases and controls in UK and Netherlands (Table 2)

Genetic interaction

When the global sample was analyzed, 16 patients (2.91%) who meet both *p53* and *MDM2* genotypes (Pro variant plus G variant carriers) (8 patients from Spain and Portugal, and 8 patients from the UK and the Netherlands) were found. A significant difference in the distribution of genotypes between the controls and cases ($p<0.0001$) was found in the global sample. The odds ratio of Pro and G carriers in global sample was 10.19 (95% CI: 3.2 to 31.9) (Table 5).

Significant differences in the distribution of genotypes between the controls and cases from Spain and Portugal together ($p = 0.0006$) and UK and Netherlands together ($p<0.01$) were found. The odds ratio of Pro and G carriers from the UK and the Netherlands together was 6.5 (95% CI: 1.5 to 28.2), whereas, the odds ratio of Pro and G carriers from Spain and Portugal together was 20.4 (95% CI: 2.4 to 169.5) (Table 5).

Discussion

Our results show that Spanish and Portuguese carriers of the homozygous G SNP at position 309 have a 5.4 -fold increased risk of PVR after RD than those that carry the T allele. This observation was confirmed also in Dutch and British population (odds ratio 7.3 (95% CI: 2.8 to 19.1). Interestingly, when Pro and G restriction site carriers in the global sample were analyzed, an additional effect (odds ratio 10.19 (95% CI: 3.2 to 31.9) was found. But both polymorphisms were only present in 16 patients out of 555 patients. Although significant results were also found in the latter analysis, we consider that 16 may be too small a sample to draw absolute conclusions and further studies may be necessary in order to know the absolute potential risk of this restriction site association.

PVR is considered a multifactorial disease [31,32] and may be the result of interaction between genetic and environmental factors [30–32]. The difficulty in identifying of patients at risk of developing PVR after RD by clinical characteristics [30] justifies our efforts to elucidate any genetic components [30–32]. A simple genetic test might identify higher risk patients before RD surgery, which might then be modified in a more personalized form.

Table 2. Distribution of genotypes and allelic frequencies in cases with PVR and controls.

Countries	Genotype	Controls	Cases	P-value Fishers test	Corrected P-value	Alleles	Controls	Cases	Controls (95% CI Alleles)	Cases (95% CI Alleles)	P-value Chi Square test	OR*	CI OR 95%
Spain	T/T	72 (50.0%)	18 (30.5%)	0.0037[1]	0,0039[2]	G	80 (27.8%)	52 (44.0%)	(22.6–32.9)	(35.1–53.0)	0.0012[3]	2.0	(1.31–3.19)
	T/G	64 (44.4%)	30 (50.9%)			T	208 (72.2%)	66 (56.0%)	(67.0–77.4)	(46.9–64.9)			
	G/G	8 (5.6%)	11 (18.6%)										
Portugal	T/T	27 (51.0%)	3 (20.0%)	0.0387[1]	0,0449[2]	G	32 (30.2%)	17 (56.7%)	(21.4–38.9)	(39.0–74.4)	0.0156[3]	3.0	(1.31–6.95)
	T/G	20 (37.7%)	7 (46.7%)			T	74 (69.8%)	13 (43.3%)	(61.7–78.5)	(61.0–25.6)			
	G/G	6 (11.3%)	5 (33.3%)										
United Kingdom	T/T	66 (50.0%)	7 (22.6%)	0.0047[1]	0,0059[2]	G	76 (28.8%)	31 (50.0%)	(23.3–34.2)	(37.5–62.5)	0.0015[3]	2.4	(1.40–4.35)
	T/G	56 (42.4%)	17 (54.8%)			T	188 (71.2%)	31 (50.0%)	(65.7–76.6)	(37.5–62.5)			
	G/G	10 (7.6%)	7 (22.6%)										
Netherlands	T/T	40 (43.5%)	4 (13.8%)	0.0037[1]	0,0049[2]	G	59 (32.0%)	31 (53.5%)	(25.3–38.8)	(40.6–66.2)	0.0023[3]	2.4	(1.27–464)
	T/G	45 (48.9%)	19 (65.5%)			T	125 (68.0%)	27 (46.5%)	(61.1–74.8)	(46.5–59.3)			
	G/G	7 (7.6%)	6 (20.7%)										

[1]Fishers test. Ho. Independence between genotype case/control group. Significant differences were observed between cases and controls in the G/G genotype in Spain and Portugal, and in UK and Netherlands.
[2]Permutation test for multiple comparison adjustment.
[3]G homozygote carrier analysis between different countries revealed differences in Spain and Portugal as well as in Netherlands and UK.
*OR : odds-ratio.
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Table 3. Models of inheritance in the global sample.

Model	Genotype	Controls	Cases	OR*	CI 95%	P-value	AIC*	Corrected P-value
Co-dominant	T/T	205 (48.7%)	32 (24.0%)	1.00		2.0374e-08	584.1	0,0009[1]
	T/G	185 (43.9%)	73 (54.4%)	2.53	(1.59–4.01)			
	G/G	31 (7.4%)	29 (21.6%)	5.99	(3.20–11.24)			
Dominant	T/T	205 (48.7%)	32 (24.0%)	1.00	-	2.1790e-07	590.7	0,0009[1]
	T/G-G/G	216 (51.3%)	102 (76.0%)	3.03	(1.95–4.70)			
Recessive	T/T-T/G	390 (92.6%)	105 (78.4%)	1.00	-	1.4218e-05	598.7	0,0009[1]
	G/G	31 (7.4%)	29 (21.6%)	3.47	(2.00–6,02)			
Over-dominant	T/T-G/G	236 (56.1%)	61 (45.6%)	1.00	-	3.3371e-02	613.0	0,0319[1]
	T/G	185 (43.9%)	73 (54.4%)	1.53	(1.03–2.26)			
Spain+Portugal	T/T	99 (50.3%)	21 (28.4%)	1.00	-	0.0003	307.7	0,0019[1]
	T/G	84 (42.6%)	37 (50.0%)	2.08	(1.13–3.82)			
	G/G	14 (7.1%)	16 (21.6%)	5.44	(2.30–12.7)			
Netherlands+UK	T/T	106 (47.3%)	11 (18.3%)	1.00	-	2.6566e-05	277.8	0,0009[1]
	T/G	101 (45.1%)	36 (60.0%)	3.43	(1.66–7.11)			
	G/G	17 (7.6%)	13 (21.7%)	7.30	(2.80–19.01)			

OR* (odds ratio).
AIC*(Akaike Information Criterion); The AIC is a measure of the relative goodness of fit of a statistical model. It can generally be used for the identification of an optimum model in a class of competing models.
Given a set of candidate models for the data, the preferred model is the one with the minimum AIC value.
[1]Permutation test for multiple comparison adjustment.
Results of odds ratio using a co-dominant model for Spain plus Portugal and Netherlands plus United Kingdom (UK).
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Apoptosis is critically important during various developmental processes, it is necessary to rid the body of pathogen-invaded cells and also is involved in the removal of inflammatory cells and the evolution of granulation tissue into scar tissue [50]. Moreover, inappropriate apoptosis is an important factor in many human pathologic conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer. In addition, it has been reported that a deregulation of apoptosis during wound healing can lead to pathologic forms of healing such as excessive scarring and fibrosis [50].

As mentioned, PVR is considered an abnormal wound-healing process induced by the production of a retinal break and vitreal escape of RPE cells into an appropriate intraocular pro-inflammatory environment [3–7]. It is characterized by several intraretinal and extraretinal changes. One of the commonest is the extracellular matrix (ECM) formation, produced by activated-cytokines RPE cells (RPE cells transdifferentiate into mesenchymal like α-smooth muscle actin cells) in the vitreous cavity [51,52]. This series of events culminates in the formation of a retina-associated membrane over and/or behind the neuroretina, which further contracts and thereby causes recurrent and tractional RD. Nevertheless the most severe changes are induced inside the retina by stimulating a reactive gliosis that causes a shortening of the retina preventing its surgical reattachment [17]. Some of these activating cytokines like transforming growth factor-β (TGFβ), and tumor necrosis factor α (TNFα), have been previously reported by our group in association with PVR [30–32].

Current studies have highlighted the involvement of extrinsic and intrinsic pathways of apoptosis in retinal cells after RD, and also, the existence of other pathways, such as programmed necrosis, when apoptosis are inhibited [40]. In addition, a proteomic study of human vitreous samples of RD and PVR has indicated that p53 could be involved in PVR process [53].

An association between *p53* Arg72Pro polymorphism and PVR has been recently reported by our group [33]. According to our results, carriers of the Pro allele of the *p53* gene, which are

Table 4. Distribution of *MDM2* T309G polymorphism in the whole sample.

Genotypes	T/T	T/G	G/G	Total	CI 95%*	P-value Fishers test	Corrected P-value	OR[2]	CI OR 95%[2]
Cases	32 (23.9%)	73 (54.5%)	29 (21.6%)*	134 (100%)	(42.9–54.8)*	1.6738e-08	0.0009[1]	2.3	(1.73–3.05)[2]
Controls	205 (48.7%)	185 (43.9%)	31 (7.4%)	421 (100%)	(26.2–32.4)*			-	-
Total	237	258	60	555					

1. Permutation test for multiple comparison adjustment.
2. OR. (Odds ratio).
*Analysis of G homozygote carriers between case/control group.
Fishers test.
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Table 5. Genetic interaction between *p53* Pro72Arg SNP (rs1042522) and *MDM2* T309G SNP (rs2279744) in the global sample and in sub-samples from different countries.

Genotype	Sample	Controls	Cases	P-value	Corrected P-value	OR*	95% CI OR
Pro/Pro + G/G	Global	4	12	2.1139e-05	0.0009 [1]	10.19	(3.2–31.9)
	Spain+Portugal	1	7	0.0005	0,0009[1]	20.4	(2.4–169.5)
	Netherlands+UK	3	5	0.0130	0,0169[1]	6.5	(1.5–28.2)

[1]Permutation test for multiple comparison adjustment.
OR*: odds ratio.
Results of patients (2.91%), who meet with both genotypes (Pro variant plus G variant carriers).
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associated with a decrease in apoptotic function of p53, have higher risk of PVR after RD. Furthermore it has been reported that suppression of p53 expression might be a necessary event in the development of RD and PVR, and maintaining levels of p53 with agents such as Nutlin-3, which prevents the interaction between p53 and MDM2, might be effective in the prophylaxis of RD and also PVR vitreous-induced contraction [43].

The *MDM2* T309G polymorphism affects binding of transcription factor Sp1 and is associated with an increased expression of the MDM2 mRNA and attenuation of the p53 pathway [41,42,46,47]. Interestingly, the elevated levels of MDM2 do not reduce the levels of p53 in non-stressed cells and the blockage of p53 binding to MDM2, using inhibitors of MDM2, promotes apoptosis [54].

Also, it has been reported that in contrast to other retinal cell types, the RPE cells are resistant to apoptosis, to TNFA and oxidative stress, which trigger apoptosis in wide range of cells [55,56]. These factors, at normal range are generally ineffective in this cell type, however, it has also been reported that the use of inhibitors of MDM2 produces an increase of expression of proapoptotic targets capable of overcoming this inherent RPE resistance to apoptosis [54].

The *MDM2* T309G polymorphism has been associated to several cancers [44–47] and also to inflammatory processes [57]. Moreover, the association with other polymorphisms of *p53* (i.e. *p53* codon 72 polymorphism) increases the risk of development of several tumors [45,46].

Results of this work suggest that carriers of the G allele of the *MDM2* gene, associated with a decrease in apoptotic function of p53, have higher risk of PVR after RD. We can speculate that the reduction in the levels of apoptosis in retinal cells may activates other cell death pathways, like programmed necrosis, which would increase the intraocular inflammation after RD, thus generating a cascade of tissue responses that generate and amplify the hostile microenvironment in which activated RPE can trans-differentiate. It is also possible that the decline in the levels of p53 is a crucial checkpoint in RD and PVR, and the recently developed ocular formulation of Nutlin-3, which can be administered by sub-conjunctival injection [58], could be an effective approach to achieve a prophylaxis in PVR disease.

This study had some limitations. One important issue in an association study is the sample size [59]. Probably, unlike other association studies, our sample is too small and the power sample is not enough to draw absolute conclusions, nevertheless, the sample collection to achieve greater power would be an extremely challenging for a low prevalence condition such as PVR. Cases were younger than controls and this could be considered a confounding factor. However, this difference was only of 6 year, which makes unlikely that this responds to a certain genetic profile. It is important to point out that functional polymorphisms are considered of interest because they allow us to shed light on the molecular basis of different pathologies. They also could be targets in the development of new therapeutic strategies. In this case-controlled study, we have identified one SNP within the *MDM2* gene that shows a strong association with PVR across several groups. Although we have carried out the study in two phases these findings must be interpreted with caution until these results are confirmed with further replication studies in order to confirm its association, because one of the major pitfalls of genetic association studies are the false positives [59,60].

In summary, this study indicates that the p53 pathway could be implicated as a significant risk factor for PVR after RD and also it highlights the role of these SNPs (rs1042522 and rs2279744) as possible markers of PVR risk.

Acknowledgments

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Conceived and designed the experiments: SPI IRH JR RGS JCP. Performed the experiments: SPI IRH. Analyzed the data: SPI IF RGS JCP. Contributed reagents/materials/analysis tools: RGS JCP. Wrote the paper: SPI JR RGS JCP. Sample collection and updating patient information: MTGG JMRM ARS YR SH REM DC JCV.

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Chapter 4

BAX and BCL-2 polymorphisms, as predictors of proliferative vitreoretinopathy development in patients suffering retinal detachment
The Retina 4 Project

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BAX and BCL-2 polymorphisms, as predictors of proliferative vitreoretinopathy development in patients suffering retinal detachment: the Retina 4 project

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ABSTRACT.

Purpose: **To compare the distribution of BCL-2 -938C>A (rs2279115) and BAX -248G>A (rs4645878) genotypes among European subjects undergoing rhegmatogenous retinal detachment (RRD) surgery in relation to the further development of proliferative vitreoretinopathy (PVR).**

Methods: **A case–control gene association study, as a part of Retina 4 project, was designed. rs2279115 and rs4645878 polymorphisms were analysed in 555 samples from patients with RRD (134 with PVR secondary to surgery). Proportions of genotypes and AA homozygous groups of BCL-2 and BAX polymorphisms between subsamples were analysed in two phases. Genotypic and allelic frequencies were compared in global sample and in subsamples.**

Results: **BAX: Differences were observed in the genotype frequencies and in AA carriers between controls and cases in the global series. The odds ratio (OR) of A carriers in the global sample was 1.7 (95% CI: 1.23–2.51). Proportions of genotypes in Spain + Portugal were significant different. The OR of A carriers from Spain and Portugal was 1.8 (95% CI: 1.11–2.95). BCL-2: No significant differences were observed in genotype frequencies. However, proportions of genotypes in Spain + Portugal were significant. A protective effect (OR: 0.6 95% CI: 0.43–0.96) was found in A carriers from Spain and Portugal.**

Conclusions: **Results suggest that A allele of rs4645878 could be a biomarker of high risk of developing PVR in patients undergoing RD surgery. The possible role of BCL-2 (inhibitor of necroptosis pathway) as a possible new target in PVR prophylaxis should be investigated.**

Key words: apoptosis – bax – Bcl-2 – necroptosis – proliferative vitreoretinopathy – retinal detachment

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Introduction

Proliferative vitreoretinopathy (PVR) is still the major cause of failure in retinal detachment (RD) surgery (Machemer et al. 1991), affecting 5–10% of RD and accounting for approximately 75% of all primary failures after RD surgery (Machemer et al. 1991; de la Rua et al. 2008).

PVR is a complex process, involving not only ischaemic tissue damage but also inflammation and proliferation of several types of intraretinal cells. Currently, it is considered a complex disease (Sanabria Ruiz-Colmenares et al. 2006; Rojas et al. 2010, 2013; Pastor-Idoate et al. 2013a,b), in which there is an interaction between environmental factors (clinical variables) and the genetic profile of each subject (Brennan 2002; Hinton et al. 2002). Despite the facts that the exact mechanisms responsible of PVR are not completely understood, it is widely accepted that inflammation plays a crucial role in its pathogenesis (Delyfer et al. 2011).

PVR is characterized not only by uncontrolled cell proliferation and migration into the retinal surface, the subretinal space and vitreous cavity but also by deep changes inside of the

retinal tissue with the disappearance of neurons and a reactive gliosis by Müller cells and astrocytes (Pastor et al. 2006). The RPE cell is thought to be one of the key cell types in this disease. And factors responsible for unwanted survival, migration and proliferation of RPE cells in this condition have not been clearly defined.

Over the recent years, some papers have highlighted that apoptosis and other cell death pathways, such as programmed necrosis, play an important role in the photoreceptor degeneration and subsequent visual loss (Arroyo et al. 2005; Trichonas et al. 2010; Lo et al. 2011; Murakami et al. 2011; Ricker et al. 2011a) and also in the development of PVR after RD (Charteris et al. 2007). It has been reported that levels of p53 (one important regulatory factor of apoptosis) expression could be a checkpoint in the development of RD and PVR. And how preventing decline in the level of p53 using inhibitors of mdm2 could be a promising approach as a prophylaxis in experimental RD and also in experimental PVR (Lei et al. 2012).

There are other mediators of apoptosis that are also involved in retinal cell death after retinal ischaemia, including endonucleases (Rosenbaum et al. 1997), caspases (Singh et al. 2001) and B-cell lymphoma 2 (Bcl-2) family (Kaneda et al. 1999; Hahn et al. 2003; Yang et al. 2004; Zhang et al. 2002), whose role in PVR development has not yet been completely studied.

The Bcl-2 family is divided into two classes of members that exert opposed effects on cell death. Anti-apoptotic members such as Bcl-2 and Bcl-x_L and pro-apoptotic members, such as Bax and Bak (Adams & Cory 2001). When Bax and/or Bak are activated, they trigger mitochondrial outer membrane permeabilization by a mechanism that has yet to be identified. This leads to the release of Cytochrome C and apoptotic regulatory proteins into the cytoplasm resulting in the activation of the executioner caspases. In contrast, Bcl-2 and/or Bcl-x_L block this process and thus inhibit programmed cell death. The pro- and anti-apoptotic members of Bcl-2 family can neutralize each other by heterodimerization or forming homodimers, but it remains unclear which complex of these serves as the functional moiety in regulating apoptosis (Knudson & Korsmeyer 1997).

Additionally, Bcl-2-related gene products have been shown to be critically involved in numerous central nervous system diseases and degeneration (Hetts 1998) and also in developmental and pathological retinal cell death processes (Mosinger Ogilvie et al. 1998).

After a RD, the outer retina layers can suffer from ischaemia. It has been reported that retinal ischaemia is one of the triggers to induce the expression of Bcl-2 proteins, but also is the responsible factor for the upregulation of other mediators such as p53 (Hinton et al. 2002) or tumour necrosis factor alpha (TNFA) (Campochiaro et al. 1996; El-Ghrably et al. 2001; Banerjee et al. 2007). And it is likely that Bcl-2 family could be also involved in RD-associated photoreceptor cell loss and may be in the development of PVR. This later idea has never been investigated. Bcl-2 and Bax not only are related with p53, as both are transcriptional targets for p53 protein, but also rs2279115 and rs4645878 in the *BCL-2* and *BAX* genes, respectively, are associated with a decrease in the apoptosis levels, being both involved in the control life or death of a cell, and in the cellular proliferative response.

Previous studies have highlighted the possible role of apoptosis in the PVR development (Charteris et al. 2007), and recent studies performed suggest that a deregulation in the apoptosis pathway could be one of the possible mechanisms in the pathogenesis of PVR (Pastor-Idoate et al. 2013a,b).

Thus, the purpose of this study has been to analyse the distribution of these 2 promoter polymorphisms (rs2279115 and rs4645878) in the *BCL-2* and *BAX* genes, respectively, in a sample of patients undergoing primary rhegmatogenous RD surgery, with and without postoperative PVR, recruited from several European clinical centres through the project named Retina 4.

Materials and Methods

Candidate gene association study

DNA samples from the Retina 4 project were analysed. The study was approved by the institutional research committee of each centre and followed the tenets of the Declaration of Helsinki. All patients gave a written informed consent before entering in the study.

Design and study population

The association studies were carried out among 555 patients from seven centres: 3 in Spain, 2 in Portugal, 1 in the United Kingdom (UK) and 1 in the Netherlands. The global sample was divided in subsamples, according to the country, for the analysis. This study was carried out in two phases. In the first one, subsamples from Spain and Portugal were analysed. After significant results were found in this first cohort, subsequent samples from the UK and the Netherlands were analysed (second phase). To compare if there were differences in the odds ratio with respect to geographical localization, Spain and Portugal were considered as southern countries and the UK and the Netherlands as northern countries. Genotypic and allelic frequencies were also compared between cases and controls in the global series.

Detailed explanation of the exclusion and inclusion criteria for classification of patients has been provided in previous publications (Pastor-Idoate et al. 2013a,b; Rojas et al. 2013). In brief, all participants were patients with a primary rhegmatogenous RD who underwent surgery (pars plana vitrectomy). Exclusion criteria were as follows: age under 16 years; traumatic, tractional, exudative or iatrogenic RD; RD secondary to macular hole or giant retinal tears (larger than 3 clock hours) and preoperative PVR grade higher than B. Those who did not develop clinical signs of PVR after 3 months of follow-up were included in the control group. Those who developed PVR grade C1 or higher, according to Machemer classification (Machemer et al. 1991), were included as cases.

Genotyping

BCL-2 -938C>A (rs2279115) and BAX -248G>A (rs4645878) polymorphisms were assessed at the Molecular Medicine Unit, at the University of Salamanca, (Salamanca, Spain) blinded to the clinical status of patients, by TaqMan 5′-exonuclease allelic discrimination assays (Applied Biosystems, Foster City, CA, USA) using a

Step-One Plus Real-time PCR system according to the manufacturer protocol (Applied Biosystems). Briefly, PCR was carried out with mixes of 15 ng of genomic DNA, 5 μl of TaqMan®SNP genotyping Mastermix (Applied Biosystems) and 0.25 μl of TaqMan® SNP Genotyping Assay (SNP ID C_3044428 for rs2279115 BCL-2 polymorphism and SNP ID C_27848291 for rs4645878 BAX polymorphism, Applied Biosystems) in a final volume of 10 μl. PCR conditions were 95°C for 10 min followed by 40 cycles at 95°C for 15 seconds, and 60°C for 1 min and finally 60°C for 1 min. For quality control purposes, each sample was processed by duplicate for each SNP.

Statistical analysis

Genotypes of the SNPs were analysed in the subsamples and in the global sample separately. Also, the characteristics of the patients were explored. The quality of data was evaluated in control subsamples by Hardy–Weinberg equilibrium using the chi-square test. Genotypic frequencies were estimated in each subsample for each SNP. The proportions of genotypes and the AA homozygous group of BCL-2 and AA homozygous group of the BAX polymorphisms between subsamples were analysed for each SNP. Also, the genotypic and allelic frequencies were compared between cases and controls in the global sample and in the subsamples for each SNP.

Association was assessed using the chi-Square and the Fisher's tests. The strength of association was measured using odds ratio (OR) and 95% confidence intervals (CIs).

Two inheritance models were considered in the BAX analysis: dominant model, in which the heterozygous (GA) and homozygous (AA) genotypes have the similar risk, as a single copy of A is sufficient to alter the risk. Hence, these two possible genotype G/A+A/A together in combination is compared to the homozygous G/G. And the additive model, in which the risk conferred by an allele is increased r-fold for heterozygotes (G/A) and 2r-fold for homozygous (A/A). In this model, each copy of A allele alters the risk in an additive form.

Dominant and overdominant models were considered in the BCL-2 analysis. In the dominant model, heterozygous (CA) and homozygous (AA) genotypes have similar risk, because a single copy of A is sufficient to alter the risk. Hence, these two possible genotypes C/A+A/A together in combination were compared to the homozygous C/C. In the overdominant model, heterozygous (CA) was compared to a pool of both allele homozygous (AA and CC). The C/A was compared with A/A+C/C).

The Akaike information criterion (AIC) was used to choose the inheritance model that best fitted the data. The statistical analyses were performed using SPSS 16.0 (IBM Inc, Armonk, NY, USA) for Macintosh and R software (Software Foundation's GNU project).

All the statistical analysis has been made by Itziar Fernández (Sct, PhD) from the statistical unit of IOBA, the Eye Institute of the University of Valladolid, Valladolid, Spain.

Results

Candidate gene association study

A total of 555 peripheral DNA blood samples including 134 cases and 421 controls were analysed, 203 from Spain (36.57%), 68 from Portugal (12.25%), 121 from the Netherlands (21.80%) and 163 from the UK (29.36%).

Regarding clinical information, some significant associations were observed as follows: control group was significantly older than cases ($p < 0.0001$) with a difference between median of 6 years (95% CI: 3.39–8.31). A significant association in patients with history of PVR in the fellow eye was also found in cases. The status of the lens was determined because aphakia has been related to a higher incidence of developing PVR after RD (Pastor 1998; Ricker et al. 2012) (Table 1). There were no significant associations with sex, race, affected eye or history of cataract surgery. There were no differences regarding the geographical localization.

There were no relevant failures for the genotyping process, with a global call rate of 96.21% for the BCL-2 and 97.13% for the BAX. Additionally, to ensure accuracy of allele-specific results, a randomized selection of PCR samples was assessed by an independent researcher unaware of the condition of the patient. All control subsamples verified the Hardy–Weinberg equilibrium.

Phase I

Genotypic distribution of rs2279115 and rs4645878 polymorphisms in Spain and Portugal.

1 rs2279115-BCL-2 polymorphism

The frequencies of the genotypes in each country for this polymorphism are shown in Table 2A. The comparison of proportions of genotypes between subsamples showed no significant differences ($p > 0.05$) between cases and controls in Spain and Portugal. Also, no significant differences in AA homozygous carriers between subsamples in controls (CI AA homozygous: Spain [34.9–46.5], Portugal [45.7–65.2]) and cases in groups (CI AA homozygous: Spain [24.0–41.5], Portugal [25.9–62.3]) were found.

Regarding geographical localization, a significant difference ($p < 0.05$) in the comparison of proportions of genotypes between cases and controls was found in southern countries. Also, a significant difference in AA homozygous carriers between cases and controls was found in southern countries. Control group (CI AA homozygous: Spain plus Portugal [39.7–49.7]) and the case group (CI AA homozygous: Spain plus Portugal [26.9–42.7]).

The OR of A carriers from Spain and Portugal together considering a dominant model (A/A, C/A and C/C) (AIC = 315.4 versus 317.3 of a codominant model) was 0.50 (95% CI: 0.29–0.86) (Table 3).

2 rs4645878-BAX polymorphism

The frequencies of the genotypes in each country for this SNP are shown in Table 2B. The comparison of proportions of genotypes between subsamples did not show significant differences ($p > 0.05$) between cases and controls neither in Spain nor in Portugal. Also, no significant differences in AA homozygous carriers between subsamples in the control group (CI AA homozygous: Spain [10.0–18.1]) and the case group (CI AA homozygous: Spain [15.4–30.8) were found. However, regarding geographical comparison, a significant difference ($p < 0.05$) in the proportions of genotypes between cases and controls was found in southern countries. Also, a significant difference in AA homozygous carriers between cases and controls was found in southern countries. Control group (CI AA homozygous: Spain plus Portugal [10.1–17.0]) and the case group (CI AA homozygous: Spain plus

Table 1. Clinical characteristics of the whole sample.

		Controls		Cases						
Characteristics		*n*	%	*n*	%	Total	% Total	p-Value	OR	CI 95% OR
Race	Unknown	27	4.86%	2	0.36%	29	5.23%	0.2368	0.6	0.314–1.336
	Caucasian	370	66.67%	120	21.62%	490	88.29%		2.5	0.740–5.998
	Hispano-American	6	1.08%	5	0.90%	11	1.98%		1.7	0.599–6.145
	Hindu	7	1.26%	4	0.72%	11	1.98%		0.2	0.012–4.031
	Arabic-North-African	6	1.08%	0	0%	6	1.08%		1.4	0.134–16.63
	Sub-Saharan	2	0.36%	1	0.18%	3	0.54%		2.0	0.331–12.13
	Asian	3	0.54%	2	0.36%	5	0.90%			
	Total	421	75.86%	134	24.14%	555	100%			
Sex	Unknown	20	3.60%	7	1.26%	27	4.87%	0.4866	1.1	0.760–1.777
	Male	258	46.49%	86	15.50%	344	61.98%			
	Female	143	25.77%	41	7.39%	184	33.15%			
	Total	421	75.86%	134	24.14%	555	100%			
Status of the Lens (Phakia)	Unknown	23	4.14%	7	1.26%	30	5.41%	0.2419	1.2	0.846–1.937
	Yes	267	48.11%	78	14.05%	345	62.16%			
	No	131	23.60%	49	8.83%	180	32.43%			
	Total	421	75.86%	134	24.14%	555	100%			
RD in fellow eye	Unknown	17	3.06%	5	0.90%	22	3.96%	0.9506	0.9	0.466–2.046
	Yes	32	5.77%	10	1.80%	42	7.57%			
	No	372	67.03%	119	21.44%	491	88.47%			
	Total	421	75.86%	134	24.14%	555	100%			
PVR in fellow eye	Yes	0	0%	5	0.90%	5	0.90%	0.0157	35	1.96–651.86
	No	4211	75.86%	129	23.24%	550	99.10%			
	Total	421	75.86%	134	24.14%	555	100%			
Geographical location	Southern	197	35.50%	74	13.33%	271	48.83%	0.0926	1.4	0.948–2.072
	Northern	224	40.36%	60	10.81%	284	51.17%			
	Total	421	75.86%	134	24.14%	555	100%			

OR = odds ratio, RD = retinal detachment, PVR = proliferative vitreoretinopathy.

Portugal [15.4–29.2]).
The OR of A carriers from Spain and Portugal together considering an additive model (A/A double risk than G/A) (AIC = 316.6 versus 316.7 of a dominant model) was 1.75 (95% CI: 1.09–2.83) (Table 3).

Phase II
Genotypic distribution of rs2279115 and rs4645878 polymorphisms in the UK and the Netherlands.
1 rs2279115-BCL-2 polymorphism
The frequencies of the genotypes in each country for this SNP are shown in Table 2A. The comparison of proportions of genotypes between subsamples did not show significant differences ($p > 0.05$) between cases and controls neither in the UK nor in the Netherlands. Also, no significant differences in AA homozygous carriers between subsamples in the control group (CI AA homozygous: UK [34.9–47.1], Netherlands [28.0–42.1]) and the case group (CI AA homozygous: UK [26.8–51.9], Netherlands [27.3–53.3]) were found. Regarding geographical comparison, no significant differences in the proportions of genotypes and in AA homozygous carriers analysis between cases and controls were found.
The OR of A carriers from the UK and the Netherlands together considering a dominant model (A/A, C/A and C/C) (AIC = 296.8 versus 298.4 of a codominant model) was 0.95 (95% CI: 0.53–1.68) (Table 3).
2 rs4645878-BAX polymorphism
The frequencies of the genotypes in each country for this SNP are shown in Table 2B. The comparison of proportions of genotypes between subsamples did not show significant differences ($p > 0.05$) between cases and controls in the UK and the Netherlands. Also, no significant differences in AA homozygous carriers between subsamples in the control group (CI AA homozygous: UK [8.5–16.8], Netherlands [9.6–20.2]) and the case group (CI AA homozygous: UK [9.6–29.9], Netherlands [12.9–35.6]) were found. Also, in the geographical comparison, no significant differences in the proportions of genotypes and in AA homozygous carriers analysis between cases and controls were found.
The OR of A carriers from UK and the Netherlands together considering an additive model (A/A double risk than G/A) (AIC = 293.4 versus 294.1 of a dominant model) was 1.66 (95% CI: 0.98–2.80) (Table 3).
3 rs2279115 and rs4645878 polymorphisms in the global sample
When all samples were grouped, (Table 4A), significant difference in the distribution of genotypes between the controls and the cases ($p < 0.05$) was found in BAX polymorphism analysis, but not in the BCL-2 polymorphism analysis. Also, homozygous carriers of the A variant were more frequent in PVR cases (CI: 16.3–26.4) than in controls (CI: 10.9–15.6) in the BAX analysis, but not in the BCL-2 analysis. The OR of the A variant in the global sample in the BAX analysis using an additive model (AIC = 608.8 versus 609.7 of a dominant model) was 1.72 (CI: 1.21–2.44) (Table 3). Whereas the OR of the A variant in the global sample in the BCL-2 using a dominant model (AIC = 614.1 versus 615.7 of a codominant model) was 0.69 (CI: 0.47–1.02) (Table 3).

Allelic frequencies comparison

1 rs2279115-BCL-2 polymorphism
Only significant differences in the BCL-2 analysis of the allelic frequencies were

Table 2. (A) Distribution of genotypes and allelic frequencies of BCL-2 in subsamples and in southern (Spain + Portugal) and northern (UK + Netherlands) countries. (B) Distribution of genotypes and allelic frequencies of BAX in subsamples and in southern (Spain + Portugal) and northern (UK + Netherlands) countries.

Countries	Genotype	Cases		Controls		p-Value Fisher's test	Alleles	Cases		Controls		95% CI Alleles Cases	95% CI Alleles Controls	p-Value Chi Square test	OR	CI OR 95%
Spain	A/A	9	15.2%	24	16.6%	0.1126*	AA	38	32.2%	117	40.6%	24.0–41.5	34.9–46.5	0.1305†	0.7	0.44–1.09
	C/A	20	33.9%	69	47.9%		CC	80	67.8%	171	59.4%	69.2–84.8	81.8–90.1			
	C/C	30	50.8%	51	35.4%											
Portugal	A/A	4	26.6%	18	33.9%	0.4376*	AA	13	43.3%	59	55.6%	25.9–62.3	45.7–65.2	0.3438†	0.6	0.26–1.37
	C/A	5	33.3%	23	43.4%		CC	17	56.7%	47	44.4%	37.6–74.0	34.8–54.2			
	C/C	6	40.0%	12	22.6%											
UK	A/A	7	22.5%	27	20.4%	0.6670*	AA	24	38.7%	108	40.9%	26.8–51.9	34.9–47.1	0.7943†	0.9	0.51–1.60
	C/A	10	32.3%	54	41.0%		CC	38	61.3%	156	59.1%	48.0–73.1	52.8–65.0			
	C/C	14	45.2%	51	38.6%											
Netherlands	A/A	6	20.7%	15	16.3%	0.7822*	AA	23	39.6%	64	34.7%	27.3–53.3	28.0–42.1	0.5689†	1.2	0.67–2.26
	C/A	11	38.0%	34	37.0%		CC	35	60.4%	120	65.3%	46.6–72.6	57.8–71.9			
	C/C	12	41.3%	43	46.7%											
Spain + Portugal	A/A	13	17.6%	42	21.3%	0.0409*	AA	51	34.4%	176	44.7%	26.9–42.7	39.7–49.7	0.0405†	0.6	0.43–0.96
	C/A	25	33.8%	92	46.7%		CC	97	65.6%	218	55.3%	57.2–73.0	50.2–60.2			
	C/C	36	48.6%	63	31.9%											
UK + Netherlands	A/A	13	21.6%	42	18.7%	0.7834*	AA	47	39.1%	172	38.4%	30.5–48.5	33.9–43.0	0.9232†	1.0	0.68–1.56
	C/A	21	35.0%	88	39.3%		CC	73	60.9%	276	61.6%	51.4–69.4	56.9–66.1			
	C/C	26	43.4%	94	41.2%											
(B)																
Spain	A/A	3	5.08%	5	3.47%	0.0659*	AA	26	22.0%	39	13.5%	15.4–30.8	10.0–18.1	0.0479†	1.8	1.04–3.13
	G/A	20	33.9%	29	20.1%		GG	92	78.0%	249	86.5%	69.2–84.8	81.8–90.1			
	G/G	36	61.0%	110	76.3%											
Portugal	A/A	1	6.67%	0	0.00%	0.2343*	AA	6	20.0%	13	12.2%	8.4–39.1	6.9–20.4	0.3622†	1.7	0.61–5.19
	G/A	4	26.6%	13	24.5%		GG	24	80.0%	93	87.8%	60.8–91.6	79.6–93.0			
	G/G	10	66.7%	40	75.5%											
UK	A/A	1	3.23%	2	1.52%	0.3459*	AA	11	17.7%	32	12.1%	9.6–29.9	8.5–16.8	0.2989†	1.5	0.73–3.30
	G/A	9	29.0%	28	21.2%		GG	51	82.2%	232	87.8%	70.0–90.4	83.1–91.4			
	G/G	21	67.7%	102	77.2%											
Netherlands	A/A	2	6.9%	2	2.1%	0.2411*	AA	13	22.4%	26	14.1%	12.9–35.6	9.6–20.2	0.1645†	1.7	0.83–3.69
	G/A	9	31.0%	22	23.9%		GG	45	77.6%	158	85.9%	64.4–87.0	79.8–90.4			
	G/G	18	62.0%	68	73.9%											
Spain + Portugal	A/A	4	5.41%	5	2.54%	0.0492*	AA	32	21.6%	52	13.2%	15.4–29.3	10.1–17.0	0.0212†	1.8	1.11–2.95
	G/A	24	32.4%	42	21.3%		GG	116	78.4%	342	86.8%	70.7–84.5	82.9–89.9			
	G/G	46	62.2%	150	76.2%											
UK + Netherlands	A/A	3	5.0%	4	1.79%	0.1414	AA	47	39.2%	172	38.4%	15.4–29.3	10.1–17.0	0.9232†	1.6	0.99–2.84
	G/A	18	30.0%	50	22.2%		GG	73	60.8%	276	61.6%	70.7–84.5	82.9–89.9			
	G/G	39	65.0%	170	75.9%											

OR = odds ratio, UK = United Kingdom.

* Fisher's test. Ho. Independence between genotype case/control group. Significant differences were observed between cases and controls in the southern (Spain + Portugal)countries.

† AA homozygous carriers analysis between different countries revealed differences in southern (Spain + Portugal) countries.

Table 3. Models of inheritance in the global sample. Results of odds ratio using a dominant model for *BCL-2* and additive model for *BAX* in Spain plus Portugal and Netherlands plus United Kingdom (UK).

Global sample		*BCL-2*				*BAX*			
Model	Genotype	OR	95% CI OR	p-Value	AIC	OR	95% CI OR	p-Value	AIC
Co-dominant	C/C	1.00	–	0.1452	615.7	1.00	–	0.0124	610.8
	C/A	0.65	0.42–1.00			1.72	1.11–2.66		
	A/A	0.78	0.46–1.33			2.93	1.06–8.09		
Dominant	C/C	1.00	–	0.0655	614.1	1.00	–	0.0052	609.7
	C/A-A/A	0.69	0.47–1.02			1.83	1.20–2.77		
Recessive	C/C-C/A	1.00	–	0.8893	617.5	1.00	–	0.0812	614.5
	A/A	0.97	0.59–1.58			2.52	0.92–6.91		
Over-dominant	C/C-A/A	1.00	–	0.0817	614.5	1.00	–	0.0283	612.7
	C/A	0.70	0.47–1.05			1.63	1.06–2.51		
Additive	–	0.84	0.64–1.10	0.1951	6.15.9	1.72	1.21–2.44	0.0030	6.08.8
Spain + Portugal		1.00	–	0.0119	315.4	1.75	1.09–2.83	0.0229	316.6
		0.50	0.29–0.86						
Netherlands + UK		1.00	–	0.8489	296.8	1.66	0.98–2.80	0.0626	293.4
		0.95	0.53–1.68						

OR = odds ratio, AIC = Akaike information criterion.
The AIC is a measure of the relative goodness of fit of a statistical model. It can generally be used for the identification of an optimum model in a class of competing models.
Given a set of candidate models for the data, the preferred model is the one with the minimum AIC value.

found between cases and controls in the Spain plus Portugal group (Table 2A).

2 rs4645878-BAX polymorphism

Significant differences in the BAX analysis of the allelic frequencies were found between cases and controls in Spain as a subsample, in Spain plus Portugal in the geographical comparison (Table 2B) and in the global sample (Table 4).

Discussion

Inappropriate apoptosis is an important factor in many human pathologic conditions including neurodegenerative diseases, ischaemic damage, autoimmune disorders and many types of cancer (Hetts 1998; Elmore 2007). In addition, it has been reported that a deregulation of apoptosis during wound-healing process can lead to pathologic forms of healing such as excessive scarring and fibrosis (Elmore 2007).

Current studies have highlighted the involvement of extrinsic and intrinsic pathways of apoptosis in retinal cells after RD, and also, the existence of other death pathways, such as programmed necrosis (which are more inflammatory pathways) and is enhanced when apoptosis is inhibited (Lo et al. 2011; Murakami et al. 2011).

PVR, as a multifactorial disease (Sanabria Ruiz-Colmenares et al. 2006; Rojas et al. 2010, 2013; Pastor-Idoate et al. 2013a,b), shows many similarities to the wound-healing response in other tissues where inflammation plays an important role (Pastor et al. 2002; Ricker et al. 2011b). However, what exactly initiates the development of PVR still remains speculative.

Bax is a death-promoting protein shown to be a tumour suppressor that stimulates cellular apoptosis *in vivo* (Zhang et al. 2000; Bellosillo et al. 2002). The *BAX* gene is located on chromosome 19 and consists of six exons and a promoter region with four p53-binding sites (Saxena et al. 2002). Sequence variations in the promoter region and coding sequence can abolish its pro-apoptotic function. The G(-248) A BAX promoter polymorphism (rs4645878) is associated with decreased cell Bax expression (Saxena et al. 2002; Starczynski et al. 2005).

Our results show that Spanish carriers of the homozygous AA genotype at position (-248) in the *BAX* gene (which is associated with a decrease in apoptotic function) have a 1.8-fold increased risk of PVR after RD than those carrying the GG genotype. This observation was confirmed also in the analysis of the southern countries (Spain plus Portugal). Results also showed a significant association between PVR risk and G(-248)A BAX promoter polymorphism when the global sample was analysed. The OR in the global sample was 1.72 (CI: 1.21–2.44).

Pro-cell-death bcl-2 proteins such as Bax are required for mitochondrial dysfunction in response to apoptotic and necroptotic agonists (Irrinki et al. 2011). It has been reported that reduced expression of *BAX* or even its deficiency, in *BAK* and *BAX* in knockout models, protects against apoptosis (Janssen et al. 2009). However, it does not compromise necrosis induction or the activation of other non-apoptotic pathways such as endoplasmic reticulum stress-induced cell death or autophagy increased as well in stress oxidative and hypoxia situations (Janssen et al. 2009).

The *BCL-2* gene consists of three exons and two promoters. The SNP rs2279115 is located in the inhibitory P2 promoter of *BCL-2* gene (Park et al. 2004). The second promoter, P2, is located 1400-bp upstream of the translation initiation site and decreases the activity of the P1 promoter, thus functioning as a negative regulatory element (Nuckel et al. 2007). The -938C allele in comparison with the A allele displayed significantly increased inhibition of BCL-2 promoter activity and binding of nuclear proteins (Nuckel et al. 2007). Thus, the BCL-2-938 AA genotype is associated with an increase in Bcl-2 expression.

This SNP (rs2279115) has been associated with an improved survival rate in some type of tumours such as breast or renal cancer (Faderl et al. 2002; Masago et al. 2013). In addition, it has

Table 4. Distribution of rs2279115 and rs4645878 in the whole sample. Fisher's test. Allelic frequencies comparison and analysis of AA homozygous carriers between case and control group in *BCL-2* and *BAX*. Chi square test.

Global sample	Genotype	Cases		Controls		p-Value Fisher's test	Alleles	Cases		Controls		95% CI Alleles Cases	95% CI Alleles Controls	p-Value Chi Square test	OR	CI OR 95%
BCL-2	A/A	26	19.4%	84	19.9%	0.1455*	AA	98	36.6%	348	41.3%	30.8–42.6	37.9–44.7	0.2064†	0.8	0.61–1.08
	G/A	46	34.3%	180	42.7%		GG	170	63.4%	494	58.7%	57.3–69.1	55.2–62.0			
	G/G	62	37.7%	157	37.3%											
BAX	A/A	7	5.2%	9	2.1%	0.0084*	AA	56	20.9%	110	13.0%	16.2–26.3	10.9–15.5	0.0029†	1.7	1.23–2.51
	G/A	42	31.3%	92	21.8%		GG	212	79.1%	732	86.9%	73.6–83.7	84.4–89.1			
	G/G	85	63.4%	320	76.0%											

OR = odds ratio.
* Fisher's test. Ho. Independence between genotype case/control group. Significant difference was observed between cases and controls in BAX in global sample.
† AA homozygous carriers analysis revealed significant difference in BAX in global sample analysis.

been reported that Bcl-2 overexpression significantly improved neuron survival in cerebral ischaemia models (Zhao et al. 2003). And also, previous studies in retinal degeneration models showed that in pathologic photoreceptor apoptosis, the bcl-2 overexpression mediates a transient protection (Adams & Cory 2001). Moreover, bcl-2 overexpression has been associated to a substantial reduction in the elimination of ganglion cells in cell death induced by optic nerve axotomy (Zhao et al. 2003).

Our results showed no significant differences in the analysis of subsamples and in the global one. However, the results showed a protective effect in the analysis of homozygous AA carriers in the southern countries. The OR of AA carriers (which is associated with an increase in Bcl-2 expression) from Spain and Portugal was 0.50 (95% CI: 0.29–0.86).

Although an overexpression of bcl-2 is also related with a decreased apoptotic response, unlikebax, the overexpression in bcl-2 is able to induce an inhibitory effect in the programmed necrosis cell death and other non-apoptotic pathways such as autophagy. In fact, some studies have suggested the use of inhibitors of bcl-2 as a new target in cancer therapy (Kang & Reynolds 2009). In addition, it has been reported that an increase of bcl-2 attenuates the TNFA-induced necroptosis pathway (Irrinki et al. 2011).

These two promoter SNPs in the *BAX* and *BCL-2* genes are particularly interesting because they are located within 100 bases from the TP53-binding element in the BAX promoter region and Tp53 responsive element in the BCL-2 promoter region, respectively. Thus, these SNPs may affect the interaction between the Tp53 protein and the Tp53-regulated sequences in the promoters (Chen et al. 2007). An association between the Tp53 Arg72-Pro polymorphism and the PVR has been already reported by our group (Pastor-Idoate et al. 2013a,b).

Besides, it has been reported that in many cells, as RPE cells, the activation of different anti-apoptotic factors such as Bcl-2 family induces a cell proliferation and transdifferentiation (Yang et al. 2005); however, the role of anti-apoptotic factors such as MDM2 (Pastor-Idoate et al. 2013a,b) or Bcl-2 family in RPE transdifferentiation in PVR after RD has not yet been completely studied.

Thus, deregulation in the apoptosis during wound healing and the activation of other cell death pathways could lead to pathologic forms of healing, such as excessive scarring and fibrosis. It can be speculate that the reduction in the levels of apoptosis in retinal cells may activate other cell death pathways, such as programmed necrosis, which would increase the intra-ocular inflammation after RD, thus generating a cascade of tissue responses that generate and amplify the hostile microenvironment in which activated RPE can transdifferentiate.

This study had some limitations. One important issue in any association study is the sample size (Dempfle et al. 2008). Unlike other association studies, our sample could be too small and the power sample could be not enough to draw absolute conclusions. Nevertheless, the results found in this study are strongly consistent with the previous reported findings by our group. And the sample collection to achieve greater power would be an extremely challenging for a low prevalence condition such as PVR.

It is important to point out that functional SNPs are considered of interest because they allow a better understanding of the molecular basis of different pathologies. They also could help to identify new targets in the development of new therapeutic strategies. In this case–control study, we have identified the rs4645878 SNP within the *BAX* gene that shows an association with PVR in Spain, in Spain plus Portugal and in global sample analysis. Although we have carried out the study in two phases, these findings must be interpreted with caution until these results are confirmed with further replication studies to confirm its association, because one of the major pitfalls of genetic association studies are the false positives (Crawford & Nickerson 2005; Dempfle et al. 2008).

Regarding clinical information, recent studies have highlighted that there is no clear association between retinal detachment and gender (Ho et al. 2009; Day et al. 2010; Hajari et al. 2014). Our results showed a predominance of males in the ratio of retinal detachments (approximately 1.8:1). However, we have considered

that this fact should not affect to the genetic analysis, as there were no differences in the percentage between cases and controls regarding distribution between males and females (75% and 77%, respectively), and there was no significant association between gender and group ($p = 0.4866$).

In the present data, we found no association between rs2279115 BCL-2 polymorphism (which is associated with an increase in anti-apoptotic Bcl-2 expression) and PVR in the whole sample, or when results were subdivided into subsamples. However, we found that BCL-2 has a protective effect in the southern countries probably because it has the ability, contrary to BAX, to inhibit both cell death signals (apoptosis and necrosis).

In summary, this study highlights the role of genetic factors as a useful tool in the identification of high-risk patients to suffer PVR and indicates that reduced apoptosis could be implicated as a significant risk factor for PVR after RD. Also, it highlights the role of the SNP rs4645878 as a possible marker of PVR risk or the role of SNP rs2279115 as a possible new target in the PVR prophylaxis. But further studies are necessary to analyse the role of these SNPs in PVR development.

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Chapter 5

Proliferative vitreoretinopathy: a new concept of predictors of disease pathogenesis and practical consequences

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Proliferative vitreoretinopathy: A new concept of disease pathogenesis and practical consequences

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ABSTRACT

During the last four decades, proliferative vitreoretinopathy (PVR) has defied the efforts of many researchers to prevent its occurrence or development. Thus, PVR is still the major complication following retinal detachment (RD) surgery and a bottle-neck for advances in cell therapy that require intraocular surgery. In this review we tried to combine basic and clinical knowledge, as an example of translational research, providing new and practical information for clinicians. PVR was defined as the proliferation of cells after RD. This idea was used for classifying PVR and also for designing experimental models used for testing many drugs, none of which were successful in humans. We summarize current information regarding the pathogenic events that follow any RD because this information may be the key for understanding and treating the earliest stages of PVR. A major focus is made on the intraretinal changes derived mainly from retinal glial cell reactivity. These responses can lead to intraretinal PVR, an entity that has not been clearly recognized. Inflammation is one of the major components of PVR, and we describe new genetic biomarkers that have the potential to predict its development. New treatment approaches are analyzed, especially those directed towards neuroprotection, which can also be useful for preventing visual loss after any RD. We also summarize the results of different surgical techniques and clinical information that is oriented toward the identification of high risk patients. Finally, we provide some recommendations for future classification of PVR and for designing comparable protocols for testing new drugs or techniques.

1. PVR: concept and pathogenesis

Proliferative vitreoretinopathy (PVR) is a term coined by the Retina Society Terminology Committee (Committee, 1983) for describing a complication that can follow rhegmatogenous retinal detachments (RD). It is still the major cause of failure after RD surgery, and no relevant advances in clinical management have been made since the initial description (Pennock et al., 2014a). PVR is estimated to occur in 5–10% of all RD cases. While it can occur before surgery, it more commonly occurs after any surgical intervention for RD (Pastor et al., 2002; Pennock et al., 2014a) (Fig. 1).

According to the original description, PVR was characterized by the growth of membranes on both surfaces of the detached retina and on the posterior hyaloids (Committee, 1983). Posterior contraction of these membranes causes distortion of the retina and keeps it detached, transforming a rhegmatogenous RD into a tractional one. The pathogenesis of this complication is divided into several steps: 1) migration of cells, mainly retinal pigment epithelial (RPE) and glial cells; 2) proliferation of the migrating cells; 3) membrane development; 4) contraction of the cellular membrane; 5) extracellular collagen production; and 6) creation of fixed folds in the retina. The authors emphasized that cellular proliferation was the essential point of this disorder.

Based on the idea that cellular proliferation is the main feature of PVR, many researchers have tried for more than 40 years to solve the problem by inhibiting the proliferation of cells. Nevertheless, the problem remains unsolved, and we believe it is time to revise

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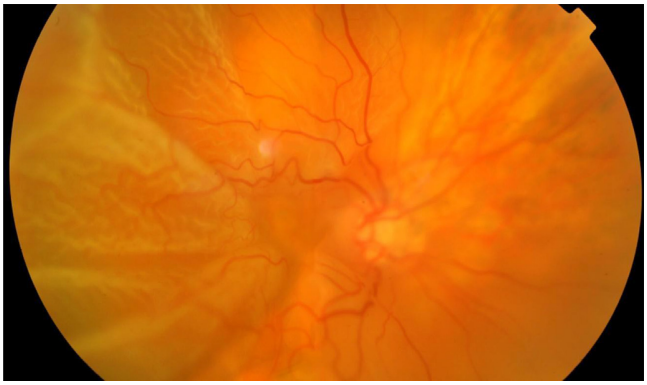

Fig. 1. Funduscopic image showing full-thickness retinal folds (star fold) and subtotal RD in a patient with history of chronic RD and PVR. Fold is created by an epiretinal membrane (epiretinal PVR). Patient was solved by simple 25G PPV and membrane peeling.

our understanding of the pathogenesis of PVR. In doing so, we can now focus on some other mechanisms involved in RD and in PVR with the aim of finding a useful prophylaxis or treatment for this complication.

Our personal approach is different from the original description of the pathogenic origins as it is based on several questions that we have asked during the last 15 years. First, if PVR appears after a RD, why are the events elicited in any RD, which include ischemia, photoreceptor death, astrocyte reactivity and others, not incorporated into the pathogenesis concept of PVR? Second, are RPE cells really the main effectors of PVR, or are there some other cell types that are more relevant for the characteristic tissue changes observed in this complication? Third, do the classification schemes in the literature have any real value for establishing the efficacy of the medical interventions? Fourth, because the pathogenesis of PVR is so poorly understood, what is the real value of experimental models that are mainly based on the original ideas of cell proliferation into the vitreous cavity?

We have structured this major review to provide updated information on all of these questions by using not only the reference databases, but also the vast experience of The Retina Group, IOBA (Eye Institute), University of Valladolid, Valladolid, Spain, both in clinical management and in PVR research (Pastor, 1998; Pastor et al., 2002; Rojas et al., 2015).

1.1. Mechanisms of tissue damage triggered by RD

Due to the scarcity of human tissue samples in the early stages of RD, most of our knowledge derives from experimental models. In the late 1960's, Machemer initiated a series of experimental RD studies in owl monkeys to understand the tissue changes (Machemer, 1978). Early alterations included intraretinal edema (mostly in the inner nuclear layer), disorganization of the photoreceptor outer segments, enlargement of some RPE cells that separate from Bruch's membrane, and reactivity of Müller and other glial cells (Geller et al., 2001; Ghazi and Green, 2002). These changes have been reproduced in other animal models (Fisher et al., 1995, 2005; Lewis et al., 1994; Lewis et al., 2005; Linberg et al., 2002). Our group has documented similar changes in organotypic cultures of pig and human neuroretinas (Fernandez-Bueno et al., 2012, 2008). These models have the potential to be useful in analyzing early changes after RD and to test some possible treatments (Fernandez-Bueno et al., 2012, 2008) (Figs. 2,3).

When the retina separates from the RPE, the outer retinal layers become ischemic. Surprisingly, despite the high metabolic demands of the neural retina, detachment from the choroidal vascular supply does not lead to immediate neuron death. Intrinsic protective mechanisms are activated during the early stages of RD. Specifically, stress-response genes and signaling pathways are activated that enable the photoreceptors to survive the acute phase of RD (Zacks et al., 2006). The breakdown of these protective mechanisms leads to the cell death, principally by apoptosis (Cook et al., 1995; Ghazi and Green, 2002).

Apoptosis has two major signaling cascades, the extrinsic and intrinsic pathways, both involving caspases, and leading to DNA fragmentation and cell death (Lo et al., 2011). Initiation of the apoptotic response may be mediated in part by the release of cytokines from the stressed and damaged tissues. The chemotactic properties of the cytokines can attract and activate macrophages and microglia. Activation of these cell types can then generate oxidative stress that could contribute to the cytotoxic effect on the photoreceptors after RD (Lo et al., 2011).

Although apoptosis is the principal mechanism of photoreceptor loss after RD, other cell-death mechanisms also exist (Lo et al., 2011). Programmed necrosis of photoreceptors also occurs after RD. Necroptosis, as this form of cell death is known, is mediated by a receptor-interacting protein (RIP) kinase (Lo et al., 2011; Murakami et al., 2011; Trichonas et al., 2010).

In this situation, programmed necrosis is less frequent than apoptosis, but it is enhanced when caspases are inhibited (Murakami et al., 2011). Another non-apoptotic pathway for cell death can be triggered by prolonged or excessive stress placed upon the endoplasmic reticulum. This programmed, endoplasmic reticulum stress-mediated pathway is important in neuronal death in neurodegenerative disorders and has been identified in experimental RD (Liu et al., 2010). Additionally, autophagy, a form of cellular recycling, is also upregulated in RD (Chinskey et al., 2014; Cook et al., 1995).

The pro-survival autophagy pathways inhibit apoptosis temporarily until the cell eventually dies (Besirli et al., 2011). An experimental model has demonstrated that photoreceptor necroptosis is associated with the activation of autophagy (Dong et al., 2014). This finding opens new possibilities for protection of these neurons by, for example, administration of necrostatin-1, which down-regulates RIP-1 phosphorylation and the autophagic biomarker LC-3ll (Dong et al., 2014).

Having a better understanding of the neuronal self-protective pathways and the self-destructive pathways is essential for developing new neuroprotective strategies as adjuncts for the surgical repair of RD (Lo et al., 2011). With careful management and innovative approaches, it may be possible to prevent the development of PVR altogether.

1.2. Initially, are there any differences between RD and PVR?

As mentioned, PVR is a complex process involving not only ischemic tissue damage, but also inflammation and proliferation of several types of cells and the production of local factors (Garweg et al., 2013; Pennock et al., 2014a). Tissue trauma, as in any RD, is triggered by the separation of the neuroretina from the RPE. According to our current knowledge on PVR, glial cells initiate the process as part of a nonspecific tissue repair response that leads to a remodeling of the retina (Garweg et al., 2013). Soon after RD, RPE cells de-differentiate into fibroblast- or macrophage-like cell morphology, driven by factors not yet fully understood. In the process, contractile cellular or fibrocellular membranes are created, preventing retinal reattachment. These membranes have been considered the most characteristic feature of PVR (Garweg et al.,

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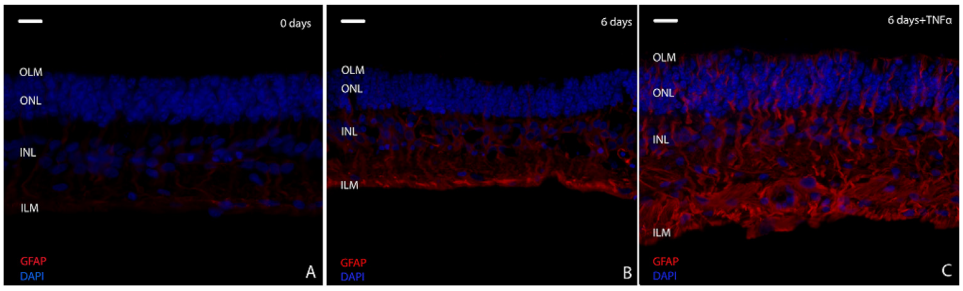

Fig. 2. Sections of porcine neuroretina organotypic culture immunostained for glial fibrillary acidic protein (GFAP). In fresh specimens (A), GFAP (glial fibrillary acidic protein) (red) expression was present in glial cells at the innermost retinal layers. Retinal structure and cellular organization were adequately preserved before culturing. In control cultures at 6 days (B) GFAP expression increased in the glial cells. Some Müller cell branches at the outer nuclear layer (ONL) expressed GFAP. In cultures with external added tumor necrosis factor alpha (TNFα; C) GFAP was markedly upregulated at 6 days culture. It appeared in Müller cell processes at the ONL and outer limiting membrane. Retinal tissue started to lose its characteristic organization. DAPI: 4′,6-diamino-2-phenylindole dihydrochloride staining (blue); ILM: inner limiting membrane INL: inner nuclear layer; OLM: outer limiting membrane; ONL: outer nuclear layer. Scale bars: 20 μm. Images courtesy of Dr. Ivan Fernandez-Bueno (IOBA, Spain).

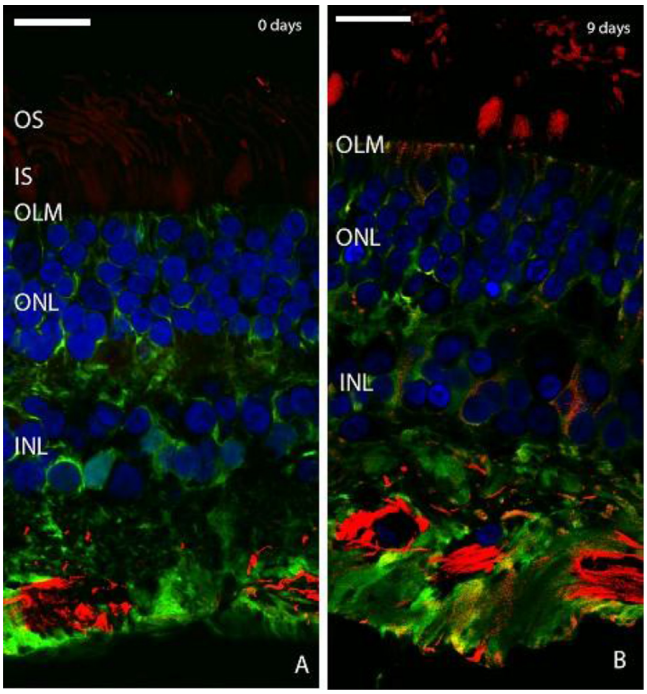

Fig. 3. Sections of human neuroretinas organotypic culture immunostained for glial fibrillary acidic protein (GFAP) and cellular retinaldehyde-binding protein (CRALBP). At day 0 (A) of culture immunostaining for CRALBP (green) showed Müller cells with normal morphology. The absence of Müller cell immunostaining for GFAP (red) indicates that these cells were not activated. At day 9 of culture (B) astrocytes had increased immunoreactivity to GFAP. Müller cells also expressed GFAP at the OLM level. OS: outer segments; IS: inner segments; OLM: outer limiting membrane; ONL: outer nuclear layer; INL: inner nuclear layer; ILM: inner limiting membrane. Scale bar: 20 μm. Images courtesy of Dr. Ivan Fernandez-Bueno (IOBA, Spain) and Dr. Nicolas Cuenca (University of Alicante, Spain).

2013). However, except for periretinal membrane formation, the glial hyper-reactivity is not different from the one elicited by any RD. Thus an as yet unidentified, critical distinctive difference must be present to direct these events towards a PVR.

Because early changes after RD are difficult to analyze in human samples, much of our current understanding of PVR development has been ascertained from experimental models. In some animal, the retinal architecture and vascularization is different from that in humans; therefore, some results could not be directly extrapolated. In cats after experimental RD, photoreceptors degenerate within 24 h (Fisher and Lewis, 2003; Lewis et al., 2003). The degeneration reaches a peak at 3 days and continues for as long as the retina is detached. In cats, 80% of photoreceptors are definitively altered after 3 months of detachment (Erickson et al., 1983); (de Souza et al., 2012) confirmed these changes in three samples of human retinal operculums or flaps. They also characterized functional changes, concluding that RD leads to alteration of the glutamate pathway.

Two days after RD in cats, rod synaptic terminals withdraw toward the cell bodies, leaving the outer plexiform layer disorganized (Lewis et al., 2003). At 7 days after DR, many ganglion cells become immunopositive for neurofilament protein and growth-associated protein 43. Furthermore, Müller cells quickly become activated. Fifteen minutes after the retina is detached, the Müller cells proliferate and hypertrophy, reaching a peak 3–4 days after RD, often extending their processes into the subretinal space.

RD also induces proliferation of some non-neuronal cells, including astrocytes, endothelial cells, pericytes, and microglia. The greatest period of proliferation for these cells is between 3 and 4 days after RD (Geller et al., 1995). Some of these changes, e.g., proliferation of endothelial cells and pericytes, are reversed by reattachment, especially if they occur within 1 day (Lewis et al., 2002). However, other changes are profound and may permanently affect the photoreceptors and glial cells. This could explain why in some cases the restoration of vision takes months or even years when the macula has been affected (Lewis et al., 2003).

Rod axon elongation towards the inner retina, formation of epiretinal membranes, Müller cell growth into the subretinal space, and stimulation of Müller cell growth into the vitreal surface of the retina after retina reattachment are events also identified in experimental models of RD.

While these events could be part of PVR pathogenesis (Lewis et al., 2003), they are also present after any RD, including those that do not have the distinctive characteristics of PVR.

1.3. The role of RPE cells

During the 1990's, many papers described the cellular types implicated in PVR by analyzing the vitreous and, more frequently, the removed epiretinal membranes (Campochiaro, 1997). RPE and glial cells were identified as the main participants. In cats, RPE cells initiate changes 24 h after RD (Anderson et al., 1981). They dedifferentiate, lose their polarity, and migrate into the subretinal space where they phagocytize outer-segment debris (Campochiaro, 1997). In RD, blood-retinal barriers are disrupted, and there is an increase of chemotactic and mitogenic activity in the vitreous

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cavity (Campochiaro, 1997). These factors stimulate the further migration and proliferation of RPE and glial cells (Campochiaro, 1997).

The RPE deserves more attention than it has previously received. Mature RPE cells are mitotically quiescent under physiological conditions, but when the neural retina suffers an injury, RPE cells start to proliferate while undergoing transformation (Chiba, 2014). After RD, RPE cells are exposed to serum factors because of the damage in the blood-retinal barriers. They become detached from Bruch's membrane, lose their epithelial morphology, and migrate into the vitreous through breaks in the neuroretina. There, they participate in the formation of epiretinal membranes (Chiba, 2014).

This process involves an epithelial-mesenchymal transition of the RPE cells, a biological process through which the detached polarized cells lose their epithelial characteristics. In doing so, the RPE cells acquire a mesenchymal phenotype that includes enhanced migratory capacity, invasiveness, resistance to apoptosis, and production of extracellular matrix (Chiba, 2014). In PVR, RPE cells become fibroblast-like cells. They can also transform into some other mesenchymal cells such as adipocytes, bone and cartilage cells, as well as reproduce RPE cells themselves, although their capability of producing neural retinal cells is very limited (Chiba, 2014).

The onset mechanism of proliferation is not fully understood although plenty of studies have been made (Campochiaro et al., 1985; Kirchhof et al., 1989; Osusky and Ryan, 1996). For instance, isolated RPE cells acquire responsiveness to proliferating factors such as platelet derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and transforming growth factor β (TGF-β) (Chiba, 2014). One of the important findings is that cell-to-cell contact seems to suppress the ability of cells to respond to mitotic factors. Thus the loss of cell-to-cell contact might be an important step in PVR development. Cadherin is a crucial adhesion protein, and its disruption results in the loss of cell-to-cell contact. In a rat RD model, Chen and Ma (2007) demonstrated that normal RPE cells do not express N-cadherin, but after RD, RPE cells, photoreceptors, and the outer limiting membrane exhibited intense immunolabeling for this protein. This type of cadherin is expressed in mesenchymal cells, and these changes were reversed by retinal reattachment.

Once RPE cells are trans-differentiated into fibroblast-like cells, they express alpha-smooth muscle actin, glial fibrillary acidic protein (GFAP), vimentin, etc. and become the main component of the epiretinal membranes (Chiba, 2014). This and other recent studies have confirmed the important role of RPE cells in one of the most characteristics findings of PVR: the membranes on the inner retinal layer and on the hyaloid.

1.4. Role of glial cells

As described above, PVR could be considered as an exaggerated response of the active remodeling process triggered by the injured retina (Garweg et al., 2013). In this process, glial cells have an important role, and tissue remodeling implicates Müller cell hypertrophy. Changes in Müller cells are observed 1 day after RD (Wickham and Charteris, 2009). Within 3 days, Müller cell bodies migrate to the outer nuclear and outer plexiform layers, occupying the spaces left by dying photoreceptors and extending their processes into the subretinal space (Wickham and Charteris, 2009). These cells, along with some RPE cells, microglia, and macrophages, contribute to create subretinal membranes, an uncommon finding in PVR. The presence of subretinal membranes is often seen after ocular trauma, and we propose to refer to this condition as subretinal PVR (Fig. 4).

In 1992, the hypertrophy of Müller cells was detected by our group in a rabbit model of PVR (Pinon et al., 1992). However, we were not able to recognize the importance of this finding, probably because we were under the influence of the pathogenesis ideas of the original description of PVR. Nevertheless in 2006, we detected these Müller cell changes in samples obtained from patients having PVR in which a retinectomy was necessary to reattach the retina (Pastor et al., 2006b). Although these changes are not specific to PVR, they lead to a shortening of the retina, a crucial point in PVR. Shortening of the retina constitutes, in our opinion, the most severe form of PVR (Pastor et al., 2003). Müller cells constitute only 20% of the retinal volume; therefore, even if they undergo hypertrophy, they cannot counteract the important loss of neurons after RD with PVR. Consequently, the retinal tissue becomes “shorter” (Pastor et al., 2003). The important role of the glial cells, especially Müller cells, of increasing PVR severity has been documented by other authors (Sethi et al., 2005). Thinning of the retina has also been demonstrated after 9 days in organotypic retina cultures (Fernandez-Bueno et al., 2012). These intraretinal changes were not described in the original pathogenesis description of this disease (Committee, 1983). But once again, most of these changes affecting glial cells are observed in any experimental model of RD as well as other retinal degenerative diseases. Therefore they cannot be considered specific for PVR.

Glial activity is very important for neuron survival (Fischer et al., 2015). Müller cells are crucial for photoreceptor survival and vascular integrity in the rodent retina. Nevertheless, glial cells can exacerbate neuronal death following excitotoxic injury (Fischer et al., 2015). For example, N- methyl-D-aspartate stimulates tumor necrosis factor alpha (TNF-α) production by Müller cells, and this cytokine diminishes neuron survival (Fischer et al., 2015). Conversely stimulation of the glia provides enhanced neuroprotection. In chickens, the elimination of microglia and macrophages prevented the development of RD and retinal folds (Fischer et al., 2015). In the central nervous system (CNS), the activation of microglia and macrophages can be detrimental to neuronal survival. In Parkinson's disease there is significant evidence that inflammation and microglial reactivity promotes neuronal degeneration (Tansey and Goldberg, 2010). Further, persistent changes in multiple sclerosis appear as the result of activated microglia and invading macrophages (Fischer et al., 2015).

Interestingly, in a porcine model of RD (Iandiev et al., 2006), the reactivity of Müller and microglial cells were not restricted to the detached areas, but were also observed in the intact regions of the

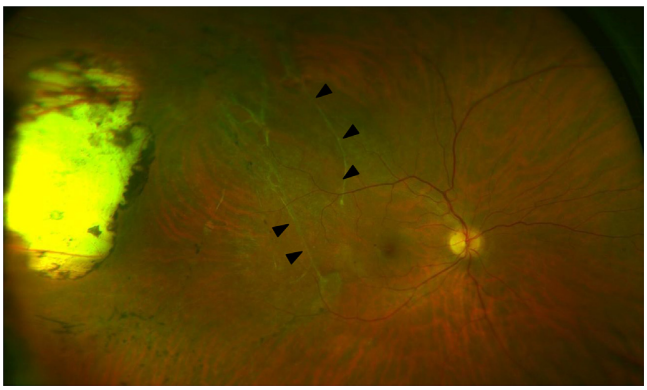

Fig. 4. Ultra-widefield fundus photograph showing subretinal PVR. Arrowheads showing subretinal bands. Courtesy of Prof. Stanga (Manchester Royal Eye Hospital).

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retina. This finding could have an implication in the loss of vision detected in some patients even after successful surgery for macula-on RD. It also might support the use of adjunctive treatments for preventing visual loss in most cases of RD.

Thus with regard to Müller and other glial cells, it is clear that they play an important role in the remodeling process elicited after RD. Hypertrophy of these cells and replacement of the lost retinal neurons causes the shortening of the retina in advanced cases of PVR (Figs. 5 and 6).

1.5. The role of macrophages

Macrophages are also considered important players in PVR (Campochiaro, 1997). Because of the breakdown of the blood-retinal barrier that follows any RD, they migrate into the sub-retinal space and to the vitreous cavity. The presence of macrophages in the vitreous is associated with a high risk of developing PVR. In 2003, our group demonstrated that they were more frequent in the vitreous of patients who developed PVR after surgery compared to RD patients that did not (Martin et al., 2003). These cells were also found around the retinal vessels and inside the retinal tissue in human PVR samples (Pastor et al., 2006b). Now it is recognized that macrophages not only release pro-inflammatory agents (Garweg et al., 2013), but they also mediate photoreceptor apoptosis through monocyte chemoattractant protein-1 (MCP-1) (Nakazawa et al., 2007).

In summary, three major cell types are implicated in RD and therefore in PVR: RPE, glial cells, and macrophages. The principal role of these cells in PVR is the remodeling of the retina after neuronal death caused by ischemia. However the unsolved question is why in some patients this mechanism exceeds the regular limits of this reparative process.

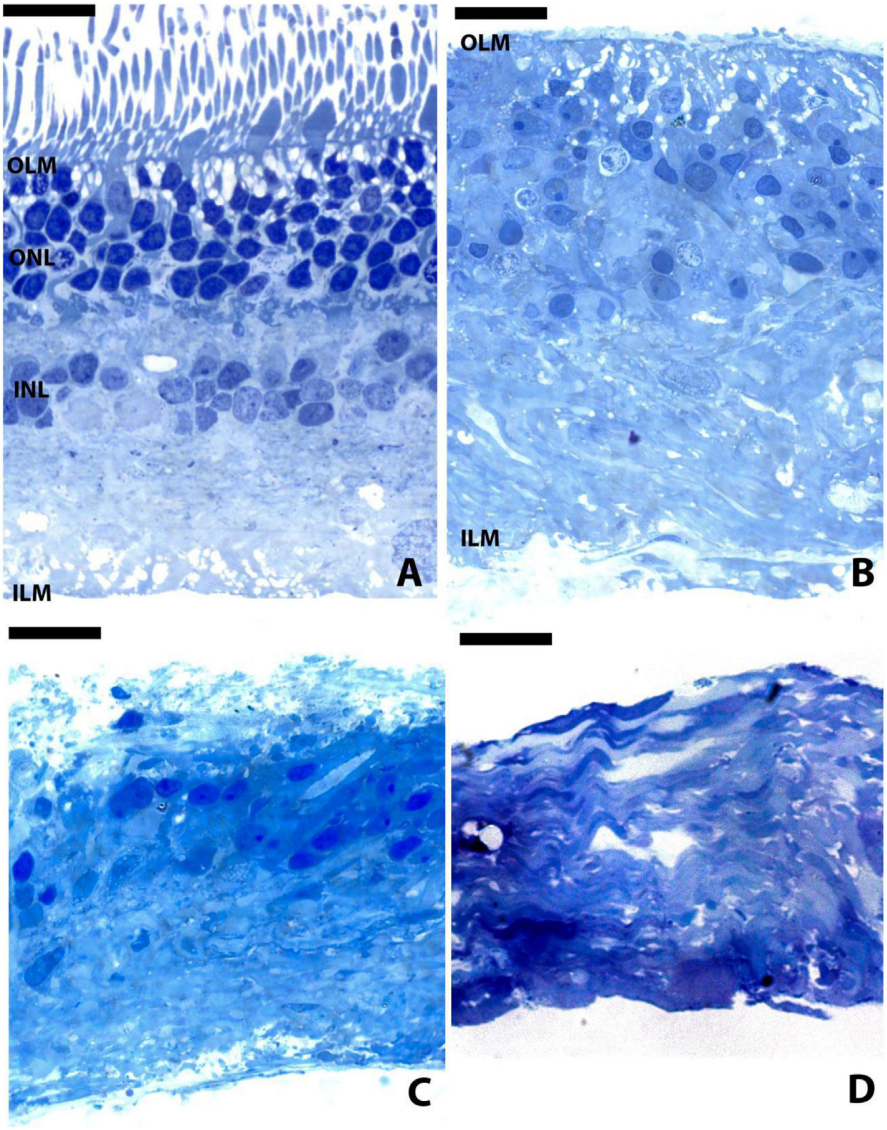

Fig. 5. Toluidine Blue staining of human retina specimens. Fresh human retina and retinal samples obtained from patients with intraretinal PVR where retinectomy was necessary to reattach the retina (100×). The images show progressive degenerative stages from a normal neuroretina in which retinal layers are perfectly organized (A) to a complete degenerate retina in which gliotic response dominate and the normal architecture is lost (D). Following retinal detachment, the photoreceptors are lost and there is a generalized loss of normal retinal architecture with progressive replacement for reactive gliosis (B and C). In the final stage, the neuroretina lacks of neurons and thick glial cells prolongations replace the normal retinal structure (D). Scale bar: 10 μ. Courtesy of Dr. M. Gayoso. University of Valladolid, Valladolid, Spain.

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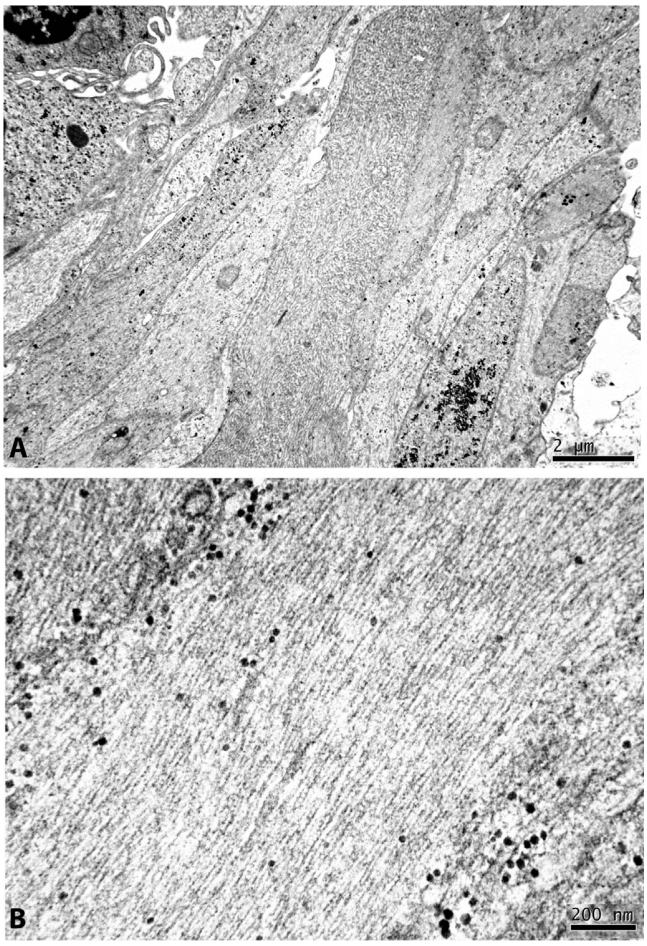

Fig. 6. Electron microscope images of intraretinal PVR samples obtained during surgery in which retinectomy was necessary to reattach the retina. Ultrastructural detail of a gliotic retinal area. (A) Most of the retina presents thick glial cells prolongations containing intermediate filaments (B). Courtesy of Dr. M. Gayoso. University of Valladolid, Valladolid, Spain.

1.6. Cytokines and other mediators

Many growth factors, signaling pathway mediators, and receptors have been described in patients suffering from PVR (Pennock et al., 2014a; Rouberol and Chiquet, 2014). Some of these have also been implicated in the stimulation of RPE and glial cells. Among them, PDGF, HGF, VEGF, EGF, transforming growth factor α (TGF α), TGF-β, granulocyte colony stimulating factor (G-CSF), acidic and basic FGF, insulin-like growth factor 1 (IGF-1), connective tissue growth factor (CTGF), and mothers against decapentaplegic homolog (SMADs) are the most important (Flanders, 2004; Saika et al., 2008, 2007). They are synthesized by a variety of cells and induce many actions. For example, PDGF is synthesized by glial cells and by RPE cells when they lose contact with photoreceptors. PDGF stimulates proliferation by its own receptor, and it is also a potent mitogen and chemoattractant for glial cells (Rouberol and Chiquet, 2014).

Other examples of molecular mediators found in PVR are TGF and the associated signaling pathway molecules SMAD3 and SMAD7. These are strongly implicated in apoptosis, the stimulation of epithelial-mesenchymal cells, fibroblast and myofibroblast conversions, and the enhanced expression of extracellular matrix proteins (Flanders, 2004; Saika et al., 2008, 2007). The following molecules have also been associated with this disease: interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), TNF-α and -β, interferon gamma (INF- γ) and MCP-1 (Pennock et al., 2014a; Rouberol and Chiquet, 2014).

The presence and concentration of cytokines have been proposed as biomarkers for predicting development and severity of PVR (Kon et al., 2000, 1999; Ricker et al., 2012; Rusnak et al., 2013). However once again, these cytokines are not specific for PVR and have been found in RD without PVR as well as in other intraocular disorders such as proliferative diabetic retinopathy (Pennock et al., 2014a; Rusnak et al., 2013).

1.7. Our ideas for the pathogenesis of PVR

To our knowledge, the initial mechanisms implicated in PVR are not different from those of any RD repair. After retinal separation, the retinal outer layers become ischemic and the photoreceptors start to die by several pathways, but mainly apoptosis. This loss of neurons stimulates the hypertrophy of retinal glial cells, mainly Müller cells, but also astrocytes and microglia, that begin the remodeling of the retina and perhaps the neuroprotection of the remaining neurons. However these intraretinal changes, if they are excessive, cause the substitution of neurons by glial tissue and the shortening of the retina. We propose to identify and name this phenomenon as intraretinal PVR (Fig. 7).

At the same time, and because of the breakdown of the blood-retinal barriers, microglia and macrophages migrate into the subretinal space and into the vitreous cavity where they release inflammatory products. RPE cells, after losing contact with photoreceptors and after being stimulated by growth factors produced by a variety of cells, including Müller cells, enter into a mesenchymal transformation. Through the retinal breaks or tears, the transformed cells migrate into the vitreous cavity. There they play an important role in the development of epiretinal PVR by the formation of contractile membranes at the inner surface of the retina and in the vitreous surface (Fig. 1). These activities are considered the most typical clinical feature of PVR (Committee, 1983; Pastor et al., 2002). Inflammation then plays an important role in PVR, but once again the mediators do not differ from those present in any RD. It is possible that the degree of inflammation is crucial for PVR development, and most of the identified clinical risk factors for PVR have an important inflammatory component (Moysidis et al., 2012; Rodriguez de la Rua et al., 2005; Tosi et al., 2014) as we will discuss further.

Our hypothesis is that when inflammation reaches a certain

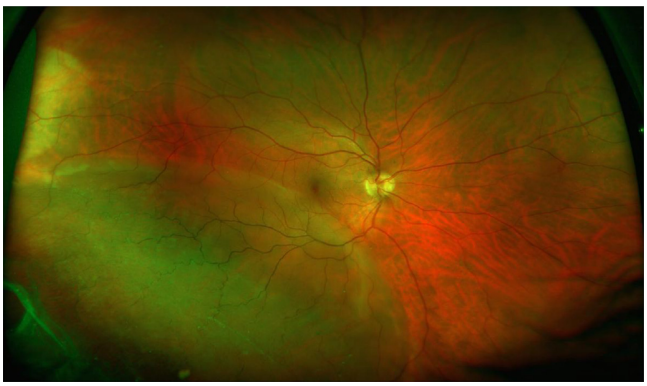

Fig. 7. Localized PVR in a RD. Whitish appearance of the surface and rolled edge of the tear suggest intraretinal changes (intraretinal PVR). Nevertheless, this finding should be confirmed during surgery (difficulties in flattening the retina). Courtesy of Prof. Stanga (Manchester Royal Eye Hospital).

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level, the remodeling mechanisms elicited by the detached retina are exaggerated and amplified, causing entry into the PVR process. There are two important questions regarding this hypothesis: "What is this level of inflammation beyond which PVR is initiated?" and "What compels the inflammatory process to exceed this level of inflammation?" Regulation of transcription factors, inhibition of kinases, stimulation of apoptosis, among others, are all processes involved in inflammation that are not perceived by the surgeon's eye. It is probable that all of these clinical factors that we evaluate in the office prior to surgery are telling us that parts of these mechanisms have been launched since the neuroretina and RPE lost contact with one another. The final outcome is played at a molecular level, which is probable determined by the genetic profile of the patient.

We have begun to explore the genetic profile of PVR patients (Pastor-Idoate et al., 2013a, 2013b; Rojas et al., 2009; Rojas et al., 2013; Sanabria Ruiz-Colmenares et al., 2006). We are convinced that genetics play an important role because the production of cytokines is a gene-regulated process (Zacks et al., 2006) and variations in these genes could be an important factor for PVR development. Additionally, the influence of gene composition probably extends further than the inflammatory process. Very recently, Matsumoto et al. (2014) demonstrated in mice that the genetic background affects the photoreceptor cell death rate in response to experimental RD. Modifying the expression of the SMAD7 gene in mice after RD suppresses fibrogenic responses to TGF-β2 by RPE cells (Saika et al., 2007).

In summary, pathogenic mechanisms implicated in PVR are not different from those elicited in any RD. If the response to RD occurs in a pro-inflammatory environment, whether by external situations or by the genetic profile of the patient, it is diverted from restoration of the injured retinal tissue. In those cases, the response is re-directed towards the onset of PVR. Obviously prophylactic measures or treatments based upon epiphenomena like intravitreal cell proliferation or extracellular matrix formation have had little success in solving the problem as has been demonstrated through the years. It now seems more likely that effective prophylaxis or treatment of PVR will be achieved by the regulation of mediators of inflammation such as transcription factors, receptors, cytokines, etc., or maybe even better, by a combination of both strategies.

2. Why should we investigate PVR in 2015?

Currently PVR management is basically restricted to the surgical repair of the detached retina. However, anatomical and functional success rates of surgical treatment remain unsatisfactory, although many efforts have been made to propose new pharmacological approaches to improve these results (Asaria and Charteris, 2006; Pennock et al., 2014a, 2011). Based on the original ideas of PVR pathology (Committee, 1983) and after testing them in experimental models, several medical treatments have been proposed. However, most of the strategies that showed positive results in animal models have failed to prove their efficacy in human clinical trials (Moysidis et al., 2012), and currently there is no other accepted treatment beside surgery. As we will discuss later, the discordant results obtained in experimental models and human clinical trials could be due to dissimilarities in the causes of PVR in the models and humans (Moysidis et al., 2012; Sadaka and Giuliari, 2012).

Without an appropriate comprehension of the biological processes involved in PVR, including the role of different mediators, it seems impossible to develop an efficient prophylaxis or treatment (Garweg et al., 2013). Therefore, to improve current outcomes in the treatment of PVR, a better understanding of its pathogenesis is mandatory and a greater cooperation between clinicians and basic scientists is needed.

2.1. There is not an appropriate treatment, yet

For the past 40 years, the pharmacologic agents proposed to prevent PVR have been mainly anti-inflammatory, anti-proliferative, anti-neoplastic, anti-growth factor, and antioxidant agents (Sadaka and Giuliari, 2012). However, none of them have been incorporated routinely into clinical treatments. Corticosteroids were the first drugs tested for PVR (Chandler et al., 1985). Experiments in animals found some efficacy with intravitreal administration of triamcinolone acetonide or topical and systemic corticosteroids (Pastor et al., 2000), but patients treated in clinical trials with these agents showed a poor response (Moysidis et al., 2012; Sadaka and Giuliari, 2012). In addition to the direct application of the corticosteroids, silicone oil was suggested as a reservoir for the drugs and some other substances with the aim of reducing the "re-proliferation". Unfortunately most of the drugs were not soluble in silicone oil (Pastor et al., 2008a).

The anti-neoplastic or anti-proliferative agents suggested for PVR prevention or treatment included compounds as 5-fluorouracil (5-FU), daunorubicin, taxol, colchicine, retinoic acid, ribozymes, vincristine and others. 5-FU is an antimetabolite that inhibits synthesis and fibroblast proliferation and has been one of the most tested agents for the treatment of PVR. In animal models it showed beneficial results, but in human clinical trials the results were poor with some important side effects (Moysidis et al., 2012). Also, a combination of this agent with low-molecular-weight heparin (LMWH), an anticoagulant that binds many growth factors, was proposed. However, in a large, randomized, controlled trial using this combination versus placebo, the combination did not improve anatomical or visual success rates after PVR (Wickham et al., 2007).

The principle for the use of anti-growth factors and antioxidant agents is to inhibit several growth factors and associated pathways. Many of them are currently being studied in experimental models, but currently there are no results from human clinical trials (Alex et al., 2010; Chan et al., 2013).

One of the latest approaches is the use of antiangiogenic drugs in PVR. Ranibizumab, an agent that neutralizes vascular endothelial growth factor A (VEGF-A) reduces vitreous bioactivity in patients and in experimental models of PVR (Pennock and Kazlauskas, 2012; Pennock et al., 2013). Similar results have been obtained with aflibercept (VEGF Trap-Eye) in animal models. These are interesting approaches but should be considered cautiously before transferring the results into the clinic. The rabbit model for these studies was based on the intravitreal injection of conjunctival fibroblasts (Pennock et al., 2014b, 2013). As we will discuss further, this model has little in common with the human disease.

One of the major drawbacks with many potential treatments, especially with the anti-proliferative agents, is the existence of undesirable side effects (Pastor, 1998; Scheer et al., 2004). In some cases these risks could be worth taking if there is a clear potential benefit. Such use could be justified only in patients with a high risk of developing PVR and with a high probability to respond to the treatment. This accurate identification is still a challenge, despite much research on the preoperative clinical risk factors, intraocular biomarkers, and genetic background. Unfortunately, there is still no accurate procedure to identify those RD patients (Rojas et al., 2009, 2015).

The early identification of at-risk patients is crucial for obtaining better outcomes because once PVR develops, the anatomical and functional results are very unsatisfactory. Currently PVR interventions are usually performed in an advanced stage of the disease, and there is no real prophylaxis (Pastor et al., 2002). Even

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more, many PVRs develop after RD surgery (Pennock et al., 2014a), and because in many cases clinical characteristics do not provide useful information, first surgeries are planned without having in mind the possibility of developing PVR. Thus, identifying patients with high susceptibility for PVR at RD presentation could also persuade the ophthalmic surgeon to decide in favor of a more conservative treatment, avoiding aggressive surgical trauma that is considered a clinical risk factor (Pastor et al., 2002).

2.2. There is little information on clinical outcomes, mainly functional

There is little information on the functional results after PVR, and this topic will be discussed later in the review. Most reports emphasize the anatomical reattachment of the retina, which is still a big challenge in most cases. Reattachment rates for RD cases that develop PVR are very low compared to those that do not develop PVR. The anatomical success rate after RD complicated with PVR is 60–80% in the most favorable cases, whereas the reattachment rate is up to 90% in RD without PVR (Mitry et al., 2010; Pastor et al., 2012, 2008b). Thus, it is not surprising that functional results are also very disappointing (Berker et al., 2007). The poor visual outcomes generate a huge burden in the quality of life of these patients (Asaria and Charteris, 2006; van de Put et al., 2014).

2.3. Multiple surgeries increase the cost of treatment

Another important aspect to be considered is the costs of treatment and current management of PVR. Patients who develop postoperative PVR (grade C or greater) double the costs of management in terms of higher number of surgical procedures and a more extensive follow-up period compared with patients without PVR (Patel et al., 2004). A recent study compared the outcomes of twenty-five gauge (25-G) surgery in RD complicated with grade C PVR with or without anterior PVR (Sato et al., 2014). The mean number of operations was 1.7 in patients with anterior PVR, classically associated with worse prognosis, and 1.3 in patients without anterior PVR. According to these results, this situation generates a heavy burden not only on these patients but also on the health care system.

2.4. PVR is not only important after RD but it also complicates other intraocular surgeries and prevents therapeutic advances

Finally, PVR is not only a complication of RD, but it also plays an important role in other ocular pathologies, such as penetrating globe trauma or RD after giant retinal tears. It can also appear after techniques such as 360-degree retinectomies that were used for macular translocation in wet AMD (Asaria and Charteris, 2006). Although these and others therapeutic attempts have been abandoned, possible new cell therapies that are already in development would require surgical approaches that could result in PVR (Fernandez-Bueno et al., 2013b).

In summary, to achieve more favorable cost-effective results that diminish the burden on health care systems and to decrease the vast impact on patient quality of life, efforts should be focused on obtaining a reliable method to identify patients at high risk for PVR and who could benefit from effective prophylactic treatment. Novel clinical strategies may require multimodal, combinatorial approaches targeting different signaling pathways involved in this disease (Moysidis et al., 2012; Rojas et al., 2009, 2015; Sadaka and Giuliari, 2012). Moreover, drugs proposed for treatment of PVR must be well considered because their application may be associated with potentially harmful side effects. Finally, correct optimization of dosing and administration of these drugs is needed. For all these reasons further investigation of this disease is still fully justified.

3. The problem of PVR classification and a new proposal

3.1. Historical review

The first widely recognized classification for PVR was published in 1983 (Committee, 1983). The Retina Society Terminology Committee based its work on the condition formerly called “massive vitreous traction” or “massive periretinal proliferation” (Havener, 1976; Machemer, 1978; Scott, 1975). This classification was based on the idea that cellular proliferation was the main feature of PVR. Thus the only clinical factors considered were the extent of fixed retinal folds and the overall configuration of the funnel shaped RD. Other important clinical features, such as the presence of visible subretinal membranes, vitreous mobility, location of trans-vitreous membranes, severity of traction on the anterior retina, equatorial retinal folds, and intraretinal membranes were not incorporated. This approach resulted in a simple classification, easy to use, but not useful in practice as has been demonstrated. In fact, this classification is not used currently by clinicians although there are still some papers dealing with medical treatments that use it (Ganekal and Dorairaj, 2014).

According to the revised classification scheme developed by The Retina Society Terminology Committee, a minimal PVR was designated Grade A and defined as the presence of vitreous haze and pigment clumps, a finding that is not unique of PVR. They can be present in many forms of granulomatous uveitis and in long standing RD without PVR (Rouberol and Chiquet, 2014). Grade B, or moderate PVR, included the presence of surface retinal wrinkling and/or rolled edges of the retinal break that could be accompanied by retinal stiffness and vessel tortuosity. However, these features can be present in some RDs that do not further develop an “extensive” PVR. In fact many surgeons obtained excellent results with the scleral techniques 10 years ago (Afrashi et al., 2005).

Grade C, or marked PVR, was defined as the presence of full thickness retinal folds in one (C-1), two (C-2), or three (C-3) quadrants. Grade D, or massive PVR, was defined as fixed retinal folds in four quadrants that result in a wide funnel shape (D-1), a narrow funnel shape (D-2), or a closed funnel without view of the optic disc (D-3).

These four stages also provided a false idea of the increasing severity of the disease. For example, a D grade PVR could be caused by a localized epiretinal membrane that would be easily managed with surgery. In contrast, a grade C PVR could be associated with intraretinal changes that have a worse prognosis (Pastor et al., 2002).

This classification missed many important clinical characteristics, such as the number and location of retinal breaks and the existence of pre-equatorial forms of PVR. With the idea of solving some of these problems, a new classification was proposed as part of the Silicone Study (Lean et al., 1989). Previous classifications emphasized the involvement of the post-equatorial retina (posterior PVR). The most important contribution of the new classification scheme was the inclusion of proliferative membranes in the pre-equatorial region and on the vitreous base (anterior PVR) that are more frequent after surgical attempts to reattach the retina. Furthermore, attention was paid to the quantitative assessment of PVR by recording the number of clock hours of the retina involved by membranes. In this classification, minimal (Grade A) and moderate (Grade B) classifications remained unchanged, but Grades C and D were replaced by Grades P and A (posterior and anterior forms). Moreover, Grades P and A were further defined by the presence of “types” of contraction. The extension of each grade was

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assessed by the number of clock hours, not necessarily contiguous, of the involved retina. As stated by the authors, this classification does not attempt to predict the PVR severity because factors that influence the results of surgical treatment were not fully understood (Lean et al., 1989).

To meet the need to incorporate changes proposed by the Silicone Study Group (Lean et al., 1989) and modifications proposed also by other authors (Heimann and Wiedemann, 1989), the Retina Society updated its classification in 1991 (Machemer et al., 1991). Three grades of increasing severity were described, emphasizing the posterior and anterior locations of proliferation. The new classification kept Grades A and B, modified Grade C, and eliminated Grade D (RD in funnel configuration). Following the Silicone Study classification, a more detailed description of Grade C (posterior and anterior) PVR was made by adding the types of contraction, the extent of which was detailed by using clock hours instead of quadrants.

In the classification revised by the Retina Society, Grade C was defined as full-thickness retinal folds and/or subretinal bands and pathologic changes that could be posterior, anterior, or both. Posterior grade C PVR was further divided into focal contractions resulting in starfold membrane formation (Type 1) and/or diffuse contractions resulting from confluent starfolds that can result in a closed-funnel configuration (Type 2). Grade C PVR also included anterior or posterior subretinal bands (Type 3). Anterior grade C was divided into circumferential contraction (Type 4) along the posterior margin of the vitreous base and anterior displacement (Type 5) of the peripheral retina (Machemer et al., 1991).

This classification may be too complex to be used in clinical practice and does not offer any indication for selecting the most appropriate management of the disease. Major drawbacks are found in its complexity and inability to be easily reproduced by different clinicians, therefore it has been rarely incorporated into the clinic.

Since the revision by the Retina Society, no other classification scheme has been popularized. Probably for all these reasons, most of clinicians do not use any classification or, alternatively, refer to the 1983 classification scheme (personal unpublished data). There are papers that still consider that the older classification is appropriate for defining the severity of the disease. This can cause controversial results that are, in part, attributable to the subjective assessments of PVR from surgeon to surgeon (Adelman et al., 2013).

3.2. Problems derived from the lack of an accepted classification

This lack of an appropriate and uniform grading system is a crucial point to resolve for evaluating new treatments. It is essential to uniformly evaluate similar cases and thereby facilitate proper comparison of alternative surgical or pharmacologic treatments and clinical results. Currently, efficient communication between clinicians and comparison of different studies are problematic.

One important problem is that all available classifications are purely a description of the ophthalmoscopic findings, and no attempts have been made to add new clinical information based on new imaging techniques. Only a few papers have tried to analyze the role of vitreous posterior detachment as a prognostic factor for PVR development. However, this type of analysis has not been incorporated into either routine examinations for PVR patients or any PVR classification (Capeans et al., 1998; Rezende et al., 2007). Even more, current classifications do not provide information on the activity of the disease, the prognosis, or the functional and/or anatomical outcome after successful treatment.

3.3. Some ideas for a possible new classification

PVR is a relatively acute process. Most of the cases are produced within one month after the retina is detached and surgically repaired. According to our experience, 77% of postoperative forms appear within one month after surgery and 95% in the first 45 days (Pastor, 1998; Pastor et al., 2002). Therefore it should be possible to find information on the stage of the disease, active or quiescent, which is important from the therapeutic point of view. During the 1990's, some surgeons recommended avoiding surgery at the early stages of the disease because it was demonstrated that after PVR was initiated, it could be stimulated by additional surgery (Campochiaro, 1997). The added degree of inflammatory activity could stimulate the so called “re-proliferation”, an important cause of failure after PVR surgery (Berker et al., 2007; Pastor, 1998; Pastor et al., 2002).

While cell proliferation in PVR peaks during the firsts 4 days after RD, it is reduced around day 7 and then continues thereafter at a lower rate (Fisher et al., 1991; Lewis et al., 2003). These events persist over the time in PVR, but currently we are not able to quantify the activity of these processes. The dynamics of retinal remodeling may have important practical implications if we were able to treat the disease at the right time. All this information is crucial when comparing different treatments and series, but currently our understanding of retinal remodeling dynamics and the forces that control it is very poor.

Finally, all of the classifications miss crucial aspects of the pathogenesis of the disease. For instance, they do not incorporate the presence and extension of intraretinal changes that are directly related to poor anatomical outcomes and functional prognoses (Pastor, 1998; Pastor et al., 2002, 2006b, 2003). The classification schemes also do not include the presence and importance of inflammation (Table 1) (Martin et al., 2003; Moysidis et al., 2012; Pastor et al., 2002; Rojas et al., 2010; Sanabria Ruiz-Colmenares et al., 2006; Symeonidis et al., 2012; Zhang et al., 2012).

3.4. Intraretinal changes are crucial

It is now clear that intraretinal PVR is the most severe form in which there are major changes in retinal architecture leading to significant dysfunction (Figs. 5, 6 and 8). Furthermore, these forms have a huge influence in the surgical complexity and in the anatomical and functional outcome, especially when the posterior pole is involved. In contrast, epiretinal or subretinal membranes can be relatively easily removed by surgery, and the prognosis of a macular epiretinal membrane is obviously better than an intraretinal one (Pastor et al., 2002, 2003). However until now, intraretinal changes have been detectable only during surgery when the surgeon could not appose the retina to the eyewall. Also, little attention has been paid to the identification of these changes before surgery or in the postoperative period. Even in recent papers these retinal changes have not been recognized (Rouberol and Chiquet, 2014). Nevertheless, there is hope that the use of new imaging technologies could add relevant information regarding the intraretinal changes (Boroomand et al., 2013).

Considering these facts, we believe that attention should be focused on changes in the retinal tissue rather than membrane extensions. We propose a simplified classification scheme with four major PVR forms: epiretinal, intraretinal, subretinal (rare) and mixed (most frequent). (Table 2) Further, we propose that the research efforts in the coming years focus on acquiring more information regarding the severity and extension of intraretinal changes. To achieve these goals, major components of these studies should include clinical information on intraretinal PVR extension, the role of intraocular inflammation, and the contribution of

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Table 1
Summary of the most relevant facts of the previous PVR classifications.

Classification	Most important features	Most critical disadvantages
1983[a]	Identifies PVR as an independent clinical entity	Based on ophthalmoscopic appearance Provides false idea of severity and progressiveness Does not recognize intraretinal changes
1989[b]	It added anterior forms and a detailed description of PVR extension an types of membranes and contraction	Too complex to be used in clinic Does not provide information of progressiveness Does not recognize intraretinal changes
1991[c]	Quite similar to the Silicone Study classification Simpler to use	Does not provide information of progressiveness Does not recognize intraretinal changes

Grades A and B are common in all classifications. They do not provide specific information on the further development of extensive PVR.
[a] Committee, T.R.S.T., 1983. The classification of retinal detachment with proliferative vitreoretinopathy. Ophthalmology 90, 121–125.
[b] Lean, J.S., Stern, W.H., Irvine, A.R., Azen, S.P., 1989. Classification of proliferative vitreoretinopathy used in the silicone study. The Silicone Study Group. Ophthalmology 96, 765–771.
[c] Machemer, R., Aaberg, T.M., Freeman, H.M., Irvine, A.R., Lean, J.S., Michels, R.M., 1991. An updated classification of retinal detachment with proliferative vitreoretinopathy. Am J Ophthalmol 112, 159–165.

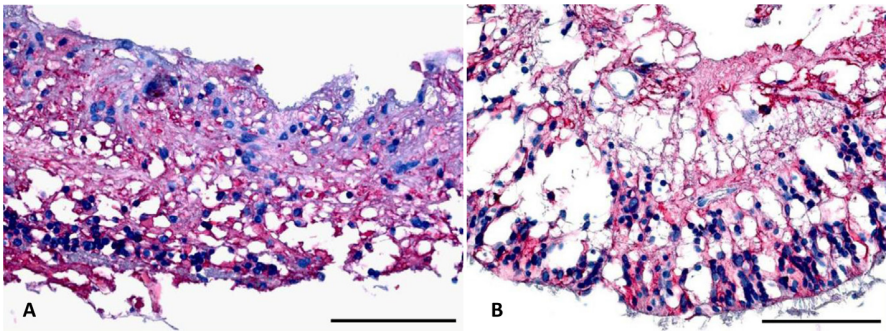

Fig. 8. Immunohistochemistry staining of PVR retinal samples obtained by retinectomy (A–B). The images show high glial fibrillary acidic protein (GFAP) immunoreactivity (red) resulting in gliotic retina (intraretinal PVR) with complete disorganization of the retinal tissue. (20× images. Scale bar: 100 μm).

Table 2
Critical elements for a new classification.

Type of morphologic changes	
Epiretinal	Membranes on retinal or vitreous surface
Intraretinal	Retinal shortening without epiretinal membranes
Subretinal	Any type of subretinal membrane, band, etc
Extension of changes	
By quadrants	
Retinal thickness by OCT	
Signs of severity and progressiveness	
Presence of SNP of risk	
Clinical risk factors clearly identified	
Time of evolution after onset	
Still unidentified signs	
New biomarkers able to determine presence of intraocular inflammation	

OCT: optic coherence tomography.
SNP: single nucleotide polymorphisms.

genetic profiles that influence the inflammatory response. Such information is critical to the ophthalmologist in deciding on the timing of surgical intervention and the use of adjuvant treatments.

4. Criticism of the current experimental models

From a theoretical point of view, experimental models should provide a systematic and controlled way to study cellular changes that occur in PVR. They can help to expand our knowledge of the pathobiological processes and also allow testing of new therapeutic agents. Unfortunately, most of the published models are based on the idea that pathogenic cellular proliferation into the vitreous cavity is the main feature in PVR, and the models have tried to reproduce this event.

Initially, models were created simply by injecting cells, mainly dermal fibroblasts, into the vitreous cavity. These efforts were followed by adding some other components such as inflammation, blood, cytokines, and so on, with or without cells (Agrawal et al., 2007). Obviously most of these models were used for testing anti-proliferative and anti-growth factor drugs. In some cases with positive results, these efforts stimulated researchers to transfer protocols into the clinic (Charteris et al., 2004). Unfortunately, these kinds of treatments failed, and the research was re-oriented towards other pathways. Nevertheless in some specific ways, these models could still have value, thus we have summarized them below.

4.1. *In vitro* models of PVR

In vitro systems can be used to analyze the behavior of defined cell populations under certain conditions. RPE cell culture has been widely used in the study of PVR, as they have been considered the major cell type involved in PVR pathogenesis (Bastiaans et al., 2014; Rouberol and Chiquet, 2014). Other *in vitro* models are based on vitreous explants and retinal organ cultures (Agrawal et al., 2007). However, the results of these studies are considered speculative and must be followed by *in vivo* studies.

A major criticism of *in vitro* model systems, mainly those based on cell culture, is that they do not adequately replicate a relatively complex tissue such as the retina, including the relationship with blood vessels. They also do not take into account the importance of the glial responses in RD and PVR, a common problem cited for organotypic cultures (Wickham and Charteris, 2009).

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4.2. In vivo models of PVR

Over 27 animal models of PVR have been described. For a recent review, see Agrawal et al. (2007). Intravitreal injections of different cell types or factors reported to play a role in this disease have been performed to reproduce the pathological processes that lead to PVR. The most common of these models consists of the injection of fibroblasts, which are not present in the healthy human retina but are present in PVR membranes (Moysidis et al., 2012; Pennock et al., 2011; Trese et al., 1985). However the fibroblast-like cells observed in human intravitreal membranes are now identified as the products of mesenchymal transformation of RPE cells (Chiba, 2014). Most of these experimental models used fibroblasts from dermal origin (Khawly et al., 1991). These cells are very different from the cells present in human PVR, although they may be somewhat similar to the intraocular proliferation of cells that occurs after ocular penetrating trauma (Hsu and Ryan, 1986). The problem with these models is that they depend on the type of cells that are chosen for injection into the vitreous rather than the cells that are actually present in PVR, i.e., RPE cells and glial cells that have transdifferentiated into fibroblasts.

Other models involve the intravitreal injection of different cell types such as RPE cells (Fastenberg et al., 1982; Radtke et al., 1981; Wong et al., 2002), macrophages (Hui et al., 1989), and platelets (Garcia-Layana et al., 1997; Pastor et al., 2000), among others. These models may have value for analyzing some specific features and for better understanding the pathogenesis of the disease. Unfortunately they have mostly been used for testing new proposed drugs, mainly anti-proliferative agents. As was the case for other studies of this type, positive results in animal models did not presage successful treatment for patients.

Other *in vivo* models have placed more emphasis on reproducing intraocular inflammation and the subsequent release of cytokines and growth factors (Pastor, 1998). These have included surgical manipulations such as lensectomy, vitrectomy, induction of RD, cryotherapy, and also penetrating trauma (Agrawal et al., 2007; Garcia-Layana et al., 1997; Goldaracena et al., 1997). These models could still have some value in terms of analyzing the preliminary changes occurring in PVR, but attention should be paid to the types of animals used for them. The retinal vascularization, glial components, and others elements of PVR are not identical among the different animal models, and the critical points in the development of experimental PVR could be unique to each species and different from that in humans.

Regarding the different animal species, rabbit is the most frequently used because rodents have a small eye and a relatively wide lens that interferes with the manipulation to induce PVR. In terms of retinal vasculature, ideally animal models should have a holoangiotic retina, which means the whole retina is vascularized like that of most mammals. The opposite retinal vascularization scheme is merangiotic, such as the rabbits, where blood vessels are present only in a smaller part of the retina. The disparate vascular patterns may play a critical role in the different behavioral responses to retinal trauma present in animal models and humans (Trivino et al., 1997).

Besides the vascular component, the presence and distribution of retinal glial cells is also an important factor for developing adequate PVR models. The retina contains Müller cells that are specialized radial glia that make extensive contacts with retinal neurons. As mentioned, in humans, Müller glial cells occupy up to 20 percent of the overall volume of the retina, and the density of these cells approaches 25,000 per mm^2 of the retinal surface area (Bringmann et al., 2006). Each Müller cell forms contacts with a clearly defined group of neurons organized in a columnar fashion. In humans, a single Müller cell supports approximately 16 neurons, while in rodents each one supports up to 30 neuronal cells (Bringmann et al., 2006).

Moreover, it is well known that biological responses differ among the species. For instance, post-transcriptional regulation of human NO synthetase 2 (NOS2) is very different from that in mouse. This simple step makes the resulting levels of NO produced by activation of human NOS2 lower than levels produced by mouse NOS2. This difference has very important implications in the tissue redox environment and immune responsiveness (Hoos et al., 2014).

Because PVR is a multifactorial disease and it results from the interaction of genetic and environmental factors, its pathogenesis may include many different events and signaling pathways (Pastor-Idoate et al., 2013b; Pennock et al., 2014a). Therefore, those experimental models that try to simulate a series of events that are not yet completely understood and which may involve pathways that are not present in human PVR pathogenesis, may in fact be very poor and misleading models of the human disease (Moysidis et al., 2012).

For all these reasons, the results of many of the above cited studies have limitations in their interpretation because there are important pathological differences between these animal model and human diseases. Nevertheless, despite these limitations, experimental models are especially important in complex diseases and in those with unknown pathogenesis like PVR, and they may provide crucial steps for the development of new therapeutic options if they are properly oriented. (Table 3).

5. Clinical risk factors

Clinical risk factors for developing PVR following a RD have been assessed since the early stages in the study of this process. Theoretically, identification of these factors could be useful for elucidating the mechanisms of pathogenesis, providing guidelines for the management of RD, and perhaps for making the decision of adding an adjuvant treatment (Cowley et al., 1989). Also, the aim of understanding the risk factors is to identify those patients at high risk and to avoid the preventable risks if possible, or to modify the surgical options (Bonnet, 1984, 1988; Chignell et al., 1973; Cowley et al., 1989; Tolentino et al., 1967; Yoshida et al., 1984). The identification of risk factors seems today very important because we are facing new therapeutic options. Thus it is necessary to identify the patients who may be prime candidates for pharmacologic treatments to prevent the occurrence and re-occurrence of PVR (Asaria and Gregor, 2002; Kon et al., 2000).

5.1. Types of clinical studies

Several attempts have been made to predict the risk of PVR development based on the clinical characteristics of RD patients (Asaria and Gregor, 2002; Kon et al., 2000; Rodriguez de la Rua et al., 2005). Unfortunately, the results so far have not been very consistent, and several formulas based on these factors have failed in demonstrating usefulness for routine clinical use (Asaria et al., 2001; Kon et al., 2000; Rodriguez de la Rua et al., 2005; Sala-Puigdollers et al., 2013; Wickham et al., 2011). However, the majority of these studies have been made retrospectively, and the results are often contradictory and inconclusive (Kon et al., 2000). It is worth noting that many differences in these studies exist in design, methodology, definitions, and statistical analysis, and therefore they cannot be directly compared (Asaria and Gregor, 2002). Other studies were oriented to discern the influence of a single specific factor or were focused on the identification of risk factors associated with specific circumstances such as aphakia, preoperative PVR, or subretinal PVR. However, these approaches are not appropriate for a multifactorial disease like PVR (Rodriguez

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Table 3
Experimental models of PVR used in recent studies. RPE: retinal pigment epithelium. EMT: epithelial mesenchymal transition. PDGF: platelet-derived growth factor. PDGFR: platelet-derived growth factor receptor.

Intervention		Animal species used	Advantages	Disadvantages
Injection	Fibroblasts + platelet rich plasma[a]	Pigmented rabbit	More rapid development, more consistent and severe compared to simple fibroblasts injection. Easy to perform, reproducible	Use of external fibroblasts. Exogenous influx of cells. Cells must be cultured to obtain sufficient number, which may cause lost of *in vivo* characteristics. No primary alteration of RPE. Too rapid time course may not be useful to test therapeutic agents
	RPE (Autologous, homologous)[b]	Pigmented rabbit	Use of RPE cells, which are primarily involved in PVR. Easy to perform, reproducible	Exogenous influx of cells. Cells must be cultured to obtain sufficient number, which may cause lost of *in vivo* characteristics. Cells may not suffer EMT
	RPE + Platelet rich plasma[c]	Wistar rat	Use of RPE cells, which are primarily involved in PVR. More rapid development, more consistent and severe compared to simple RPE injection. Easy to perform, reproducible. Suitable animal species for research	Exogenous influx of cells. Cells must be cultured to obtain sufficient number, which may cause lost of *in vivo* characteristics. Cells may not suffer EMT
	Dispase[d]	Mouse	Suitable animal species for genetic manipulation. Easy to perform, reproducible	Epiretinal membranes lack RPE-derived cells Small size of the animal limits surgical procedures
Surgical intervention + injection	RPE cell injection + gas vitreous compression	Albino rabbits[e] Pigmented rabbits[f]	Use of RPE cells, which are primarily involved in PVR. Gas compression provides a reliable technique to generate vitreous alteration, and seems easier than vitrectomy	Exogenous influx of cells. Cells must be cultured, which may cause lost of *in vivo* characteristics.
	RPE cell injection + Posterior vitreous detachment + Retinal detachment[g]	Pig	Use of RPE cells, which are primarily involved in PVR. Reproduces posterior vitreous detachment and retinal detachment, as it occurs in human disease. Animal species used more accurately resemble human retina	Absence of glial cell contribution in the membranes. Relative rapid time course may interfere with study of early stages of disease
	Blood (autologous) injection + Scleral incision + partial vitrectomy + wound closure, avoiding lens and retinal injury[h, i]	Pigmented rabbit	No need to culture cells. Reproduces posterior vitreous alteration and wound healing	Model resembles ocular trauma. Red blood cells impede ophthalmoscopic visualization. Total lack of pigmentation suggests glial origin of membranes rather than RPE
Surgical intervention	Lensectomy + vitrectomy + peripheral retinotomy through corneal incision[j]	Mouse	Reproduces vitreous alteration. Suitable animal species for genetic manipulation. Suitable for study early stages of PVR	Small size of the animal limits surgical procedures. No RPE migration into vitreous cavity
Growth factor model	Mouse fibroblasts expressing PDGFRs + Platelet rich plasma + partial vitrectomy + gas compression[k]	Pigmented rabbit	Allows the study of PDGF, which is supposed to play an important role in PVR. Gas compression provides a reliable technique to generate vitreous alteration	This factor of interest is the main one expressed, which may differ from human disease and may interfere with response of cells. Complex cell obtention

[a] Nakagawa, M., Refojo, M.F., Marin, J.F., Doi, M., Tolentino, F.I., 1995. Retinoic acid in silicone and silicone-fluorosilicone copolymer oils in a rabbit model of proliferative vitreoretinopathy. Investigative ophthalmology & visual science 36, 2388–2395.
[b] Radtke, N.D., Tano, Y., Chandler, D., Machemer, R., 1981. Simulation of massive periretinal proliferation by autotransplantation of retinal pigment epithelial cells in rabbits. American journal of ophthalmology 91, 76–87.
[c] Zhao, H.M., Sheng, M.J., Yu, J., 2014. Expression of IGFBP-6 in a proliferative vitreoretinopathy rat model and its effects on retinal pigment epithelial cell proliferation and migration. International journal of ophthalmology 7, 27–33.
[d] Canto Soler, M.V., Gallo, J.E., Dodds, R.A., Suburo, A.M., 2002. A mouse model of proliferative vitreoretinopathy induced by dispase. Experimental eye research 75, 491–504.
[e] Lee, J.J., Park, J.K., Kim, Y.T., Kwon, B.M., Kang, S.G., Yoo, Y.D., Yu, Y.S., Chung, H., 2002. Effect of 2'-benzoyl-oxycinnamaldehyde on RPE cells *in vitro* and in an experimental proliferative vitreoretinopathy model. Investigative ophthalmology & visual science 43, 3117–3124.
[f] Kuo, H.K., Chen, Y.H., Wu, P.C., Wu, Y.C., Huang, F., Kuo, C.W., Lo, L.H., Shiea, J., 2012. Attenuated glial reaction in experimental proliferative vitreoretinopathy treated with liposomal doxorubicin. Investigative ophthalmology & visual science 53, 3167–3174.
[g] Umazume, K., Barak, Y., McDonald, K., Liu, L., Kaplan, H.J., Tamiya, S., 2012. Proliferative vitreoretinopathy in the Swine-a new model. Investigative ophthalmology & visual science 53, 4910–4916.
[h] Cleary, P.E., Ryan, S.J., 1979a. Experimental posterior penetrating eye injury in the rabbit. I. Method of production and natural history. The British journal of ophthalmology 63, 306–311.
[i] Cleary, P.E., Ryan, S.J., 1979b. Experimental posterior penetrating eye injury in the rabbit. II. Histology of wound, vitreous, and retina. The British journal of ophthalmology 63, 312–321.
[j] Saika, S., Kono-Saika, S., Tanaka, T., Yamanaka, O., Ohnishi, Y., Sato, M., Muragaki, Y., Ooshima, A., Yoo, J., Flanders, K.C., Roberts, A.B., 2004. Smad3 is required for dedifferentiation of retinal pigment epithelium following retinal detachment in mice. Laboratory investigation; a journal of technical methods and pathology 84, 1245–1258.
[k] Andrews, A., Balciunaite, E., Leong, F.L., Tallquist, M., Soriano, P., Refojo, M., Kazlauskas, A., 1999. Platelet-derived growth factor plays a key role in proliferative vitreoretinopathy. Investigative ophthalmology & visual science 40, 2683–2689.

de la Rua et al., 2005). In addition, the high incidence of PVR found in some of these studies suggests that samples were composed of complex cases, and thus they are not representative of the population of non-complicated RD (Rodriguez de la Rua et al., 2005). Nevertheless, they have contributed valuable information because they have helped to unravel the complex nature of the disease. Most of the identified clinical risk factors are consistent with the inflammatory nature of PVR. While inflammation plays an important role in every case of PVR, it is not a unique factor (Pastor et al., 2002; Rodriguez de la Rua et al., 2005).

Some studies have been specifically designed to prove a particular hypothesis regarding the pathological origin of PVR, a decision that could implicate an important bias (Asaria and Charteris, 2006). For example, RPE cells are considered crucial in the development of PVR; therefore, many circumstances related to the high release of these cells have been investigated, such as the

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size of the retinal break, the use and intensity of cryo-application, the use of vitrectomy before or after the cryotherapy, etc. However, any risk factor should be identified as an independent parameter. Unfortunately most of the suspected risk factors are clearly related to each other, and it is not possible to isolate them from other circumstances (Rodriguez de la Rua et al., 2005).

5.2. Preoperative, intraoperative, and postoperative risk factors

Risk factors are classified as preoperative, intraoperative, or postoperative. Preoperative factors associated with a higher risk of PVR include ocular trauma (Girard et al., 1994), a history of prolonged intraocular inflammation (previous uveitis), prior infectious retinitis, and low intraocular pressure (IOP) secondary to intraocular inflammation (Girard et al., 1994; Pastor et al., 2002; Rodriguez de la Rua et al., 2005; Rodriguez de la Ruz Franch et al., 2000; Wickham et al., 2011; Yoshino et al., 1989). Retinal tear characteristics such as large size and number and RD extension are also some of the clinical factors almost constantly associated with the development of PVR (Rodriguez de la Rua et al., 2005; Rodriguez de la Ruz Franch et al., 2000; Wickham et al., 2011; Yoshino et al., 1989). Other preoperative risk factors include RD associated with vitreous hemorrhage (Duquesne et al., 1996), aphakia, previous intraocular surgery, previous choroidal detachment, and preoperative grade A or B PVR. All of these preoperative risk factors are in concordance with the inflammatory nature of PVR (Asaria et al., 2001; Bonnet, 1984, 1988; Yoshino et al., 1989).

Intraoperative risk factors include vitreous or subretinal bleedings, inability to fully close retinal tears, intraoperative choroidal detachments (Cowley et al., 1989), pigment release during endodrainage (Lleo Perez et al., 2000), and excessive cryotherapy (Bonnet, 1988; Cowley et al., 1989) or endolaser (Rodriguez de la Rua Franch et al., 2000). Both strategies of retinopexy induce a pro-inflammatory environment that could explain the high risk as shown in experimental models (Garcia-Layana et al., 1997; Goldaracena et al., 1997; Pinon et al., 1992). Indeed, in a prospective, randomized, placebo-controlled, double-blind clinical trial performed to determine if prolonged administration of systemic corticosteroids would attenuate early stages of PVR, the application of transcleral cryocoagulation was associated with more cases developing epiretinal membranes in the placebo than the steroid group (Koerner et al., 2012). These findings reinforce the idea that the anti-inflammatory strategy could be one of the best options for preventing PVR. While other clinical trials have not reported the same results (Dehghan et al., 2010), it is possible that the time of application or intraocular levels of steroid may not have been the most appropriate.

Postoperative risk factors for inducing PVR include prolonged inflammation or uveitis, new or persistent vitreous hemorrhage, postoperative choroidal detachment, the use or air or sulphur hexafluoride (SF_6), repeated surgical procedures, loss of vitreous during drainage of subretinal fluid, persistent traction of breaks, and the presence of unadverted or non-appropriated closed tears or holes. (Asaria et al., 2001; Pastor et al., 2002). Most of these are clearly related to inflammation. Table 4 summarizes some of the clinical risk factors identified by large clinical studies.

5.3. Some other problems and formulas

The design of many clinical retrospective studies for identifying clinical factors that predispose the onset of PVR is very weak. Often the many factors that contribute to PVR development are closely related to others and cannot be analyzed in an independent way. Nevertheless, there are a few studies with the appropriate methodology. One prospective study included 409 eyes and tested the influence of 14 categories of clinical variables (Duquesne et al., 1996). Using single and multiple logistic regression analysis, only four variables were found to independently and jointly be associated with the risk of postoperative PVR. Among them, two have been confirmed by further studies: 90° or greater circumferential extent of the retinal tears and the use of cryopexia (Sadaka and Giuliari, 2012).

In 2005, our group performed an observational case-controlled study among 335 patients (201 control and 134 cases) with non-complicated RD (Rodriguez de la Rua et al., 2005). Risk factors for PVR were identified by multivariate analysis, and the influence of variables was assayed according to the surgical approach. Once again, breaks larger than “1 clock hour” and extension of RD were found to be risk factors for PVR. Age and lower IOP, scleral surgical techniques, aphakia/pseudophakia when associated with scleral buckle and re-interventions were also risk factors. We proposed a statistical model that was independent of the surgical procedure employed for RD repair to estimate the probability of developing PVR for any patient. The best values of sensitivity and specificity obtained with this model were 78.0% and 75.6% respectively, which were higher than those obtained by previous studies (Asaria et al., 2001; Kon et al., 2000). The area under the receiver operating characteristic curve was 0.86. Nevertheless, those values were not sufficiently high for routine clinical use (Rodriguez de la Rua et al., 2005).

More recently, Wickham et al. (2011) devised a simplified formula that used preoperative clinical data to estimate the risk of PVR following primary RD repair by vitrectomy. Vitreous hemorrhage, grade C PVR, and the extent of detachment were related to failure due to PVR. There was good agreement between risk estimates produced by the point system and those calculated directly using a multivariate regression model. The area under the receiver operating characteristic (ROC) curve for the model was 0.84 (Wickham et al., 2011). Nevertheless, in general the low sensitivity and specificity of these formulas has rendered them unsuitable for routine clinical use (Asaria et al., 2001; Kon et al., 2000; Rodriguez de la Rua et al., 2005; Wickham et al., 2011).

There is another critical problem regarding the usefulness of the predictive formulas. These models tend to perform better with the data from which they were constructed rather than with new data (Bleeker et al., 2003). Predictive formulas require external validation with a new sample before being fully validated and implemented in clinical practice (Bleeker et al., 2003; Terrin et al., 2003). Our group performed an external validation of the four previously published formulas for predicting PVR development after RD surgery, each of which were developed with different criteria (Sala-Puigdollers et al., 2013). All four formulas (Asaria et al., 2001; Kon et al., 2000; Rodriguez de la Rua et al., 2005; Wickham et al., 2011) had limited ability to prospectively identify patients who would develop PVR, and therefore they are not reliable for general use in the clinic (Sala-Puigdollers et al., 2013). Thus, it is apparent that clinical factors alone do not provide sufficient predictive power to identify patients at high risk of PVR.

In fact, it is only when additional variables such as genetics or biochemical biomarkers are considered that performance of predictive models improves (Ricker et al., 2012; Rojas et al., 2015). These new formulas, after appropriate validation, could provide novel tools in our current clinical practice to identify those patients at high risk of developing PVR. This is a crucial step for designing new clinical trials. As we will discuss further, the inclusion in clinical trials of only those patients with a high risk of PVR will reduce the sample size and increase the power of the results.

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Table 4
"Risk factors for PVR". Main papers analyzing risk factors for developing PVR. "**X**" means that the variable evaluated in the study was significantly associated to PVR. RD. retinal detachment. SB: scleral buckle. PPV: pars plana vitrectomy. Pros: prospective study. Retrosp: tretrospective study. E: evaluated. NE: non evaluated.

Design of the study	Bonnet M. 1984	Bonnet M. 1988	Yoshida A. 1984	Cowley M. 1989	Malbran E. 1990	Girard P. 1994	Duquesne N. 1996	Kon CH. 1999
	354 eyes, complicated and non-complicated RD (included trauma), SB and/or PPV	Prosp. 70 eyes, SB	Retrosp. 521 eyes, complicated and non-complicated RD, SB	Retrosp. 607 eyes, complicated and non-complicated R (included trauma) D, SB and/or PPV	Retrosp. 1180 eyes, complicated and non-complicated RD	Retrosp. 1020 patients, complicated and non-complicated RD	Prosp. 409 eyes, RD	Prosp. 140 eyes, complicated RD, PPV
Preoperative PVR	X	X	X	X	E	X	X	NE
Tear/break characteristics	X	E	X	E	X	X	X	NE
Size of RD	NE	E	NE	E	NE	X	NE	NE
Aphakia/pseudophakia	NE	NE	NE	E	NE	E	E	NE
Genetic profile of patients	NE	NE	NE	NE	NE	NE	NE	NE
Vitreous cytokines/proteins	NE	NE	NE	NE	NE	NE	NE	X
Pre/intraoperative vitreous hemorrhage	NE	X	X	E	NE	X	E	NE
Reintervention	X	NE	E	E	NE	E	NE	NE
Postoperative choroidal hemorrhage	NE	NE	X	X	NE	X	NE	NE
Preoperative choroidal hemorrhage	NE	NE	E	X	NE	X	NE	NE
Technical procedure (PPV or SB)	NE	NE	NE	X	NE	E	E	NE
Tamponade	NE	NE	NE	NE	NE	X	E	NE
Cryopexy	NE	NE	NE	X	NE	NE	X	NE
Laser	NE	NE	NE	E	NE	E	E	NE
Uveitis	NE	NE	NE	E	NE	X	NE	NE
Age	NE	NE	NE	E	E	E	E	NE
Cytokines /proteins in subretinal fluid	NE	NE	NE	NE	NE	NE	NE	NE

Shading in Table 4 highlights PVR associated risk factors.

6. Designing new strategies for PVR treatment based upon genetics and biomarkers

6.1. Monogenic and complex diseases

The human genome contains an estimated 20,000–25,000 genes that encode all of our proteins (Consortium, 2004). Protein-coding sequences account for only a very small fraction of the genome, approximately 1.5%. The remainder includes non-coding sequences such as introns, short and long interspersed elements, and the rest of the genome for which no function has yet been elucidated (Lobo, 2008).

Inherited human diseases can be classified as monogenic or complex. Monogenic diseases are the result of a mutation in a single gene and are inherited in a Mendelian fashion. These diseases are relatively rare, but they have a high penetrance despite a very low allele frequency. In contrast, complex inherited diseases, which are relatively common, are multifactorial in nature. Most multifactorial diseases have low penetrance and a common allele frequency, occurring in at least 5% of the population (Lobo, 2008). Multifactorial diseases can occur in isolation but environmental influences can increase or decrease the risk of the disease. In this sense, a given *noxa* could induce different responses in different subjects. Thus, an individual's genetic profile could determine if he/she has a greater or lesser susceptibility to a disease under the influences of the same environment (Dempfle et al., 2008).

An important ocular example for the role of genetics is age-related macular degeneration (AMD). In this disease, the importance of the genetic profile in the development of the disease and the response to treatment has been demonstrated (Gemenetzi and Lotery, 2014; Horie-Inoue and Inoue, 2014; SanGiovanni and Chew, 2014; Schramm et al., 2014).

6.2. Usefulness of genetic studies: unraveling the pathogenesis of diseases and biomarkers of risk

Single nucleotide polymorphisms (SNPs), variations at a single isolated nucleotide position, are the most frequent polymorphisms in nature. They are almost always biallelic, involving only one of two choices, such as A or T, at a given site within the population. SNPs have a wide variation in frequency in different populations and may occur anywhere in the genome: exons, introns, or intergenic regions. The SNPs that occur in exons are more likely to be important because they could alter the gene product and predispose a change in phenotype or susceptibility to a disease (functional SNP). However much more commonly, SNPs have no real functional significance but rather serve as markers that are co-inherited with a disease-associated gene as a result of physical proximity. In other words, the SNP and the causative genetic factor are in linkage disequilibrium, and the SNP can be an identifying character when looking for the causative gene (Kumar and Cotran, 1994).

The genetic study of any multifactorial disease can help to unravel the pathogenesis in different ways. For example, in the identification of causative genes, functional SNPs or SNPs in linkage disequilibrium with causative genes could point out genes that have never been implicated in the disease, or they could confirm the role of other genes previously identified (Rojas et al., 2013).

Groups of biomarkers such as SNPs can serve as indicators of risk for multigenic diseases. As such, they could be used to select high-risk patients for recruitment into clinical trials for assessing new procedures or therapeutic agents. This could reduce the required sample size in a dramatic way because it would enable the selection of a more homogenous population of subjects, as discussed below.

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Kon CH. 1998	Kon CH. 2000	Rodriguez de la Rua E. 2000	Rodriguez de la Rua E. 2005	Rojas J. 2009	Rojas J. 2010.	Wickham L. 2011	Ricker LJ. 2012	Rojas J. 2015.	Times found it as risk factor
Prosp. 140 eyes, complicated RD, PPV	Prosp. 136 eyes, complicated RD, PPV	Retrosp. 298 eyes, complicated and non-complicated RD, PPV and/or SB	Prosp. 335 eyes, non-complicated RD, PPV and/or SB	Retrosp. and prosp. 450 eyes, non-complicated RD, PPV and/or SB	Retrosp. and prosp. 450 eyes, non-complicated RD, PPV and/or SB	Prosp. 615 patients, complicated and non-complicated RD, PPV	Retrosp. 75 eyes, primary RD, SB.	Retrosp. and prosp. 546 eyes, non-complicated RD, PPV and/or SB	
NE	X	X	E	NE	NE	X	X	X	11
NE	NE	E	X	NE	NE	E	E	NE	6
NE	E	X	X	NE	NE	X	E	NE	4
NE	X	E	X	NE	NE	E	X	NE	3
NE	NE	NE	NE	X	X	NE	NE	X	3
X	X	NE	NE	NE	NE	NE	NE	NE	3
NE	E	E	E	NE	NE	E	E	NE	3
NE	NE	X	X	NE	NE	NE	NE	NE	3
NE	NE	E	E	NE	NE	NE	NE	NE	3
NE	NE	E	E	NE	NE	NE	NE	NE	2
NE	NE	E	X	NE	NE	NE	NE	NE	2
NE	E	X	E	NE	NE	NE	E	NE	2
NE	E	E	E	NE	NE	NE	E	NE	2
NE	E	X	E	NE	NE	NE	NE	NE	1
NE	E	E	E	NE	NE	E	NE	NE	1
NE	E	E	X	NE	NE	E	E	NE	1
NE	NE	NE	NE	NE	NE	NE	X	NE	1

Another advantage of studying the genetic component of any multifactorial disease is the possibility of identifying biomarkers of the response to treatment.

6.3. Genetics of PVR

With these concepts in mind and taking into consideration the weak ability of primary RD patient clinical characteristics to predict the risk for developing PVR, we thought that the genetic composition of these patients could have an important role. Our working hypothesis was that RD can evolve in different ways depending on the genetic profile of each patient (Sanabria Ruiz-Colmenares et al., 2006).

Most genetic studies of PVR have been performed by analyzing monogenic diseases such as Norrie disease and familial exudative vitreoretinopathy among others (Poulter et al., 2012; Robitaille et al., 2011; Yang et al., 2012). Because single genes are responsible for each of those diseases, the pathogenesis of these vitreoretinopathies has nothing in common with PVR secondary to a primary RD.

As mentioned, some years ago we performed a preliminary genetic study that reinforced the idea that the genetic component could have a role in the risk of PVR following RD (Sanabria Ruiz-Colmenares et al., 2006). Later, a replicated candidate gene association study confirmed the implication of the SMAD7 gene and the TNF locus in the pathogenesis of PVR (Rojas et al., 2010, 2013). In addition p53 and MDM2, genes that play roles in apoptosis, have been implicated in the development of PVR (Pastor-Idoate et al., 2013a, 2013b). All of these efforts have been made thanks to a collaborative study that we named “Retina 4 Project”.

6.4. Designing new strategies for PVR treatment guided by genetics

The findings of the Retina 4 Project have resulted in recognition of the importance of early inflammation mediators such as TNF-α in the development of PVR. This and other mediators of inflammation are potentially new targets for preventing PVR. Our group has demonstrated that adalimumab, a TNF-α blocker, reduced the reactive retinal glio
sis in organotypic cultures of porcine neuroretinas (Fernandez-Bueno et al., 2013a). Also, the genetic study of PVR pointed out the relevance of TGF-β mediators in the onset of fibrosis that occurs in PVR. Our group's results support the idea that anti-TNF-α could serve as a novel way to prevent or even treat the gliosis observed in PVR.

The TGF-β pathway is another potential target pointed out by our study (Rojas et al., 2013). SMAD7 is a mediator that acts as an inhibitor of the profibrotic action of TGF-β by blocking phosphorylation of SMAD2 and SMAD3. SMAD7 is a mediator in the TGF-β pathway, and it acts as an inhibitor of the pro-fibrotic action of TGF-β by blocking phosphorylation of SMAD2 and SMAD3 (Flanders, 2004; Saika et al., 2008). In mice, overexpression of SMAD7 inhibited the RPE transition to myofibroblasts elicited by the addition of TGF-β2 (Saika et al., 2007). As a result, the RPE fibrogenic response was inhibited. The replication genetic study highlighted the possible role of SMAD7 in the development of PVR in humans, and potentially opened a new therapeutic target (Rojas et al., 2013). Finally, a Korean research group has found that pirfenidone, a small compound with combined anti-inflammatory and antioxidative action, inhibits TGF-β1-induced fibrogenesis by blocking nuclear translocation of Smads in the human retinal pigment epithelial cell line ARPE-19 (Choi et al., 2012). The authors proposed this inhibitor of the TGF-β pathway as a potential treatment for preventing PVR.

The Retina 4 Project also revealed the importance of apoptosis in PVR by the involvement of both the p53 and MDM2 genes. The

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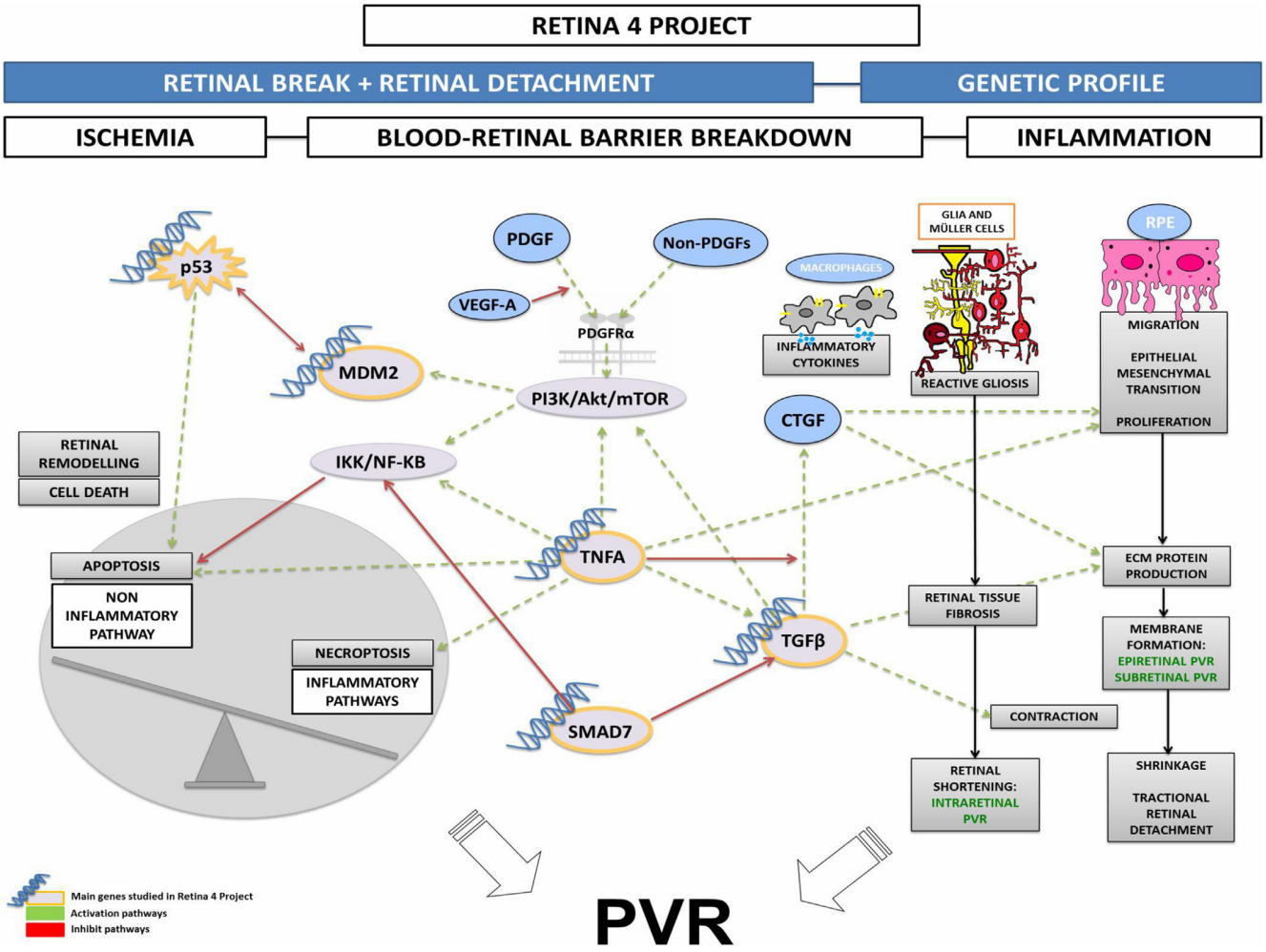

Fig. 9. This figure represents a summary of our ideas in the pathogenesis of PVR highlighting the genetic findings and their relations with the tissular changes and mechanisms of neuronal death.

tumor suppressor gene p53 is crucial for host defense against genomic mutations. It is well known for its ability to induce cell cycle arrest, apoptosis, senescence, and differentiation following cellular stress (Hede et al., 2011).

Our colleagues Gomez-Sanchez et al. from the University of Salamanca reported that the human Tp53 Arg72Pro SNP is responsible for worse functional prognoses in patients suffering stroke (Gomez-Sanchez et al., 2011). The basis for this observation was that the variant SNP was associated with an increase of apoptotic activity. The presence of neurons prone to apoptosis in the ischemic penumbra and perihematoma may account for poor prognosis (Gomez-Sanchez et al., 2011). Therefore with the assistance of the Salamanca group, the presence of this SNP was analyzed by our group for our sample of RD patients with and without PVR.

A SNP variant of the p53 gene associated with anti-apoptotic activity resulted in increased risk for developing PVR (Pastor-Idoate et al., 2013a). This suggests that the variant SNP could be associated with an enhanced inflammatory process after RD. We also found that a SNP variant of MDM2, one of several apoptosis mediators in the p53 signaling pathway, was related to a high risk of PVR (Pastor-Idoate et al., 2013b). Pro-apoptotic agents such as crocetin and other drugs currently being evaluated in early phases of clinical trials (Li et al., 2015) could be proposed in the near future as another strategy directed toward preventing PVR.

The identification of SMAD7, TNF-locus, p53, and MDM2 in PVR or any other gene associated with human diseases provides the rational for research of new drugs, or better, already developed drugs, in the treatment or prevention of these diseases (Fig. 9).

6.5. Designing new strategies for PVR prevention based upon genetic biomarkers

The identification of patients at high risk for developing PVR would help to diminish the required sample size to test more specific treatments or therapeutic strategies. Our group developed three predictive models of PVR based on the analysis of genetic variables (Rojas et al., 2009). One of these models withstood external validation, offering as good a predictive accuracy as obtained prior to the validation (Rojas et al., 2015). Also, the analysis of the relevant clinical and genetic variables significantly improved the discriminatory capability of the model (Rojas et al., 2015). Our model, which incorporated the genetic analysis along with clinical data and was validated by an external sample, had much better predictive power for the risk of PVR than did other studies that did not incorporate a genetic component (Asaria et al., 2001; Kon et al., 2000; Rodriguez de la Rua et al., 2005; Rojas et al., 2015; Sala-Puigdollers et al., 2013; Wickham et al., 2011).

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Table 5
Genetic contribution to PVR development.

Retina 4 project
Genetic contribution to PVR development

GEN	Polymorphisms		Reasons for analysis/potential contribution to PVR
CTGF	rs1931002	rs928501	- Participates in fundamental biologic processes including wound healing and fibrosis
	rs4897554	rs9399005	- Under the influence of TGF-β and CTGF, the RPE become myofibroblastic and fibrosis ensues
	rs6917644	rs9483364	- Reported CTGF expression in the human PVR membranes
			- Polymorphisms in CTGF predict the risk of PVR
EGF	rs1024600	rs2237051	- Promotes Müller glia proliferatio and RPE proliferation via activation of the β catenin signaling pathway
	rs11568943	rs4698803	- Contributes to cell-growth regulation in PVR
	rs17238095	rs6533485	- EGF receptor exists in the early stage of PVR
	rs9999824	rs718768	- EGF stimulates phosphorylation of the phosphatidylinositol 3'-kinase (PI3K)-dependent effector kinase Akt, MEK- Dependent
	rs1860129		mitogen- activated kinase (MAPK), and extracellular signal-regulated kinase (ERK)
			- Polymorphisms in EGF predict the risk of PVR
FGF2	rs1048201	rs308435	- Basic FGF (bFGF) in the vitreous of PVR is overexpressed
	rs1476217	rs3804158	- bFGF mRNA, bFGF peptide and FGF receptor are involved in epiretinal membrane formation in PVR
	rs167428	rs7683093	- Promotes RPE proliferation via activation of the β catenin signaling pathway and stimulates the production of interferon gamma
	rs1982569	rs9990554	- Participates in the activation of Smad/ZEB1/2 signaling responsible of proliferation and epithelial-mesenchymal transition (EMT)
	rs308417	rs308428	of RPE (hallmarks of PVR)
			- Polymorphisms in FGF2 predict the risk of PVR
HGF	rs1558001	rs17501080	- Exhibits pleiotropic biologic functions in its target cells as mitogenic, motogenic, morphogenic, and angiogenic factors
	rs2074724	rs5745687	- Has profound effects on growth and migration of RPE cells
	rs917183		- HGF and its receptor (HGFR) are strongly expressed in epiretinal membranes associated with PVR and in the vitreous of patients with PVR
			- Polymorphisms in HGF predict the risk of PVR
IFNG	rs12306852	rs2098395	- Inflammatory cytokine significantly increased in vitreous samples from patients with PVR
	rs2069718	rs2193049	- Mediates in cellular mechanisms of migration, proliferation, and differentiation, which are involved in PVR membrane formation
	rs2069727	rs2430561	- Participates in the induction of ICAM-1 by RPE cells
			- Polymorphisms in IFNG predict the risk of PVR
IGF1	rs1019731	rs5742629	- Are present in biologically active quantities in the vitreous fluids of patients with fibrocontractive diseases
	rs1520220	rs6214	- Increase the contraction stimulating effect of Müller cells and has a stimulating effect for traction on RPE cells
	rs2195240	rs7136446	- Have a significant physiopathological role in fibro-contractile disorders like PVR
	rs2971575	rs9308315	- Upregulated expression in vitreous of patients with PVR
	rs35767		- Involved in the progression, stimulation of epiretinal membrane contraction and inflammation process in PVR
IGF2	rs1003483	rs3213221	- Potent promoters of RPE cell tractional force generation
	rs2585	rs3741208	- IGF ligands and binding proteins are known to be present in the vitreous, and in the environment that drives RPE responses in PVR
	rs3213221	rs1003483	- The concentration of some IGF proteins are correlated with the severity and prognosis of PVR
	rs3741212		- Polymorphisms in IGF-IR predict the risk of PVR
IGF-IR	rs10794486	rs2684787	
	rs12899533	rs4305005	
	rs1568501	rs4966035	
	rs1879613	rs7166287	
	rs2048641	rs7173191	
	rs2229765	rs8038015	
IL1A	rs1304037	rs2856836	- Broad spectrum of activity in inflammation and wound healing
	rs17561	rs3783516	- Associated with the initiation of inflammatory mediators cascade and inflammatory reaction in PVR
	rs1800587	rs2048874	- Have the ability to activate T- lymphocytes, stimulate the secretion of immunoglobulin, induce neuronal differentiation, and
	rs3783550		trigger the release of acute phase proteins
IL1B	rs1143634	rs16944	- Are synthesize by a variety of cells, including monocytes, synoviocytes, fibroblasts and RPE cells
	rs3917368	rs4848306	- Stimulate the migration of RPE cells
	rs7596684		- Are increased in vitreous samples from patients with PVR
IL1RN	rs1688072	rs1794066	- Stimulate the proliferation of fibroblast and glial cells
	rs3087270	rs315946	- Stimulate the synthesis of collagen
	rs315949	rs315952	- Total levels of some ILs in the vitreous are predictive risk factors for postoperative PVR development
	rs315958	rs446433	- Reported ILs expression in the human PVR membranes
	rs973635		- The interaction between extracellular matrix- bound cytokine and inflammatory leucocytes or resident cells of the retina may
IL6	rs11766273	rs12700386	promote the development and perpetuation of PVR
	rs1474347	rs1800795	- Participate in the transdifferentiation, migration, proliferation, survival, and extracellular matrix formation in fibro-contractile
	rs2056576	rs2069840	disorders like PVR
	rs2140543	rs4719713	- IL-10 limits the inflammatory response by blocking IFNG, IL-2, TNFA and IL-4 production. IL1RN binds the IL-1 receptor, Inhibiting
IL8	rs2227306	rs2227543	its union to IL1A and B, neutralizing their action
	rs4073	rs4694178	- Polymorphisms in IL-10 and IL1RN predict the risk of PVR
IL10	rs10494879	rs17015767	
	rs1800871	rs1800872	
	rs1800890	rs2222202	
	rs3024493	rs3024498	
	rs4390174		
MCP1	rs1024611	rs2530797	- Potent chemotactic factor for monocytes
	rs2857653	rs3091316	- Is present in a substantial percent of vitreous samples from eyes with proliferative vitreoretinal disorders stimulating the
	rs3760396	rs4795893	infiltration of monocytes and macrophages
			- Is induced by TNF alpha in retinal glial cells during post- ischemic inflammation
			- Polymorphisms in MCP1 predict the risk of PVR

(*continued on next page*)

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Table 5 (*continued*)

Retina 4 project Genetic contribution to PVR development			
GEN	Polymorphisms		Reasons for analysis/potential contribution to PVR
MIF	rs1007888	rs1984309	- Involved in cell-mediated immunity, immunoregulation, and inflammation
	rs2096525	rs4820571	- Plays a role in the regulation of macrophage function in host Defense
	rs4820571	rs738806	- Increased levels in the vitreous samples of patients with PVR
	rs755622		- Polymorphisms in MIF predict the risk of PVR
MMP2	rs1561220	rs243864	- MMPs and their natural inhibitors (TIMPs) play an important role in matrix remodeling and their involvement in the formation of
	rs2192853	rs243866	scar-like tissue in PVR
	rs243840	rs7201	- Presence of MMPs and TIMPs in epiretinal and subretinal membranes in patients with PVR
	rs243845	rs9928731	- Significant correlations between PVR grade and MMPs in sub retinal fluid and proMMPs, MMPs-and TIMPs levels in vitreous
MMP9	rs2250889	rs3787268	- Polymorphisms in MMPs predict the risk of PVR
	rs2274756	rs3918241	
	rs3918253	rs4810482	
NFKB1	rs11722146	rs4648141	- NF-KB is a transcription factor that plays an important role in biological processes
	rs230540	rs4698858	- NF-KB can be activated by exposure of cells to pro-inflammatory cytokines such asTNF-a and IL-1/3 or by exposure to several other
	rs28362491	rs7674640	stimuli including bacteria, viral proteins, and hypoxia
	rs3774932	rs997476	- Induce the transcription of a variety of genes that bear KB- binding sites, including proinflammatory cytokine, chemokine, and cell
	rs4648110		adhesion molecule genes
NFKBIA	rs17103274	rs3138045	- NF-κB, in combination with GDNF receptors, are involved in the formation of the glial cell component of ERMs in PVR
	rs2007960	rs3138056	- NF-κB is expressed in human PVR membranes and vitreous samples
	rs3138045	rs696	- Polymorphisms in NFkBIA and NFkBIB predict the risk of PVR
	rs7152826		
NFKBIB	rs10410544	rs2241705	
	rs11879872	rs3136640	
	rs2053071	rs3136646	
	rs9403		
PDGFA	rs11764261	rs11768030	- Its expression is increased in RPE cells within the ERM of human patients
	rs4916944	rs7777705	- Are associated with PVR in humans and strongly promotes experimental PVR driven by multiple vitreal growth factors outside the
	rs7806249		PDGF family
PDGFRA	rs17739921	rs2114039	- Once activated, the PDGFR initiates signal relay cascades that drive biologic responses, such as chemotaxis and proliferation
	rs4289498	rs4864877	- Expression increase the ability of fibroblasts to induce experimental PVR
	rs6850748	rs7656613	- Vascular endothelial growth factor A and non- PDGFs are able to influence in the activation of PDGFRα
	rs7691129		- Polymorphisms in PDGF and PDGFR predict the risk of PVR
PIK3CG	rs3173908	rs4460309	- Important modulator of extracellular signals, including those elicited by E-cadherin-mediated cell–cell adhesion, which plays an
	rs4727666	rs4730204	important role in maintenance of the structural and functional integrity of epithelia
	rs6961244	rs757902	- Plays a pivotal role in the regulation of cytotoxicity in NK cells
	rs849380	rs849384	- Is highly activated in the RPE cells of PVR and is essential for PVR in a rabbit model of the disease
	rs849385	rs849387	- Influences in the activation of αPDGFR to mediate PVR
			- Polymorphisms in PIK3CG predict the risk of PVR
SMAD3	rs1866316	rs7169183	- SMAD3 mediates the signals from the TGF-β superfamily ligands that regulate cell proliferation, differentiation and death
	rs2033785	rs7177795	- SMAD3 is essential for EMT and fibrogenic responses by RPE cells induced by retinal detachment
	rs3743343	rs731874	- SMAD7 is a TGF-β type 1 receptor antagonist. It blocks TGF-β1 and activin associating with the receptor, blocking access to SMAD2.
	rs4776881	rs8031627	It is an inhibitory SMAD (I-SMAD) and is enhanced by SMURF2
	rs6494634	rs8032802	- Experimental PVR development after retinal detachment is inhibited by Smad7 overexpression
SMAD7	rs1873190	rs4939826	- Polymorphisms in SMAD3 and SMAD7 predict the risk of PVR
	rs2337143	rs6507877	- Genetic implication of SMAD7 in the development of PVR has been confirmed by genetic case–control studies
	rs2878889	rs7226855	
	rs9946510		
TGFB1	rs2241715	rs4803455	- Participates in the modulation of cell migration and proliferation, cell death, and protein synthesis during development, tissue
	rs2241713	rs8179181	repair, and other physiological or pathological processes.
	rs1800471		- Enhances extracellular matrix production and suppresses cell proliferation
TGFB2	rs1418556	rs2796821	- Capables of inducing several number of growth factors
	rs1891467	rs4846267	- TGFB1 induces cytoskeleton reorganization, alpha- SMA expression, increases the phosphorylation of ERK, Smad2/3, and AKT, and
	rs2000220	rs4846476	activates RhoA and Rac1 signaling pathways.
			- Concentration of TGFB2 in the vitreous humor of the eye correlates with the severity of the PVR
			- TGF-β2 contributes to transdifferentiation of hyalocytes into α-smooth muscle actin positive myofibroblast-like cells that cause
			collagen gel contraction
			- Are overexpressed in the vitreous of patients with PVR and are also detectable in the contractile membranes
			- Polymorphisms in TGFB1 and TGFB2 predict the risk of PVR
TNFA	rs1799964	rs2857602	- TNFA and TNFR2 play a pivotal role in inflammation, by activating endothelial cells to display leukocyte adhesion molecules such
	rs1800629	rs2857706	as E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1
	rs2229094	rs673	- High levels of these molecules are associated with inflammatory processes such as PVR
			- Are overexpressed in the vitreous of patients with PVR and are also detectable within the extracellular matrix
	rs2256965	rs909253	PVR membranes
	rs2256974	rs915654	- TNFα mRNA detected by RT-PCR in vitreous and Subretinal Fluid (SRF) samples of patients with PVR, indicating local production of
TNFR2	rs1061622	rs3397	these cytokines by vitreous and SRF cells
	rs1061624	rs542282	- TNFα, is able to bind to receptors on Müller cells and able to activate Müller cells, microglia and astrocytes
	rs1061628	rs590977	- TNF-induced Reactive oxygen species (ROS) production and other cell death pathways such as necroptosis
	rs652284		- Participate in the formation of fibrillar collagen and cellular proliferation in eyes with PVR
			- Genetic analysis from patients with post-rhegmatogenous retinal detachment PVR demonstrated a significant association with the
			nonsynonymous, SNP rs2229094(T→C) at the TNF locus
			- Polymorphisms in TNFA and TNFR2 predict the risk of PVR
			- Genetic implication of TNFA in the development of PVR has been confirmed by genetic case–control studies

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Table 5 (*continued*)

Retina 4 project Genetic contribution to PVR development		
GEN	Polymorphisms	Reasons for analysis/potential contribution to PVR
p53	rs1042522	- Is crucial for cell repair of genomic mutations and induces cell cycle arrest, apoptosis, senescence, or differentiation following cellular stress - The codon 72 polymorphism in p53 regulates the interaction with NF-kB and transactivation of genes involved in apoptosis, immunity and inflammation. It has been associated with several cancers and inflammatory processes - Is related to a poor functional prognosis in patients who have suffered from ischemic or hemorrhagic stroke - Seems to be a checkpoint of RD and how its local increase in the vitreous by using inhibitors of MDM2, seem to be a promising approach as a prophylaxis in experimental RD and also in experimental PVR - Pro allele of the p53 gene, associated with a decrease in apoptotic function of p53, has a higher risk of developing PVR after RD - Downregulation of p53 appeared to be a required event in PDGFRα-mediated contraction of cells in a collagen gel and retinal detachment in an animal model of PVR - The presence of soluble apoptotic molecules has been implicated in the development of PVR - Polymorphisms in P53 predict the risk of PVR
MDM2	rs2279744	- Key negative regulator of p53 and in humans - The G/G variant increases the binding affinity of the transcriptional activator Sp1 resulting in high levels of mdm2 protein; formation of transcriptionally inactive p53-MDM2 complexes and a consequent decreased activity of the p53 pathway - Carriers of the G allele of the MDM2 gene, associated with a decrease in apoptotic function of p53, have higher risk of PVR after RD - Polymorphisms in MDM2 predict the risk of PVR
BAX	rs4645878	- Pro-apoptotic members, triggers mitochondrial outer membrane permeabilization - A mediator of apoptosis that is also involved in retinal cell death after retinal ischemia - Is associated with a decrease in the apoptosis levels, being involved in the control life or death of a cell, and in the cellular proliferative response - A/A allele of rs4645878 could be a biomarker of high risk for developing PVR in patients undergoing RD surgery
BCL-2	rs2279115	- Anti-apoptotic members of Bcl-2 family able to neutralize BAX and block mitochondrial outer membrane permeabilization - The BCL-2-938 AA genotype is associated with an increase in Bcl-2 expression - Although an over-expression of bcl-2 is associated with a decreased apoptotic response, unlike bax, the over-expression in bcl-2 is able to induce an inhibitory effect in the programmed necrosis cell death and other non-apoptotic pathways like autophagy - It has been reported that increase expression of bcl-2 attenuates the TNFA induced necroptosis pathway

A total of 200 common SNPs with minor allelic frequencies >10% were selected for its analysis in the retina 4 project (Pastor-Idoate et al., 2013a, 2013b; Rojas et al., 2009; Rojas et al., 2013).

The genetic implication of TNF locus and SMAD 7 in the development of PVR have been confirmed by subsequent genetic case–control replication studies within the retina 4 project.

- 196 common SNPs from 30 candidate genes associated with inflammation and known to be implicated in PVR pathways: CTGF, PDGF, PDGFRα, PI3KCG, EGF, FGF2, MIF, MMP2, MMP7, MCP1, IGF1, IGF2, IGF1R, TNF, TNFR2, TGF-β1, TGF-β2, SMAD3, SMAD7, IFNα, IL1α, IL1β, IL1RN, IL6, IL8, IL10, NFκB1, NFκBIA, NFκBIB, and HGF.
- 4 common SNPs from 4 genes related with apoptosis and diverse cell death pathways: p53, MDM2, BAX and BCL-2.

- CTGF: Connective Tissue Growth Factor.
- EGF: Epidermal Growth Factor.
- FGF-2: Fibroblast Growth Factor 2 (bFGF).
- HGF: Hepatocyte Growth Factor.
- IFNG: Interferon Gamma.
- IGF1: Insulin-like Growth Factor 1.
- IGF2: Insulin-like Growth Factor 2.
- IGF-IR: Insulin-like Growth Factor Receptor.
- IL1A: Interleukin-1 Alpha.
- IL1B: Interleukin-1 Beta.
- IL1RN: Interleukin 1 Receptor Antagonist.
- IL6: Interleukin-6.
- IL8: Interleukin-8.
- IL10: Interleukin-10.
- MCP1: Monocyte Chemoattractant Protein-1.
- MIF: Macrophage Migration Inhibitory Factor.
- MMP2: Matrix Metalloproteinase 2.
- MMP9: Matrix Metalloproteinase 9.
- NFKB1: Nuclear Factor NF-kappa-B 1 p105 subunit.
- NFKBIA: Nuclear Factor of Kappa Light Polypeptide Gene.
- NFKBIB: Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-cells Inhibitor, Beta.
- PDGFA: Platelet-Derived Growth Factor Subunit A.
- PDGFRA: Platelet-Derived Growth Factor Receptor Alpha.
- PIK3CG: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Gamma.
- SMAD3: Mothers Against Decapentaplegic Homolog 3.
- SMAD7: Mothers Against Decapentaplegic Homolog 7.
- TGFB1: Transforming Growth Factor, Beta 1.
- TGFB2: Transforming Growth Factor, Beta 2.
- TNFA: Tumor Necrosis Factor Alpha.
- TNFR2: Tumor Necrosis Factor Receptor 2.
- p53: Tumor Suppressor p53 Gene.
- MDM2: Mouse Double Minute 2 Homolog.
- BAX: BCL2-Associated X Protein Gene.
- BCL-2: B-Cell Lymphoma 2 Gene.

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Another interesting attempt to develop a predictive model for the onset of PVR was made by Ricker et al. (2012). They examined the combined predictive value of clinical risk factors and biomarkers in the subretinal fluid collected during surgery. Once again preoperative PVR was identified as the only variable that was an independent predictor of postoperative PVR. The addition of certain biomarkers improved the area under the ROC curve. However, this interesting approach has two limitations. First, the model should be externally validated before being fully accepted. Second, from a practical point of view, obtaining the information after surgery may delay the application of prophylactic measures.

It is clear then that analysis of the genetic component of any multifactorial disease, and particularly PVR, is probably one of the most innovative approaches to understanding this entity. Knowledge of the genetic contribution to this multifactorial disease could explain many of the obstacles that until now have obscured the path to prevention and treatment of PVR. Table 5 summarizes some of the most relevant findings of the genetic component of PVR.

7. Anatomical and functional results after surgery

Currently, treatment of PVR consists of surgical repair of the retina (Pastor et al., 2002; Sadaka and Giuliari, 2012). However, despite recent advances in vitreoretinal surgery that offer a wider range of surgical techniques, the improved anatomical and functional success rates remain unsatisfactory. But as it has mentioned, presently, it is widely accepted that there is no effective treatment for PVR (Coffee et al., 2014). The final goal of RD and PVR surgeries is to reattach the retina and to prevent future re-detachment. To do this, repair of retinal breaks, relief of traction forces, and stabilization of the retina are required (Coffee et al., 2014; Pastor, 1998).

Given the high variability of this process and wide diversity of surgical procedures currently available, it is very difficult to adequately estimate anatomical and visual outcomes of this condition (Pastor et al., 2002). This variability arises principally from the difficulty of classifying the severity of disease, the existence of different surgical techniques and timing of surgery, and the great variability of the PVR process itself (Pastor et al., 2002). Nevertheless, many studies have been performed to assess these results.

7.1. Anatomical results after surgery

Anatomical success has been reported to be 60–90% depending on the severity of the disease (Aaberg, 2010; Pastor et al., 2002; Sadaka and Giuliari, 2012). In the most complex cases, when heavy silicone oil was used as a tamponade, in only 39% of the cases did the retina remain attached during the entire follow-up period (Regler et al., 2009).

7.2. Functional results after surgery

The best visual acuity results are associated with the success of the first surgery (Abrams et al., 1997). However, anatomical success does not ensure visual improvement, as will be discussed later, and there are many factors in PVR that impede an appropriate recovery of best corrected visual acuity (BCVA).

The most commonly reported rates of functional success following RD surgery show that 40–80% of the patients recover at least ambulatory vision of 5/200 or better (Pastor et al., 2002; Sadaka and Giuliari, 2012). The poor results may be due to changes in the macula (Kiss et al., 2007), but they are also related to the number of procedures performed (Pastor et al., 2002). These results could also be derived from reactive gliosis secondary to an excessive scarring process generated in this condition. This scarring can be the result of microscopic changes such as Müller cell hypertrophy and transdifferentiation of RPE cells into fibroblasts that invade the retinal layers (Pastor et al., 2006b; Pennock et al., 2011). Alternatively, they could be due to macroscopic changes with the creation of epiretinal membranes, intraretinal changes, or subretinal bands (Pastor et al., 2006b). The sum of these factors leads to a severe visual loss despite a complete reattachment of the retina. With the implementation of 25-G surgery, BCVAs of >20/60 in 51.9% of the eyes at 12 months have been achieved (Iwahashi-Shima et al., 2013). The development of PVR with recurrent RD usually requires additional surgery, and it is associated with poor visual acuity (Pennock et al., 2014a). Classically, the prognosis of anterior PVR tends to be worse than posterior PVR (Diddie et al., 1996).

The problem of poor functional results is common with RD even when it is not complicated with PVR. Although reattachment is obtained in up to 94% of the cases (Pastor et al., 2008b), functional results at 3 months of follow-up show that less than 50% achieve a BCVA of 20/40 or higher. Further, in our experience almost 15% of patients with RD macula-on and no involvement of the macula experienced a visual loss (personal unpublished data).

This decrease in visual acuity can be attributable in some cases to structural changes, as has been reported with the use of new optical coherence tomography and adaptative optic retinal imaging instrumentation (Saleh et al., 2014). However, it is also possible that the release of factors by the detached retina may affect some other areas of the retina, even if the affected regions were not within the detached retinal area (Iandiev et al., 2006). For these reasons, we are convinced that neuroprotection must be a complement of RD surgery in the very near future. These factors, obviously, will be also present in PVR.

7.3. Timing for PVR surgery

As PVR is a dynamic process, the ideal time to perform surgery is difficult to choose, and it has been a matter of controversy among vitreoretinal surgeons (Coffee et al., 2014; Pastor et al., 2002). The judgment of surgical management remains subjective because decisions are based on the expected evolution of the disease and depend on preferences and surgical skills of the surgeon. According to some authors, the presence of clinical signs of activity may point out the need to delay surgical intervention for some weeks (Pastor et al., 2002) because the controlled trauma of a new surgery could generate an extra stimulus for cellular proliferation (Coffee et al., 2014). Besides, as epiretinal proliferation takes an average of 6–12 weeks to completely develop, delay of surgery would allow an easier removal of these membranes (Coffee et al., 2014).

However, another recent study showed that delay in surgery greater than 28 days is itself an independent risk factor associated with PVR (Feng et al., 2013).

Obviously the time of evolution is an important factor for functional results, but we have the impression that in some cases repeated surgeries lead the problem towards an intractable stage. Perhaps in these cases an adjuvant treatment would be a better solution.

7.4. Anatomical and visual results of different surgical techniques

Surgical approaches must be modified according to the preoperative characteristics of the each patient. Some characteristics that increase the risk of PVR are easily detectable, such as traumatic RD or the presence of giant tears. However others may be subtle, such as the extent of RD (Wickham et al., 2011), and yet others may require special diagnostic techniques such as the analysis of genetic predisposition. Light and moderate cases of PVR, according to the

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currently existing classifications, can be managed by conventional surgery, whereas severe cases may require complex interventions, including scleral buckling (SB), pars plana vitrectomy (PPV), membrane peeling, retinotomy, retinectomy, and retinal tamponade among others (Sadaka and Giuliari, 2012). However, once again the lack of a uniform classification prevents the adequate comparison between techniques and series.

7.4.1. Scleral buckling

Scleral buckling (SB) involves the placement of a silicone band that encircles the eye to indent it from the outside. This band helps to close retinal breaks, support the vitreous base, and reduce the anterior-posterior traction (Coffee et al., 2014; Pennock et al., 2014a). Light and moderate cases of PVR can be treated with this technique, but PPV is indicated in most cases (Pastor et al., 2002). Anatomical success reported with SB is 34–47% (Coffee et al., 2014). In those patients at high risk of suffering postoperative PVR, the combination of PPV with SB was associated with significantly higher single surgery anatomical success compared with PPV alone (Storey et al., 2014). Nevertheless, some authors suggested that there is no benefit regarding the anatomical success by adding SB to PPV (Oyagi and Emi, 2004).

7.4.2. Vitrectomy

The main goals of vitrectomy are to remove sources of traction, achieve retinal reattachment, and prevent re-detachment. Careful membrane peeling is needed to achieve these goals (Coffee et al., 2014). Vitrectomy eliminates transvitreal traction in PVR and must include elimination of the vitreous base. The vitreous base may play a key role in the pathogenesis of PVR because it hosts the RPE cells that accumulate and proliferate. These cells then produce collagen and generate membranes that subsequently contract and generate new breaks and extensions of the existing RD. Thus, an anterior vitrectomy reaching the vitreous base is highly recommended (Pennock et al., 2014a).

Historically, twenty-gauge (20-G) vitrectomy has been used for the treatment of PVR. However, new transconjunctival vitrectomies (23-G and 25-G) reduce the surgical trauma and postoperative inflammation, leading to a faster visual recovery (Iwahashi-Shima et al., 2013; Recchia et al., 2010). These advantages make the new surgical procedures especially valuable in diseases like PVR in which inflammation plays a major role (Iwahashi-Shima et al., 2013). Twenty-five gauge vitrectomy is effective in selected cases of PVR, although anatomical recovery rates are difficult to compare with previous reports due to the great variability in the severity of the cases and also in the surgical procedures performed in each case (Iwahashi-Shima et al., 2013).

In one study, anatomical success rates were similar between 20-G and 25-G surgeries (Iwahashi-Shima et al., 2013). However, visual recovery was faster in eyes after 25-G vitrectomy, with BCVA improving at one month after surgery. In contrast, eyes with 20-G vitrectomy required 3 months to regain visual acuity. The authors reported that sometimes it was necessary to perform an additional 20-G sclerotomy to use 20-G instruments for specific surgical manipulations (Iwahashi-Shima et al., 2013).

7.4.3. Retinotomy and retinectomy

If adequate mobilization or complete retinal reattachment is not achieved, it may be due to a contraction and foreshortening of the retina. The prudent use of a relaxing incision (retinotomy) or removal of tissue (retinectomy) should be considered (Williamson and Gupta, 2010). According to our data, up to 50% of patients with RD complicated with PVR showed intraoperative signs of "shortening" after a careful membrane peeling (Pastor et al., 2003). In 16% of these patients, it was necessary to perform a retinectomy to flatten the retina. Retinectomies are necessary in extensive cases of intraretinal PVR. Even a 360-degree retinectomy has been proposed in cases of severe PVR to obtain functional vision and prevent new surgeries (Garnier et al., 2013). These aggressive techniques are usually indicated in severe anterior PVR and may be beneficial for some cases. Nevertheless, the number and severity of complications are very high, including PVR recurrence (up to 50%), hypotony (up to 40%), and other complications. In some cases, the resulting complications can lead to enucleation of the blind and painful eye (Garnier et al., 2013).

In some series, after PPV and retinectomy in grade C PVR, reattachment rates of 51% and 72% at the end of the follow-up have been reported (Grigoropoulos et al., 2007). Nevertheless, cutting the retina does not seem like a good solution. After retinotomies, retinal edges may lift and scroll up with subsequent visual impairment. Additionally, aggressive surgical trauma produced by these techniques may exacerbate the process by increasing stimuli that lead to PVR (Williamson and Gupta, 2010). To avoid the increase in the breakdown of the blood-retinal barrier and the release of cytokines and growth factors, it has been recommended that surgeons avoid retinotomies and retinectomies when possible (Williamson and Gupta, 2010). If they are mandatory, the surgery should be delayed until the PVR is in a quiescent state.

7.4.4. Other procedures

7.4.4.1. Membrane peeling and internal limiting membrane (ILM) peeling. As described above, epiretinal proliferation takes an average of 6–12 weeks to completely develop. Thus delay of surgery would allow an easier removal of these membranes (Coffee et al., 2014). ILM peeling is another proposed approach in PVR surgery. This technique acts by reducing retinal tension in the posterior pole. It also reduces the recurrence of posterior epiretinal membrane formation and subsequent re-detachment (Minarcik and von Fricken, 2012). Nevertheless, neither the deleterious effect of removing of the ILM nor the possible beneficial effect on PVR has been clearly evaluated.

7.4.4.2. Lensectomy. When a RD and PVR occur in a patient with a cataract, removal of the lens is a routine procedure performed with the PPV. Removal of the clear lens by pars plana lensectomy improves the intraoperative visualization and helps to complete elimination of the anterior vitreous. In some cases this procedure has significantly better anatomical results (Quiram et al., 2006), and can be performed through a 25-G system (Kiss and Vavvas, 2008). This surgical maneuver might decrease the risk of suffering postoperative hypotony by reducing membrane formation on the ciliary body (Tseng et al., 2009).

7.4.4.3. Photocoagulation. The application of endolaser along the margins of the retinotomies, retinectomies, and retinal breaks is needed to seal these lesions and to reattach the retina (Coffee et al., 2014). It is considered less likely to stimulate RPE release and intraocular inflammation (Singh et al., 1986).

7.4.4.4. Retinal tamponade. When PVR traction has been eliminated and the retina has been reattached, retinal endotamponade is mandatory (Pennock et al., 2014a). The tamponade material provides time for retinal adhesions to form firmly around tears and incisions and avoids fluid flow into the breaks (Coffee et al., 2014). Endotamponade is usually performed with long-acting intraocular gas such as sulfur hexafluoride (SF6) or perfluoropropane (C3F8), or with silicone oil (SiO). Based on findings provided by the Silicone Oil Study, the retinal reattachment rate increased to 70–85% of macular attachments at 36 months, and there were better visual outcomes with SiO or C3F8 compared with SF6 (Abrams et al., 1997;

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Schwartz et al., 2014). However less than 50% of the eyes with reattached retinas reached visual acuity of 5/200 or better in severe cases of PVR (Abrams et al., 1997).

Silicone oil and C3F8 had similar rates of success regarding anatomical and visual outcomes (Abrams et al., 1997; Schwartz et al., 2014), suggesting that success in the first surgery is more important than the choice of tamponade in most of the cases. Nevertheless, the selection of the tamponade must be individualized for each patient. This was the conclusion of a Cochrane study (Schwartz et al., 2014). One of the disadvantages of SiO compared to C3F8 is the complications derived from the silicone oil removal (Coffee et al., 2014; Jonas et al., 2001). This maneuver can induce the formation of new retinal breaks, reopen previous breaks with insufficient scarring or incomplete retinopexy, and create unresolved tractions that produce retinal re-detachment affecting from 3.5% to 34% of the eyes (Al-Wadani et al., 2014; Jonas et al., 2001).

Some patients undergo a severe visual loss when the SiO is removed (Christensen and la Cour, 2012; Shalchi et al., 2015). Based on optical coherence tomography analysis, atrophy and a significant thinning of inner retinal layers in the macular region occurred with SiO tamponade but not with C3F8 (Christensen and la Cour, 2012). The reason of this loss remains unknown, but it is hypothesized that the thinning and atrophy may be due to a direct or indirect toxic effect of the SiO (Christensen and la Cour, 2012). Some explanations for this phenomenon deal with changes of ion concentrations derived from the collapse of Müller cells, increased levels of cytokines, or phototoxicity that lead to retinal cell death (Christensen and la Cour, 2012; Shalchi et al., 2015).

In 2006 we published an analysis of organic lipophilic compounds from silicone oil used in humans eyes for repairing RD. Among the compounds, cholesterol and α-tocopherol were present in high concentrations, and they were correlated with the intraocular permanence time. We concluded that silicone oil was able to extract lipophilic compounds from intraocular tissues, and therefore it may affect retinal and other intraocular cells. This may be an alternative explanation to the retinal atrophy that can occur after silicone oil use as a tamponade (Pastor et al., 2006a). This deleterious effect on the retina was observed by our group many years ago in experimental models (Nakamura et al., 1991; Pastor et al., 1992). At that time we attributed the changes to the presence of low molecular weight components, but the changes were also present when we used highly purified silicone oil (Pastor, 1998). For special cases where there are signs of PVR in the inferior retina, there is now the option of using “heavy silicone oils” (Williams et al., 2013). However the discussion on these substances is not within the scope of this major review (Khan et al., 2015).

Considering the poor anatomical and visual outcomes obtained after very aggressive surgeries, we can conclude that there is no effective treatment for PVR. As it is well recognized that PVR is an inflammatory condition, the implementation of new surgical approaches that avoid the inflammatory response triggered by surgical trauma may help improve these unsatisfactory results. Clearly, research must be focused in preventing these complications.

8. Adjuvant therapy for the treatment of PVR: present and future

As discussed earlier in this report, PVR is a complex process involving different risk factors for development. It is comprised of events that have been considered to be similar to those of the wound healing response with inflammation, migration, and proliferation of a variety of cells (Kauffmann et al., 1994; Kosnosky et al., 1994; Limb et al., 1991; Pastor, 1998). However the retina reacts in the same manner as the CNS, and more attention should be paid to the mechanisms of repairing the brain after an inflammatory insult. With respect to the cellular aspect of the CNS inflammatory response, microglia respond within minutes to hours by proliferating, activating, and migrating to the area of injury, where they essentially function as macrophages (Hauwel et al., 2005; Schmidt et al., 2005). Increased blood–brain barrier permeability allows leukocyte infiltration from the blood to the injury site, a process that is mediated by cytokines, chemokines, and complement proteins. Neutrophils are followed by monocytes. The oxidative burst of neutrophils and macrophages is harmful because of the release of oxygen free radicals and neurotoxic enzymes; however, both activated microglia and monocyte-derived macrophages aid in clearing debris from dead and damaged cells via phagocytosis.

Over the last 20 years, vitreoretinal surgical techniques have evolved, and greater emphasis has been placed on the success of primary RD surgery to prevent PVR. Case selection has been refined and the incidence of PVR might have been expected to decline. Yet the frequency of this condition remains largely unchanged, with a postoperative incidence of PVR ranging from 4% to 34% in prospective studies (Charteris et al., 2002; Heimann et al., 2007; Leiderman and Miller, 2009). These results show that there is a need for adjunctive pharmacologic treatment that could prevent or halt progression of PVR.

Laboratory and clinical studies have suggested that pharmacological adjuvant therapy can mitigate the proliferative disease process and improve surgical success. In general, these pharmacological strategies have included anti-inflammatory, anti-proliferative, anti-neoplastic, anti-growth factor, and antioxidant agents to either modify the inflammatory cascade or interfere with proliferation. Although most of these attempts have failed, we have summarized the results because of their valuable information.

8.1. Anti-inflammatory agents

Based on the hypothesis that PVR pathogenesis is due to inflammation, steroids such as triamcinolone acetonide or dexamethasone have been considered promising prophylaxis and/or treatment candidates (Ahmadieh et al., 2008; Garcia-Layana et al., 1997; Hui et al., 1993; Koerner et al., 1982; Rubsamen and Cousins, 1997; Tano et al., 1980a, 1981, 1980b; Weller et al., 1990). However, despite the success seen with steroids in animal experiments (Hui et al., 1993; Rubsamen and Cousins, 1997; Tano et al., 1980a), human studies failed to demonstrate the same beneficial effects in terms of reattachment rate, visual acuity, recurrence of PVR, or reoperation rate (Ahmadieh et al., 2008; Pastor, 1998; Sadaka and Giuliari, 2012). It is possible that these drugs have not been applied at the proper time or they have not reached the appropriate concentration in the eye (Nguyen and Lee, 1992). In fact some authors have recommended systemic postoperative use (Koerner et al., 1982) while others have advocated for the early administration of these drugs (Hui and Hu, 1999).

There is a huge variety in the route of steroid administration. Besides the systemic route, some authors recommend intravitreal application (Jonas et al., 2000) and others subconjunctival application (Bali et al., 2010), both of which are claimed to reduce PVR development. There are some papers that have tested the efficacy of low doses of intravitreal triamcinolone after silicone oil tamponade (Chen et al., 2011; Fernandes-Cunha et al., 2014; Kivilcim et al., 2000; Szurman et al., 2009). The authors reported that it is safe and effective, a result that is difficult to reconcile with our finding that triamcinolone is hardly soluble in silicone oil (Pastor et al., 2008a), a finding confirmed by others (Spitzer et al., 2009). Further, it seems that relatively high intraocular levels of steroids would be required. In cell culture, only concentrations of dexamethasone over 200 μg/ml had an additive effect with 5-FU on the inhibition of human RPE

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proliferation (Tung et al., 2001).

8.2. Anti-neoplastic/anti-proliferative agents

To interfere with proliferative events in PVR, anti-neoplastic agents, which inhibit the cell cycle and cellular proliferation, have been extensively explored, including compounds like 5-FU, daunorubicin, taxol, colchicine, retinoic acid, ribozymes, vincristine, cisplatin, adriamycin, mitomycin, dactomycin, and others. 5-FU is one of the most tested compounds for the treatment of PVR because it is frequently used to reduce scarring in glaucoma-filtering surgeries (Sadaka and Giuliari, 2012).

Several clinical trials have tested the efficacy of heparin and its fragment, low molecular weight heparin (LMWH), in PVR by using them in combination with steroids or anti-metabolite drugs such as 5-FU (Asaria et al., 2001; Charteris et al., 2004; Wickham et al., 2007; Williams et al., 1996). The use of heparin in the vitrectomy-infusion fluid during PVR vitrectomy not only did not reduce the rate of proliferation, but it did increase the rate of postoperative hemorrhage (Williams et al., 1996). Most of the clinical trials with LMWH showed no significant or only minimal differences in anatomical and functional outcomes (Asaria et al., 2001; Charteris et al., 2004; Wickham et al., 2007). Furthermore, the combined use of 5-FU and LMWH resulted in worse visual acuity (Wickham et al., 2007), raising some toxicity concerns about this adjuvant therapy. A recent Cochrane review has concluded that there is not enough evidence to recommend the use of 5-FU and LMWH to prevent PVR (Sundaram et al., 2013).

Daunorubicin, a topoisomerase inhibitor that acts on cell proliferation and migration, has also been used to treat PVR (Wiedemann et al., 1998, 1987). The Daunomycin Study Group assessed the efficacy and safety of daunorubicin during vitrectomy in eyes with PVR and found that it produced a small reduction in the number of re-operations in patients undergoing retinal surgery with established PVR (Wiedemann et al., 1998). Although there are only a few clinical trials that have analyzed the efficacy of daunorubicin in preventing PVR, it appears to be ineffective when used as a single agent (Moysidis et al., 2012). Thus this drug has not been incorporated into the clinical routine.

Agents like taxol and colchicine that respectively stabilize and inhibit microtubule formation have the potential to reduce migration and proliferation of cells (Lemor et al., 1986a) and therefore have been tested in models for efficacy in PVR (Lemor et al., 1986b; van Bockxmeer et al., 1985). Retinoic acid, which promotes growth arrest of RPE cells *in vitro* (Campochiaro et al., 1991), increased the rate of retinal attachment (Campochiaro et al., 1991; Chang et al., 2008; Fekrat et al., 1995; Verstraeten et al., 1992). It also significantly lowered rates of macular pucker formation and produced higher rates of ambulatory vision in the treated groups (Chang et al., 2008).

In addition to these agents, there are a large number of studies that show potential benefits from a variety of pharmacological interventions to prevent the occurrence of PVR. One of these is glucosamine, an inhibitor of N-linked oligosaccharide biosynthesis and processing. It effectively suppresses RPE cell proliferation *in vitro* (Liang et al., 2010) and interferes with the TGF-β signaling pathway in RPE cells (Liang et al., 2011). DNA-RNA chimeric ribozymes that target proliferating cell nuclear antigen (PCNA) have been tested in preclinical and multicenter clinical trials for PVR (Mandava et al., 2002; Schiff et al., 2007). Unfortunately this drug was not effective in preventing PVR recurrence in patients with established grade C or worse PVR (Schiff et al., 2007). Agents such as etoposide and tacrolimus have also been tested and shown to significantly decrease the severity of experimental PVR (Kuo et al., 2007; Turgut et al., 2012).

8.3. Anti-growth factor pathway inhibitors

With our improved knowledge regarding the role that growth factors play in the pathogenesis of PVR, there has been a movement towards blocking growth factors and the respective pathways as a prophylaxis or as a treatment. These strategies include kinase inhibitors such as hypericin or herbimycin, which have both shown positive results in preclinical PVR studies (Imai et al., 2000; Machado et al., 2009; Tahara et al., 1999). Alkylphosphocholine, an inhibitor of protein kinase C, was effective against RPE cell attachment, spreading, migration, and proliferation *in vitro* (Eibl et al., 2007). It was also identified as a promising agent in reducing the number of dividing Müller cells following RD *in vivo*. AG1295, an inhibitor of PDGF receptor (PDGFR) kinase, also significantly attenuated development of RD without apparent histologic or functional damage to the retina (Zheng et al., 2003).

8.4. Antioxidants and other agents

N-acetylcysteine (NAC), an antioxidant used in a variety of clinical entities, protected rabbits from PVR by blocking the activation of PDGFR-α. It also protected rabbits from developing RD, although it did not prevent formation of epiretinal membranes (Lei et al., 2010). Also in this group of anti-oxidants, three polyphenolic agents from vegetal origin, epigallocatechin gallate (from green tea), resveratrol (from red wine), and curcumin (from turmeric), were tested *in vitro* to analyze their effects on proliferation of human RPE cells (Alex et al., 2010). Of these, resveratrol was the most potent, but we have not found further studies on this agent.

Other agents such as genistein, an isoflavone (Yoon et al., 2000), calcium antagonists (Smith-Thomas et al., 2000), and neutralizing antibodies against PDGF, TGF-beta2, and IL-10 (Carrington et al., 2000) have also been tested against PVR. However, none of these agents has been incorporated routinely into clinical practice owing to concerns about retinal toxicity and because only a few have been assessed in clinical trials (Asaria and Gregor, 2002; Pastor, 1998; Pastor et al., 2002).

Another interesting approach is based on the inhibition of Rho-kinase (ROCK) because of the effect this kinase has on retinal cell survival and glial reactivity. In a retina culture, inhibition of ROCK had neuroprotective properties by attenuating the glial cell reactivity (Tura et al., 2009). Finally, Palomid 529 (Paloma Pharmaceuticals, Jamaica Plain, MA, USA), an inhibitor of the Akt/mTOR pathway that regulates intracellular signaling important for control of cell cycle, suppressed Müller cell proliferation, glial scar formation, and photoreceptor death in an experimental model of RD in rabbits (Lewis et al., 2009).

8.5. Neuroprotection

Although neuroprotection has the primary goal of improving the functional outcome after RD surgery by preventing photoreceptor death, (Lo et al., 2011; Murakami et al., 2011), it could be also useful in PVR. One of the possibilities is the use of caspase inhibitors, of which a large number of synthetic agents exist. However, despite extensive experimental efforts, there are few clinical trials using these compounds in human diseases (Murakami et al., 2013).

8.6. Multimodal approaches

Clinical strategies to prevent PVR would probably require a multimodal, combinatorial approach (Moysidis et al., 2012) because this is a multifactorial disease. Surprisingly, aside from the combination of 5-FU and LMWH, few reports have been published using

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combinations of drugs. Most of the existing studies were made many years ago and usually combined an anti-proliferative agent and a corticosteroid (Chen et al., 1992; Hui and Hu, 1999; Pastor et al., 2000; Salah-Eldin et al., 1994; Tung et al., 2001). There is also one study that combined a protein kinase C inhibitor and melatonin in an experimental model of PVR in rabbits, using grades A and B of the original classification of PVR (Er et al., 2006).

8.7. A look into the future

The most effective treatment option currently available for PVR remains vitrectomy. However the recent elucidation of pathways important in the pathogenesis of PVR and the increased understanding of its pathobiology has led to the identification of new potential targets to prevent it or to be used in adjunctive prophylaxis. One of these approaches is the interference or the modulation of integrin activity. Integrins are transmembrane protein receptors that act as bridges for cell-to-cell and cell-to-extracellular matrix interactions. Besides their importance for keeping RPE cells attached to their basal membranes and preventing migration into the vitreous cavity, these proteins could have a potential role in the final contraction process that affects existing epi- and/or subretinal membranes. Thus it may be possible to use antagonists to endogenous integrin mediators or reagents designed to inhibit integrin activity to prevent or treat PVR. Recent preclinical studies have shown that decreasing the expression of epithelial membrane protein 2, a RPE cell integrin, or inhibition of it by directed antibodies reduces the onset of PVR formation (Morales et al., 2012; Telander et al., 2011). The applicability of these *in vitro* results to human disease is not yet known.

Other insights have emerged from studies with drugs such as fasudil or simvastatin, generally used for other systemic disorders such as diabetes or pulmonary arterial hypertension. These agents have shown protection against PVR by interfering in the Rho activated kinase pathway (Kawahara et al., 2008; Kita et al., 2008). Anti-allergic drugs such as tranilast have also shown the ability to reduce experimental PVR (Ito et al., 1999).

Recently, in multiple preclinical models, strong evidence has shown that interfering with the PDGFR-α and p53 signaling pathways by using different targets such as VEGF or mdm2 may attenuate PVR. Therefore this could be another future approach (Lei et al., 2011; Murakami et al., 2013; Pennock et al., 2014b; Pennock and Kazlauskas, 2012; Pennock et al., 2013, 2011; Rosenkranz et al., 2002).

Perhaps, one of the most promising strategies for halting PVR development could emerge from studies of other retinal diseases or of the CNS. These studies involve neuroprotection and controlling reactive gliosis, one of the major intraretinal changes in PVR. Agents such as melatonin (Iribarne et al., 2007), aspirin (Bazan et al., 2010), tauroursodeoxycholic acid (TUDCA) (Fernandez-Sanchez et al., 2011; Mantopoulos et al., 2011), lutein (Woo et al., 2013), and especially anti-TNF-α (Nakazawa et al., 2011) may provide new therapeutic neuroprotective avenues to treat photoreceptor degeneration after a RD. They may also interfere at the same time with the glial processes that occur after an ischemic event in the retina (Fernandez-Bueno et al., 2013a), much like that which occurs in the CNS (Alonso-Alconada et al., 2013; Bae et al., 2006).

We believe that neuroprotection could play an important role in not only preventing PVR but also for improving the visual results after successful RD surgery. Many drugs are being investigated along with natural products such as resveratrol. This naturally occurring polyphenol, mainly found in grapes and red wine, has shown *in vitro* and *in vivo* neuroprotective effects for a variety of experimental models of neurodegenerative diseases (Zhang et al., 2015). This and other natural products such as TUDCA (Fernandez-Sanchez et al., 2011) or safranal deserve further investigation (Fernandez-Sanchez et al., 2012). Stem cells have also demonstrated neuroprotective properties *in vitro*, and our personal approach is that some of these factors released by cells, with or without some other drugs, should be investigated (Rodriguez-Crespo et al., 2014).

Apoptosis is another approach that should be taken into consideration for preventing PVR. Although some pro-apoptotic agents have failed to demonstrate any ability to inhibit progression of CNS tumors (Bedikian et al., 2014; Hu et al., 2013), they could still be an adequate option for PVR. Crocetin, which induces apoptosis through increased expression of pro-apoptotic Bax and activated caspase 3, has demonstrated promising results with esophageal carcinoma cells and could be another potential strategy for preventing PVR (Li et al., 2015).

As mentioned previously, another interesting target is TNF-α. It is a pleiotropic cytokine that plays an important role in inflammation by prompting various responses in different cell types, such as cell survival, proliferation, differentiation, and cell death by apoptosis or necroptosis among others (Vandenabeele et al., 2010). However, very little is known about the mechanisms by which TNF-α may mediate neuroprotection (Figiel, 2008). These mechanisms should be investigated. There are currently many anti-TNF-α drugs already approved for human disease, although none for PVR. Experience has shown that in recent years, many of the most interesting drugs were not specifically developed for eyes diseases but rather they were transferred from other purposes. Thus this and other similar approaches must be investigated.

A final point that deserves attention is the duration of the treatments. Although PVR is a relatively acute complication, it can last between 30 and 45 days in most cases (Pastor et al., 2002). Proposed treatments should guarantee efficient levels of therapeutic agents at the right target during this time. There are a few older reports that propose sustained delivery systems for treating PVR (Enyedi et al., 1996; Yang et al., 1998; Zhou et al., 1998). Currently we have gained more clinical experience with drug delivery systems, and there are new possibilities that should be explored.

For those agents that in preclinical studies have been demonstrated to be safe and effective in preventing PVR, clinical trials are the logical next step. Based on the identification of new key PVR mediators, the clinical trials could address the main complication after RD surgery.

9. Coming back to the classification: improving future clinical trials

One of the functions of any system of classification is to allow the comparison of new techniques and treatments, a clear necessity for PVR. In thinking about clinical trials, it is clear that the use of any of the existent classification schemes does not guarantee uniform results, and thus makes comparisons of techniques and treatments difficult or impossible. We are not sure if a new classification could be developed, but in the meantime we have a proposal for increasing the uniformity of the clinical trials.

Patients entering clinical trials could be classified in three categories. The first category would be composed of those patients with a RD and who do not exhibit any clinical sign of PVR. We do not consider the pigmented clumps as a definitive sign of this disease, so these patients could be included within this first category. This group could be used for testing prophylactic measures. To reduce the sample size necessary for these studies and to enhance the efficiency, it would be especially important to determine the genetic profile of the patients and select only those with a high risk of developing the disease.

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The second category of patients entering the clinical trials would be those with a frank preoperative PVR, a clear postoperative PVR, and those for whom the goal is to prevent the further development or re-proliferation of membranes that occur after long term tamponade with silicone oil. In these patients, emphasis should be made on the identification of intraretinal changes. The existence of epiretinal membranes can be solved in most of the cases by surgery. Patients in this second category, especially those with tamponade, could be an appropriate population to test anti-proliferative agents, drugs that inhibit collagen deposition and drugs that inhibit contraction of the membranes, and so on. Patients with intraretinal PVR, which could be detected by incorporating new imaging techniques into the routine examination, would be excluded from this second category of patients participating the clinical trials.

Finally, the third category of patients entering the clinical trials would be composed of those with extensive intraretinal PVR. At the time of surgery, the "shortening" of the retina is pathognomonic, but it would be very important to identify them before surgery. A careful clinical examination can add valuable information pertaining to intraretinal changes such as those that modify the color and transparency of the retina and the loss of mobility of the detached retina as detected by B-ultrasound. However these techniques have serious limitations and more reliable information can probably be gained by the application of new image-analysis techniques.

For patients in the third category of clinical trials, a severity scale should be added to indicate the extension of the intraretinal changes. If the extension affects more than one quadrant, it will be difficult to reattach without a retinectomy. The activity of the disease could also be evaluated. As described above, patients with RD and PVR of less than one week duration are likely to be in an almost pure inflammatory phase. In those patients, the measure of some biomarkers could add relevant information. For patients in whom the PVR is more than one week old, we can assume that mesenchymal transformation of RPE cells towards fibroblast-like cells plays an important role. Finally, in patients with PVRs of more than two weeks duration, membrane formation and contraction would be probably the most relevant facts. Obviously, a genetic profile should be mandatory to estimate the risk in this group.

According to these ideas, different families of compounds should be tested in patients placed into each of the three categories. We still believe that most of the effort must be made in avoiding the onset of PVR, and therefore the priority of designing clinical trials should be that of patients in the first category.

10. Conclusions and outlook (future directions)

Despite our best efforts over the past 40 years, we have thus far been unable to develop effective methods to prevent and treat PVR. It is still the most frequent and severe complication of RD surgery. Because of this, it also is a bottleneck for the development of new surgeries needed for advanced treatments, mainly cell therapy. The explanation for this failure for such a long period can be attributed to several causes. With the initial focus almost exclusively on cell proliferation and formation of membranes on the surface of the retina, not enough thought was given towards the development of objective and effective schemes for classification of PVR. This is an essential development that is needed to set the appropriate framework for further research. Furthermore, our lack of understanding of the existence and timing of developmental changes within the retina, lack of appropriate animal models, and lack of information regarding the cellular, molecular, and genetic origins of PVR pathogenesis have all contributed to the slow pace of clinical advances for this disease. Thus the historical focus on cell proliferation and membrane formation, which are relatively late events in the development of PVR, has led to inappropriate experimental models that have offered positive results for some drugs, but most of which have failed in the clinic.

Now we have a better idea regarding PVR pathogenesis and how it induces the remodeling of the retina that occurs after any RD. These changes, mainly intraretinal, are probably excessively amplified by inflammation and/or by a genetic pro-inflammatory profile. New advances in understanding the pathogenesis should drive the development of new and more appropriate models, if they are needed. Older classifications of PVR should be abandoned because they do not provide useful information from the clinical point of view. Even more, they do not contribute to uniformity in the samples for comparing new treatments and/or techniques. A broad consensus from clinicians would be required to collect samples in a more uniform way. The early identification of high risk patients through appropriate biomarkers, preferably before surgery, is mandatory to reduce the sample size of new clinical trials to a manageable number.

New treatments could be directed toward other factors besides cell proliferation such as immunomodulation or genetic therapy. In this sense we believe that retinal changes in RD, PVR, and some other retinal diseases will benefit from translational research currently taking place for CNS diseases. Thus it is highly probable that this disease cannot resist prevention and treatment for other 40 years.

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Conclusions

The general conclusions drawn from this second stage of the *Retina-4 project* are the following:

- The functional SNPs have allowed a better understanding of the molecular basis on the PVR, and also highlighted the important role of genetics as a useful tool in identification of high-risk patients and in new therapeutic targets.
- Although the mechanisms responsible for the PVR are not completely understood, it is accepted that inflammation and cell death play a crucial role in its pathogenesis, and the results of these studies seem to confirm this.
- Apoptosis plays an important role in the secondary disturbances of RD, and also seems to has a decisive role in the genesis of the PVR, which should be considered when proposing new treatments.

These general conclusions are based on the following sub-conclusions from the findings of the working group:

Chapter 1:

- Although the results suggest lack of functionality of the LTA rs2229094 polymorphism, this polymorphism could be remain as a valid biomarker
- to identify high-risk patients to develop PVR after RD, due to strong association between PVR and LTA rs2229094 polymorphism previously shown by our group
- The results of this study showed lower immunohistochemical staining in sections from retinas with chronic RD, however, due to limitations of the study is not possible to draw conclusions about the role of LTA in the affected retinas. So further studies are needed in this regard.

Chapters 2, 3 and 4:

- The association studies suggested that patients who carrying the Pro allele of rs1042522 of p53 gene, the G allele of rs2279744 of MDM2 gene and allele A of rs4645878 of BAX gene have a higher risk of developing PVR, so they could be used as potential biomarkers in the identification of high-risk patients or as elements to identify future therapeutic targets.
- The A allele of the rs2279115 of the BCL-2 gene could have a protective effect against the development of PVR, since overexpression of this protein is not only associated with a decrease in the apoptotic function but also induces an inhibitory effect on other cell death pathways or autophagy.

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