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Tesis Doctoral

Estudio de factores de riesgo vascular en patologías retinianas

Beatriz Fernández-Vega Sanz

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

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Español/Otro Idioma: Estudio de factores de riesgo vascular en patologías retinianas	Inglés: Study of vascular risk factors in retinal diseases

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RESUMEN (en español)

Las enfermedades vasculares que afectan a la retina y/o al nervio óptico producen, en muchos casos, una pérdida irreversible de la visión. Son actualmente muy prevalentes y tienen origen multifactorial. De ellas, las obstrucciones venosas retinianas (OVR) y las neuropatías ópticas isquémicas (NOIAs) constituyen la segunda causa de pérdida de visión de origen vascular, mientras que la degeneración macular asociada a la edad (DMAE) es una de las principales causas de ceguera central irreversible en personas mayores de 60 años. Ambas entidades afectan por igual a hombres y a mujeres y, aunque en un porcentaje de los casos no se llega a encontrar el origen, existen factores de riesgo a nivel cardiovascular y de trombofilia que pueden estar en relación con las mismas. Por ello, el objetivo fundamental de esta Tesis Doctoral ha consistido en la investigación de la etiopatogenia de las enfermedades vasculares retinianas OVR, NOIAs y DMAE mediante estudios de asociación génica, y en la evaluación del coenzima Q10 (CoQ10) como potencial agente terapéutico en el tratamiento de la perdida de campo visual en distintos tipos de patologías retinianas y del nervio óptico causadas por trastornos vasculares.

En el primer capítulo se llevó a cabo el estudio de la asociación de los polimorfismos del gen metilentetrahidrofolato reductasa (*MTHFR*) C677T y A1298C, responsables de una actividad enzimática reducida de MTHFR, con la OVR central y de rama venosa en una población española constituida por 359 sujetos (183 pacientes diagnosticados con OVR y 176 controles sanos). Tras el genotipado de los individuos mediante secuenciación y otras técnicas de biología molecular, se observó una alta prevalencia de las variantes T del polimorfismo C677T y C del A1298C del gen *MTHFR* en nuestra población. La prevalencia de estas variantes no fue significativamente diferente al comparar pacientes diagnosticados con OVR con controles sanos y tampoco se observó asociación de factores de riesgo como dislipidemia, diabetes mellitus, glaucoma, enfermedad tiroidea y enfermedad renal. Así mismo se observó una discordancia entre los niveles de homocisteína en sangre y las distintas mutaciones genéticas del *MTHFR*, siendo normal en prácticamente todos los casos en los cuales existía una mutación en homocigosis y estando elevada en aquellos casos en que no existía dicha mutación. Sin embargo, la frecuencia de la hipertensión fue significativamente mayor en los pacientes con OVR, confirmando este factor clínico como importante factor de riesgo de esta enfermedad.

En el segundo de los capítulos se estudió la asociación de los polimorfismos genéticos C677T y A1298C de *MTHFR* con NOIA en una población española formada por 94 pacientes y 204 controles sanos. Aunque las frecuencias alélicas y genotípicas de las variantes de *MTHFR* obtenidas no fueron significativamente diferentes en los pacientes con NOIA al compararse con los sujetos control, el genotipo C677T/A1298C, que codifica la enzima no mutada, fue significativamente más frecuente en sujetos control que en pacientes con NOIA, lo que sugiere un efecto protector de la proteína de tipo silvestre. Además, la historia clínica de enfermedades cardíacas o cerebrovasculares fue significativamente más frecuente en pacientes con NOIA en comparación con los controles. Aunque a la vista de los resultados de los dos primeros capítulos las variantes de los polimorfismos C677T y A1298C del gen *MTHFR* no son por si mismas un factor de riesgo de OVR o NOIA, no se puede descartar su influencia en el

desarrollo de estas patologías en un subgrupo de la población con otras características específicas como altos niveles plasmáticos de homocisteína, junto con deficiencias nutricionales que incluyen bajo nivel de folato o vitamina B12 y la combinación de factores de riesgo sistémicos y/o locales

En el tercero de los capítulos se abordó el manejo de un paciente diagnosticado con un infarto del lóbulo occipital superior derecho y consecuente cuadrantanopsia homónima inferior izquierda que fue tratado con CoQ10 en combinación con vitaminas. El inicio del tratamiento con CoQ10 produjo una mejoría inmediata del campo visual en ambos ojos, mientras que el seguimiento anual durante un periodo de 10 años mostró un incremento exponencial del campo visual, con recuperación completa, eliminación del escotoma y mejoría del pronóstico sin signos actuales de cuadrantanopsia, lo que abre nuevas perspectivas en el abordaje de aquellos casos en los que se consideraba imposible la recuperación de la visión con los conocimientos actuales.

En el cuarto de los capítulos, y en línea con el capítulo anterior, se presentaron los hallazgos clínicos y el manejo de una serie de múltiples casos de pacientes diagnosticados de distintas patologías retinianas o de nervio óptico como oclusión de arteria central de la retina o de rama arterial, perdida de campo visual de origen central tras accidente cerebrovascular, atrofia del nervio óptico de diferentes orígenes (tóxico, post-traumática, de origen desconocido), NOIA u OVR, tratados con CoQ10 y vitaminas. En los 48 pacientes incluidos en este estudio se observó una tasa de progresión de mejoría del campo visual superior al 10% anual tras la prescripción del tratamiento oral con CoQ10 siendo en algunos casos la recuperación prácticamente total hasta la fecha. El tratamiento con CoQ10 se interrumpió en uno de los pacientes, observando una disminución significativa del campo visual que se recuperó parcialmente cuando se restableció la suplementación con este coenzima, respaldando el papel de la CoQ10 como agente terapéutico en enfermedades vasculares que afectan a la retina o al nervio óptico.

En el quinto de los capítulos se dilucidó la posible asociación de once polimorfismos de un solo nucleótido en los genes más relevantes del metabolismo lipídico con DMAE, en pacientes del norte de España. Mediante un estudio de casos y controles realizado en 323 sujetos se observó que las frecuencias de alelos y genotipos para cada uno de los once polimorfismos estudiados no mostraron diferencias significativas. Entre ambos grupos Sin embargo, la frecuencia de los genotipos portadores del alelo APOE- ε2 en pacientes con DMAE húmeda es inferior en comparación con los controles, lo que indica un posible papel protector del alelo APOE-ε2 para la forma neovascular en la población española. Por último, la frecuencia significativamente más baja del alelo T del polimorfismo rs10468017 del gen *LIPC* en los casos con DMAE seca respectó al control, sugirió un papel protector frente al desarrollo de esta forma de la enfermedad, en dicha población.

RESUMEN (en Inglés)

Vascular diseases affecting the retina and/or the optic nerve cause irreversible vision loss, are currently very prevalent and have a multifactorial origin. Of these, retinal vein occlusions (RVO) and ischemic optic neuropathies (ION) constitute the second cause of vascular origin vision loss, while age-related macular degeneration (AMD) is one of the main causes of irreversible central blindness in people over 60 years. Both entities affect equally both men and women and, although in a percentage of cases the origin is not identified, cardiovascular and trombophilic risk factors contribute to their onset. Therefore, the main aims of this Doctoral Thesis consisted in the investigation of the etiopathogenesis of the retinal vascular diseases RVO, ION and AMD by gene association studies, and in the evaluation of coenzyme Q10 (CoQ10) as a potential therapeutic agent for treatment of different types of retinal dysfunctions caused by vascular disorders.

In the first chapter, the study of the association of the methylenetetrahydrofolate reductase (*MTHFR*) genetic polymorphisms C677T and A1298C, responsible for a reduced enzymatic activity of *MTHFR*, with retinal vein occlusion (RVO) in a Spanish population involving 359 subjects (183 patients diagnosed with RVO and 176 healthy controls) was carried out. DNA sequencing, using molecular biology techniques, showed a high prevalence of the MTHFR variants T and C of the polymorphisms C677T and A1298C, respectively. The prevalence of these variants was not significantly different when comparing RVO patients and controls and no

association of risk factors such as dyslipidemia, diabetes mellitus, glaucoma, thyroid disease and renal disease was observed. Likewise, a mismatch between blood homocysteine levels and the different genetic mutations of the *MTHFR* gene was observed, being normal in practically all cases in which there was a homozygous mutation and being elevated in those cases where there was no such mutation. However, frequency of hypertension was significantly higher in the RVO patients, confirming this clinical factor as risk factor of this disease.

In the second chapter we studied the association of the *MTHFR* genetic polymorphisms C677T and A1298C with non-arteritic ION in a Spanish population involving 94 patients and 204 healthy controls. Although the allelic and genotypic frequencies of the *MTHFR* variants obtained in the ION group were not significantly different when compared with the control group, the C677T/A1298C genotype, codifying the non-mutated enzyme, was significantly more frequent in control subjects than in ION patients, suggesting a protective effect of the wild-type protein. Furthermore, clinical history of heart or cerebrovascular diseases was significantly higher in ION patients comparing to controls. Although in view of the results of the first two chapters, the variants of the C677T and A1298C polymorphisms of the *MTHFR* gene are not themselves a risk factor for RVO or NOIA, their influence on the development of these pathologies cannot be ruled out in a subgroup of the population with other specific characteristics including high plasma levels of homocysteine along with nutritional deficiencies including low folate or vitamin B12 and the combination of systemic and local risk factors.

In the third of the chapters, the management of a patient diagnosed with a right superior occipital lobe infarction and consequent left inferior homonymous quadrantanopia treated with CoQ10 in combination vitamins was studied. The initiation CoQ10 treatment produced a promptly slight improvement of the visual field in both eyes, while the successive one-year follow-up examinations over a period of 10 years the patient experienced an exponential improvement in the visual field with gradually fading of the scotoma, with complete recovery and improvement of the prognosis without current signs of quadrantanopia, opening new perspectives in the management of these cases in which it was considered impossible to recover vision with current knowledge.

In the fourth chapter, the clinical findings and management of a series of cases of patients presenting different retinal or optic nerve pathologies, including ischemic optic neuropathy, retinal artery occlusion, loss of visual field of central origin after stroke, optic nerve atrophy of different origin (toxic, posttraumatic, or unknown), ION or OVR, and treated with CoQ10 and vitamins were reported. In the 48 patients included in this study, a progression rate of visual field improvement greater than 10% per year was observed after the prescription of oral CoQ10 treatment, with almost total recovery in some cases. CoQ10 treatment was interrupted in one of the patients, observing a significant decrease in the visual field, which was partially recovered when the supplementation with the enzyme was restored, supporting the role of CoQ10 as a therapeutic agent for vascular diseases affecting the retina or the optic nerve.

In the fifth chapter the possible association of eleven single nucleotide polymorphisms in the most relevant lipid metabolism genes in northern Spanish patients with AMD was elucidated. Through a case-control study conducted on 323 subjects, it was observed that allele and genotype frequencies for each of the eleven sequence variants in the lipid metabolism genes did not show significant differences when comparing AMD cases and controls. However, APOE-ε2 carrier genotypes were less frequently less frequently in patients with wet AMD compared to controls, which demonstrated a protective role of the APOE-ε2 allele for the neovascular form. Finally, significant lower frequency of the T allele of rs10468017 polymorphism of the *LIPC* gene in dry AMD cases when compared with controls suggested a protective role against the development of the dry form of AMD in the Spanish population.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN CIENCIAS DE LA SALUD

A mi padre, por ser mi maestro y mi ejemplo a seguir tanto a nivel profesional como humano, por su bondad, su gran humildad y discreción de la que todos deberíamos aprender.

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ÍNDICE

1. INTRODUCCIÓN

Las obstrucciones venosas retinianas (OVs) tanto de vena central (OVCR) como hemicentral o de cualquiera de sus ramas, así como las neuropatías ópticas isquémicas (NOIAs), constituyen la segunda causa de pérdida de visión de origen vascular tras la retinopatía diabética. Afectan a hombres y mujeres por igual y se producen fundamentalmente en personas mayores de 65 años (Klein et al., 2000; Rogers et al., 2010; Woo et al., 2016). Ambas patologías tienen etiología multifactorial y en muchos casos no llega a conocerse nunca el origen. Clínicamente se caracterizan por pérdida brusca de visión central y de campo visual, que suele ser grave en las NOIAs y OVCR y moderada en las trombosis de rama venosa (TRV) (O'Sullivan y Graham, 2007; Karia et al., 2010; Hong et al., 2017; Baumal, 2018).

El factor de riesgo más importante en las OVs es la hipertensión arterial (HTA) pero existen otros relacionados directamente con ellas como la diabetes, la hiperlipidemia, la hiperviscosidad sanguínea y la presencia de ciertos anticuerpos circulantes, especialmente antifosfolipídicos (anticardiolipina y anticoagulante lúpico). Así mismo pueden asociarse con alteraciones genéticas en el sistema de anticoagulantes naturales (factor V de Leiden, proteína C, factor II, etc.) o con un aumento de la homocisteína en sangre (Hansen et al., 200; Wong et al., 2005; Janssen et al., 2005).

Respecto a éste último punto, existen evidencias de que la hiperhomocisteinemia (HHC), incluso moderada, juega un importante papel en la aparición de las OVs y las NOIAs. Éste aumento de la homocisteína en sangre puede ser debido en un alto porcentaje de los casos a una mutación en el gen metilentahidrofolato reductasa (*MTHFR*). Sin embargo, hemos observado que las mutaciones en este gen, incluso en homocigosis, podrían no estar directamente relacionadas con niveles anormales de homocisteína (Jacques et al., 1996; D'Angelo et al., 2000; Schneider et al., 2005; Abd-Elmawla et al., 2016). Uno de los aspectos abordados en el presente trabajo de tesis doctoral ha sido el estudio de la incidencia de las dos mutaciones genéticas más

prevalentes en el gen MTHFR (C677T y A1298C) y sus repercusiones en los niveles sistémicos de los aminoácidos relacionados con el metabolismo de la homocisteína (metionina, taurina, etc.) en una población de pacientes del noroeste de España diagnosticados con OVCR o NOIA.

Por otro lado, se abordará el estudio de la oclusión vascular y sus secuelas a nivel clínico con pérdida irreversible de visión en aquellos casos en los que va acompañada de isquemia con la consecuente muerte progresiva de los fotoreceptores. En este proceso de isquemia el estrés oxidativo desempeña un papel muy importante ya que los radicales libres pueden producir la muerte celular mediante la inhibición de enzimas clave del ciclo del ácido tricarboxílico, de la cadena transportadora de electrones mitocondrial y la homeostasis de calcio mitocondrial (Beal, 2005). Por ello, se ha decidido llevar a cabo un estudio sobre el efecto de la administración exógena de coenzima Q10 (CoQ10) en la evolución clínica de las patologías neurovasculares oculares. El racional para ello se basa en que la CoQ10 (también conocida como ubiquinona, ubidecarenona o coenzima Q) es un cofactor esencial de la cadena transportadora de electrones. Actúa manteniendo el potencial de la membrana mitocondrial, contribuyendo a la síntesis de ATP, e inhibe la generación de especies reactivas de oxígeno (Beal, 2004; Russo et al., 2008; Salama et al., 2013).

Por último, la patología retiniana de degeneración macular asociada la edad (DMAE), considerada una de las principales causas de ceguera central irreversible en los países desarrollados (Jager y Mieler, 2008; Wong et al., 2014) es una enfermedad compleja y multifactorial con cierto componente genético de algunos genes relacionados con el metabolismo lipídico (Francis y Klein, 2011; Grassmann et al., 2012). Sin embargo, no existen estudios que aborden las mutaciones de genes relacionados con el metabolismo de lípidos en nuestro medio. Por ello, en otro apartado del trabajo se aborda la asociación de los genes APOE, CTEP, ABCA1, ABCA4, CETP, SCARB1, LIPC y LPL mediante un estudio casos-control en una población española de pacientes con DMAE.

Por tanto, la presente tesis doctoral persigue contribuir al conocimiento de la etiopatogenia de algunas enfermedades vasculares retinianas (OVCR, NOIAs, patologías

del EPR y DMAE) mediante estudios de asociación génica, así como dilucidar el papel que juegan determinadas moléculas (p. ej. CoQ10) en su fisiopatología y evolución. Los resultados obtenidos podrían resultar de potencial interés para el manejo clínico de estas enfermedades y contribuir al establecimiento de nuevos tratamientos y proponer dianas terapéuticas.

El cuerpo de esta Tesis está formado por cinco trabajos de investigación, 3 publicados, 1 aceptado y 1 en revisión, relacionados entre ellos pero que representan argumentos únicos y cerrados. No obstante, los artículos que forman los capítulos 4 y 5 pueden considerarse ambos parte del mismo estudio ya que representan la continuación uno del otro.

Por tanto, cada uno de los artículos contiene en su estructura los apartados de: a) Introducción y al final de la misma los objetivos del estudio; b) Material y Métodos: lo suficientemente detallados como para poder ser replicados en cualquier laboratorio; c) Resultados: en todos los artículos apoyados en tablas o figuras que los avalan; Discusión: basada en los resultados y sin ninguna especulación.

El trabajo que se presenta cumple el Artículo 28 (*Presentación de la tesis como compendio de publicaciones*) del Reglamento de los Estudios de Doctorado aprobado el 20 de julio de 2018, del Consejo de Gobierno de la Universidad de Oviedo y publicados en Boletín Oficial del Principado de Asturias, núm. 185 de 9-viii-2018.

2.

Association study of high frequency variants of MTHFR gene with retinal vein occlusion in a Spanish population (*publicado en Ophtalmic Genetics* 2019)

Association study of high-frequency variants of MTHFR gene with retinal vein occlusion in a Spanish population.

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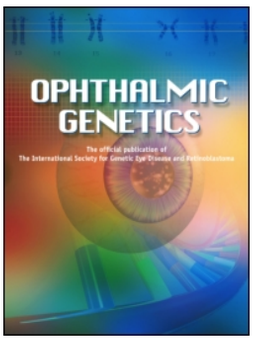

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Association study of high-frequency variants of *MTHFR* gene with retinal vein occlusion in a Spanish population

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RESEARCH REPORT

Association study of high-frequency variants of *MTHFR* gene with retinal vein occlusion in a Spanish population

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ABSTRACT

Background: To study the association of the most common methylenetetrahydrofolate reductase (*MTHFR*) genetic polymorphisms C677T and A1298C with retinal vein occlusion (RVO) in a Spanish population.

Methods: Case–control study involving 359 subjects, 183 unrelated native Spanish patients diagnosed with RVO, distributed in central or branch RVO, and 176 healthy controls. Two SNPs located in the gene *MTHFR*, C677T (rs1801133) and A1298C (rs1801131) were analyzed by DNA sequencing and TaqMan assays.

Results: A high prevalence of the *MTHFR* variants T and C of the SNP C677T and A1298C, respectively, was observed in our population. Specifically, 88.07% of controls and 85.25% of RVO patients have at least one of these variants. However, the prevalence of these variants was not significantly different when comparing RVO patients and controls. The variant T of C677T was identified in 60.65% of RVO patients and 59.10% of control subjects, while the variant C of A1298C was present in 46.45% of RVO patients and 51.14% of controls. No association of dyslipidemia, diabetes mellitus, glaucoma, thyroid disease and renal disease with RVO was observed, while hypertension was significantly higher in the RVO patients ($p < .0001$).

Conclusions: The *MTHFR* variants, T of C677T and C of A1298C, did not significantly increase the risk of suffering RVO in a Spanish population and therefore additional risk factors are contributing to the onset of the disease.

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Methylenetetrahydrofolate reductase (*MTHFR*) polymorphisms; retinal vein occlusion; genetic association study; Spanish population; world population review

Introduction

Retinal vein occlusion (RVO) is the second most common cause of visual loss due to retinal vascular disease, after diabetic retinopathy [(1).] It is a multifactorial disease occurring most frequently in elderly subjects. There are two main types of RVO: central retinal vein occlusion (CRVO) where the central retinal vein becomes blocked when it passes through the lamina cribosa of the optic nerve, and branch retinal vein occlusion (BRVO) in which the venous occlusion occurs in any branch of the central retinal vein at an arteriovenous crossing where an artery and vein share a common vascular sheath. Exceptionally, the occlusion may occur in a vein that drains half of the retina, resulting in hemiretinal vein occlusion (HRVO) onset (2).

The precise pathogenesis of RVO is still unclear. In addition to local risk factors, including open-angle glaucoma, systemic conditions such as arterial hypertension, diabetes mellitus, hyperlipidemia, cigarette smoking, and atherosclerosis have been associated with an increased risk for the development of RVO (3–5). Conflicting results have been reported for additional risk factors related with thrombophilia (deficiencies of the physiological coagulation inhibitors antithrombin III and proteins C and A), increased resistance to activated protein C (APCR), elevated levels of plasminogen activator inhibitor-1 (PAI-1) or hyperhomocysteinemia (6–8). In certain cases, these anomalies in the coagulation pathway could have a genetic component. Several polymorphisms in genes encoding proteins implicated in the coagulation system, including factor V G1691A (FV Leiden), prothrombin G20210A, methylenetetrahydrofolate reductase (MTHFR) C677T, PAI-1 4G/5G, and factor V H1299R, have been reported as potential risk factors for retinal vein occlusions (9–12).

Among the genetic prothrombotic risk factors, the two most common polymorphisms in the *MTHFR* gene, C677T and A1298C, and their association with RVO have been widely studied. The enzyme MTHFR, which catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, is a critical part of the process of homocysteine remethylation to methionine (13,14). The SNP C677T, located in the catalytic domain of the MTHFR enzyme, is a C to T substitution at nucleotide 677, which converts an alanine to a valine resulting in a thermolabile MTHFR protein with

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decreased enzymatic activity(15). Individuals carrying the allele T of this variant in homozygosis or heterozygosis have 30% or 65% of the enzymatic activity of the protein, respectively, which may lead to high levels of plasma homocysteine (hyperhomocysteinemia) mainly in the presence of low plasma folate levels (13,15,16). Interestingly, hyperhomocysteinemia has been related with vascular diseases(17), including RVO(18). On the other hand, the A1298C polymorphism consists of an A to C transition leading to the substitution of a glutamate by alanine within the C-terminal regulatory domain of the protein. This SNP has been also associated with the reduction of the MTHFR activity, but to a lesser extent than C677T mutation(19). Thereby, and according to the literature, the variants T of the SNP C667T and C of the SNP A1298C will be referred to as "risk variants."

Since the discovery of *MTHFR* polymorphisms was reported, their association with RVO has been studied in populations of diverse geographic origins, including Europe (20–22), USA(23), South America(24), North Africa (25) and Asia(26). These studies have shown that the frequency of the risk allele for each SNP varies among different ethnic groups and even between different populations within the same country. Therefore, in view of the controversial results reported in the literature and the lack of studies in the Spanish population, this work aims the association study of the *MTHFR* SNPs C677T and A1298C with RVO in 359 cases and controls from a Spanish population, and summarizes the obtained results of published works on this topic to date. In this vein, the study of the genetic risk factors associated with RVO may contribute to a deeper understanding of this disease, determining the suitability of performing complementary genetic test for its clinical management and to obtain new therapeutic strategies for this pathology.

Material and methods

Study subjects

The present case–control study involved 183 unrelated native Spanish patients diagnosed with RVO and 176 healthy controls recruited at the Fernández-Vega Ophthalmological Institute (Oviedo, Principality of Asturias, Spain). RVO group included 38 patients with CRVO, 134 with BRVO, 9 with history of both central and branch retinal vein occlusion, and 2 in which it was not possible to accurately determine the area of the vein occlusion. Complete ophthalmic examinations were performed for both patients and controls. Control subjects were selected from patients undergoing cataract surgery without a history or clinical evidence of RVO.

Diagnosis of RVO was made within 48 h from the onset of the first symptoms and all patients were again examined at least 6 months after the occurrence of the acute event. The diagnosis was based on a comprehensive clinical examination of the fundus of the eye, including direct and indirect ophthalmoscopy, optical coherence tomography (OCT) of the macula, fluorescein angiography (FA) imaging, visual field index analysis, best-corrected visual acuity and anterior segment examination. Multiple hemorrhages were found along the arcade or vascular arches affected by thrombosis, most of the times also accompanied by macular edema. FA study has been performed to assess ischemic degree, allowing the observation of the retinal perfusion and rule out the presence of neovessels. Briefly, the diagnostic criteria for CRVO included multiple retinal haemorrhages in all four quadrants of the retina, dilatation and tortuosity of the retinal veins and optic dis swelling, while the diagnosis of BRVO included the presence of retinal hemorrhages in the distribution of the occluded vein with the apex of the obstructed system located at the arteriovenous crossing(27).

Exclusion criteria for patients and controls were anterior ischemic optic neuropathy (AION), clinical evidences of atherosclerosis or peripheral vascular diseases and history of cardiovascular or cerebrovascular diseases. No subjects involved in this study presented with other relevant ocular pathologies, including age-related macular degeneration, diabetic retinopathy, or eye diseases affecting the retina or the optic nerve, except glaucoma. The clinical history of hypertension, dyslipidemia, diabetes mellitus, glaucoma, thyroid disease, and renal disease was obtained from all subjects to study its association with RVO in our population.

The study adheres to the tenets of the Declaration of Helsinki on Biomedical Research Involving Human Subjects and was approved by the Clinical Research Ethics Committee at Principality of Asturias (Oviedo, Spain). All participants signed an informed consent.

Genotyping

The present study included two SNPs located in the gene *MTHFR*, rs1801133 (C677T) and rs1801131 (A1298C). RVO patients were genotyped by DNA sequencing or by TaqMan assays. A total of 142 subjects were genotyped by DNA sequencing, 17 were genotyped using TaqMan assays, and a random subgroup of 24 patients was genotyped by both technologies, in order to validate the results. All control individuals (n = 176) were genotyped by TaqMan assays.

Sequencing

Saliva sampling and genomic DNA purification were carried out using the DANASALIVA Sample Collection Kit (DANAGEN, Barcelona, Spain). Patients must not eat, drink, smoke, or chew gum at least 30 min before collecting the sample. From 1 to 2 mL of saliva were collected in a tube, preservative solution added and finally stirred. The extraction of the genomic DNA from each primary sample of saliva extracted was carried out according to the recommendations of the DANAGEN manufactures protocols.

Two PCR reactions were carried out, designed to amplify the regions affected by the *MTHFR* variants (NM_005957): c.677C> T (exon 4) and c.1286A> C (exon 7), using the genomic DNA from saliva. The DNA sequence has been obtained by cyclic sequencing and capillary electrophoresis, using the 3130XL Genetic Analyzer (Applied Biosystems) at the Instituto de Medicina Oncológica y Molecular de Asturias (IMOMA, Oviedo, Spain). The sequence obtained was compared with that deposited in Ensembl for the *MTHFR* gene (ENSG00000177000), corresponding to the chr1 coordinates: 11846660–11866115 of the GRCh37 version of the human genome. The variants determined in each sample have been

identified following international nomenclature recommendations, according to the effect producing in the nucleotide sequence (based on the reference sequence with RefSeq ID NM_005957) or protein (based on the reference sequence NP_005948).

Taqman technology

Peripheral blood samples from 41 RVO patients and all the control individuals (n = 176) were collected in 6 mL K2EDTA tubes coated with EDTA (Vacuette, Madrid, Spain). Tubes were stored at −20°C until DNA isolation. Genomic DNA was obtained from the blood samples using a commercial DNA extraction kit (FlexiGene DNA Kit; Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Allelic discrimination of the SNPs was performed with TaqMan assays, C__1202883_20 (rs1801133, C677T) and C__850486_20 (rs1801131, A1298C), provided by the manufacturer (Applied Biosystems Inc., Foster City, CA, USA), in the 7500 Real Time PCR System (Applied Biosystems, Inc.), at the Fernández-Vega Ophthalmological Institute. All PCR amplifications were carried out with the thermal cycling conditions of 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min.

Statistical analysis

The SNPs were assessed for Hardy–Weinberg equilibrium (HWE) by a χ^2 test in both groups (cases and controls) and in the RVO subgroups (CRVO and BRVO), with HaploView 4.0 software(28). The ages of the RVO patients (CRVO and BRVO) and control subjects were compared using the Unpaired t-test (GraphPad InStat 3.0, San Diego, California). The comparison between groups of the SNPs allelic frequencies as well as of the frequencies of the additional potential risk factors considered was performed using a standard χ^2 test, with a *p*-value of less than 0.05 considered statistically significant. The comparison of genotypic frequencies between the RVO and control groups was performed using a χ^2 test (Pearson correction) with SPSS version 15.0 software (IBM Corporation, Armonk, NY). Relative risk association was estimated by calculating odds ratios (OR) along with 95% confidence intervals (CI), using the methods described by Armitage et al. (29) and the tool PLINK (version 1.07). Additionally, we used SPSS version 15.0 software (IBM Corporation, Armonk, NY) to run a logistic regression analysis in order to control for potential confounders.

Results

We have determined the allele and genotype frequencies of two *MTHFR* gene variants, C677T and A1298C, in RVO patients (n = 183) and controls (n = 176) of a Spanish population, as described in Material and Methods section. Demographics and clinical characteristics of RVO patients and controls are reported in Table 1. The distributions by gender and age did not show significant differences between groups. Regarding clinical factors, only the prevalence of hypertension was significantly higher in the RVO group compared with control subjects (64.48% and 41.48%, respectively, $p < .0001$). An additional logistic regression analysis was carried out for possible interactions among risk factors. This analysis confirmed that hypertension was independently associated with the presence of RVO.

Table 1. Demographics and clinical characteristics of RVO patients and controls.

	RVO patients (n = 183) (%)	Controls (n = 176) (%)	p Value
Men/women	87 (47.54)/96 (52.46)	70 (39.77)/106 (60.23)	0.1380
Age (mean years ± SD)	62.49* ± 11.27	64.25 ± 11.54	0.1439
Age range	20–88	35–92	
Hypertension, *n* (%)	118 (64.48)	73 (41.48)	**<0.0001**
Dyslipidemia	84 (45.90)	66 (37.50)	0.1066
Diabetes mellitus, *n* (%)	25 (13.66)	19 (10.80)	0.4078
Treatment for glaucoma	19 (10.8)	11 (6.25)	0.1572
Thyroid disease	12 (6.56)	13 (7.39)	0.7577
Renal disease	7 (3.8333)	2 (1.14)	0.1033

RVO, retinal vein occlusion; n, number of subjects; SD, standard deviation. The asterisk (*) indicates the average age at the first event.

Cases and controls were in Hardy–Weinberg equilibrium for the analyzed genetic variants ($p > 0.05$). The comparison between the allele and genotype frequencies of the *MTHFR* variants analyzed in this study did not show significant differences between RVO cases and controls (see Table 2). A total of 67 RVO patients were younger than 60 years. The allele and genotype frequencies in this younger group were not significantly different compared to older subjects (data not shown).

To assess any influence to our results of a possible misclassification of some individuals included in the control group, we reanalyzed the data excluding all individuals under 60 from this group. The number of individuals in the new control group was 116, and the average age (70.85) was significantly higher than that of the RVO patients (62.30), $p < .0001$. Similar to the previous results, the allele and genotype frequencies of the studied SNPs did not show significant differences between groups.

In addition, a genetic association study was carried out with the RVO patients according to the localization of the vascular occlusion (38 CRVO and 134 BRVO). Table 3 details the demographics and clinical characteristics of CRVO and BRVO patients. The nine patients with a history of both types of RVO and the two subjects in which the place of the RVO was unknown were excluded from this analysis. Both subgroups were in Hardy–Weinberg equilibrium for the analyzed SNPs ($p > .05$). No association was found between the C677T and A1298C *MTHFR* polymorphisms and the CRVO and BRVO subgroups when compared to control subjects (see Table 3). We also did not observe any significant difference in *MTHFR* genotypes in BRVO comparing to CRVO (data not shown). Regarding clinical factors, hypertension was found to be a significant risk factor for BRVO ($p < .0001$), but not for CRVO ($p = .0647$), while diabetes was significantly associated exclusively with CRVO patients ($p = .0326$).

Finally, the nine theoretically possible genotypes formed by the SNPs C677T and A1298C in RVO (CRVO and BRVO) and controls have been studied, as shown in Table 4. As can be observed, the C677T/A1298C genotype codifying the native MTHFR form (i.e., CC/AA) reached the 14.75% in RVO patients (7.89% of CRVO and 15.67% of BRVO) and 11.93% in control subjects.

Table 2. Allelic and genotypic association analysis of RVO patients and control subjects.

SNP ID		RVO % (*n* = 183)	Control % (*n* = 176)	*p* Value	OR (95% CI)
***MTHFR* C677T**					
Allele	C	62.30	61.93		1.0 (0.81–1.25)
	T	37.70	38.07	0.9201	1.0 (0.70–1.41)
Genotype	CC	39.34	40.91	0.5526	0.96 (0.68–1.35)*
	CT	45.90	42.05		1.09 (0.80–1.50)
	TT	14.75	17.05		0.86 (0.46–1.64)
	Total	72/84/27 (CC/CT/TT)	72/74/30 (CC/CT/TT)	0.7207	
***MTHFR* A1298C**					
Allele	A	72.13	69.32		1.04 (0.87–1.24)
	C	27.87	30.68	0.4075	0.91 (0.59–1.40)
Genotype	AA	53.55	48.86	0.7646	1.10 (0.84–1.44)*
	AC	37.16	40.91		0.91 (0.64–1.28)
	CC	9.29	10.23		0.91 (0.40–2.11)
	Total	98/68/17 (AA/AC/CC)	86/72/18 (AA/AC/CC)	0.6740	

RVO, retinal vein occlusion; n, number of subjects; OR, odds ratio; CI, confidence interval. p < 0.05, significant. Total indicates the general test of association in the 2- by-3 table of disease-by-genotype. The asterisk (*) indicates the OR values and p-values derived from comparison of the genotypic frequencies under the recessive model (TT *vs* CT + CC at rs1801133, and CC *vs* AC + AA at rs1801131).

Table 3. Overall distribution of the genotypic and clinical risk factors in cases and controls.

	Controls (*n* = 176) (%)	CRVO patients (*n* = 38) (%)	*p* Value	BRVO patients (*n* = 134) (%)	*p* Value
Men/women	70 (39.77)/106 (60.23)	19 (50)/19 (50)	0.2460	63 (47.01)/71 (52.99)	0.2019
Age (mean years ± SD)	64.25 ± 11.54	62.74* ± 13.23	0.4763	62.97* ± 10.08	0.3082
Age range	35–92	28–82		32–88	
MTHFR C677T (TT *vs* CT + CC)	30 (17.05)	7 (18.42)	0.8389*	19 (14.18)	0.4931*
MTHFR A1298C (CC *vs* AC + AA)	18 (10.23)	3 (7.89)	0.6612*	12 (8.96)	0.7075*
Hypertension, *n* (%)	73 (41.48)	22 (57.89)	0.0647	90 (67.16)	**<0.0001**
Dyslipidemia, *n*(%)	66 (37.50)	14 (36.84)	0.9394	67 (50)	0.0276
Diabetes mellitus, *n* (%)	19 (10.80)	9 (23.68)	**0.0326**	13 (9.7)	0.7538
Treatment for glaucoma, *n* (%)	11 (6.25)	5 (13.16)	0.1420	12 (8.96)	0.3680
Thyroid disease, *n* (%)	13 (7.39)	2 (5.26)	0.6420	10 (7.46)	0.9797
Renal disease, *n* (%)	2 (1.14)	2 (5.26)	0.0885	4 (2.99)	0.2418

CRVO, central retinal vein occlusion; BRVO, branch retinal vein occlusion; n, number of subjects; SD, standard deviation. The symbol (#) indicates the average age at the first event. The asterisk (*) indicates the p-values derived from comparison of the genotypic frequencies of cases and controls under the recessive model (TT *vs* CT + CC at C677T, and CC *vs* AC + AA at A1298C).

Discussion

The main goal of the present case–control study was to evaluate the relationship among the *MTHFR* C677T and A1298C polymorphisms and RVO in the Spanish population. In our study, the prevalence of *MTHFR* polymorphisms was not significantly different when comparing RVO patients and controls. Similarly, when CRVO and BRVO subgroups were considered separately, mutations in *MTHFR* were not found to be risk factors. Accordingly, as shown in Table 2, the risk allele T of C677T has been identified in the 60.65% of RVO patients and 59.10% of control subjects, while the risk allele C of A1298C has been present in 46.45% of RVO patients and 51.14% of controls, responsible in both cases for the synthesis of thermolabile MTHFR enzymes. Interestingly, the prevalence of the genotypes responsible for MTHFR thermolabile isoforms (i.e., CT/AC, CT/CC, CT/AA, TT/AA, TT/AC, TT/CC, CC/AC, and CC/CC) accounts the 85.25% in RVO patients and 88.07% in controls (Table 4). These isoforms have a decreased enzymatic activity, which may contribute to high levels of plasma homocysteine. However, the thermolabile forms are not risk factor themselves, since plasma homocysteine levels depend not only in the MTHFR activity but also in folate, vitamin B12, and creatinine levels (13,14).

Among the clinical factors classically associated with RVO (see Table 3), hypertension, dyslipidemia, diabetes mellitus, glaucoma, and renal disease, were more frequently found among RVO patients than controls, although only hypertension was a risk factor statistically significant for RVO ($p < .0001$), which is consistent with previous reports (30). Systemic disorders may be associated with RVO subtypes, but this does not necessarily imply a cause-and-effect relationship. In this regard, hypertension was also a significant risk factor for BRVO ($p < .0001$), but not for CRVO ($p = .0647$) probably due to the relatively small sample size of this subgroup (n = 38). Interestingly, diabetes disease was significantly associated only with CRVO, although a greater number of

Table 4. Genotypic frequency of RVO, CRVO and BRVO patients and control subjects.

C677T/ A1298C	RVO (*n* = 183) (%)	CRVO (*n* = 38) (%)	BRVO (*n* = 134) (%)	Control (*n* = 176) (%)
CT/A**C**	40 (21,86)	8 (21,05)	28 (20,90)	39 (22,16)
CT/CC	0,00	0,00	0,00	1 (0,57)
CT/AA	44 (24,04)	10 (26,32)	33 (24,63)	35 (19,89)
TT/AA	27 (14,75)	7 (18,42)	19 (14,18)	29 (16,48)
TT/A**C**	0,00	0,00	0,00	1 (0,57)
TT/CC	0,00	0,00	0,00	0,00
CC/A**C**	28 (15,30)	7 (18,42)	21 (15,67)	32 (18,18)
CC/**CC**	17 (9,29)	3 (7,89)	12 (8,96)	18 (10,23)
CC/AA	27 (14,75)	3 (7,89)	21 (15,67)	21 (11,93)

RVO; retinal vein occlusion, CRVO; central retinal vein occlusion, BRVO; branch retinal vein occlusion, n; number of subjects. The risk allele is indicated in bold. Heterozygotes for C677T including CT and TC subjects; AC, Heterozygotes for A1298C including CT and TC subjects.

patients with CRVO must be studied to confirm this observation.

It should be stressed that the RVO and control groups were balanced for sex and age. Considering that RVO is a late-onset disorder, some misclassification among the youngest individuals in the control group could be possible. However, the analysis of the data following the exclusion of individuals older than 60 confirmed the results previously obtained, without significant differences in the allele and genotype frequencies. Interestingly, hypertension continued to be a risk factor for RVO (p = .0087), confirming the robustness of this association (data not shown).

The extensive literature revised, and summarized in Table 5, evidence conflicting results, varying the frequency of the risk alleles among the different ethnic groups and between populations of the same country. The *MTHFR* C677T polymorphism has been associated with RVO in some populations of Mediterranean origin, including Italy (20,31–33), Tunisia(25), Greece (11), Israel (9,48) and Jordan(49) while the A1298C polymorphism has been exclusively associated in a Tunisian population(25). However, other populations of same countries did not provide genetic association (21,34–36), so it is not possible to confirm any geographic influence. Moreover, lack of association was also shown in populations from European (Ireland, UK, Sweden, Austria, and Turkey), North and South American (USA, Argentina, and Brazil), Middle Eastern (Iran) and Asian countries (China and India) (see Table 5). The great heterogeneity existing (ethnic origin, sample size, type of RVO or evaluating the risk conferred by allelic or genotypic frequencies) must be considered when comparing these studies. In this sense, meta-analyses carried out to date showed no evidence to suggest an association between TT genotype for the *MTHFR* C677T and RVO (12,18,47).'

The high frequency of the *MTHFR* variants in the Spanish population may trigger a decrease in the activity of the synthesized enzyme, contributing along with additional risk factors to the development of ocular vascular occlusions(15). Interestingly, 88.07% of our controls are carriers of at least one of these variants. To contextualize this observation over the world, the presence of these alleles in other populations of different racial/ethnic origins has been studied, based on the data collected in the 1000 Genomes Project(52). To this end, the populations (Iberian Population in Spain), CEU (Utah Residents (CEPH) with North and Western European Ancestry), PEL (Peruvians from Lima, Peru), CHB (Han Chinese in Bejing), YRI (Yoruba in Ibadan, Nigeria) and GWD (Gambian in Western Division) were

Table 5. Case–control studies of the *MTHFR* polymorphisms and RVO reported so far.

Country of origin	Disease	Cases/ Controls	C677T (rs1801133)	A1298C (rs1801131)
Italy(31)	CRVO	100/100	**TT genotype, p = .04***	NA
Italy(32)	RVO (CRVO + BRVO)	55/61	**TT genotype/T allele p < 0,005**	NA
Italy(20)	RVO	63/48	**TT genotype, p < .01**	NA
Italy(33)	RVO	109/104	**TT genotype, p = .017**	NA
Italy(34)	CRVO	31/62	No Assoc.	NA
Italy(22)	RVO (CRVO + BRVO)	105/226	No Assoc.	NA
Italy(35)	RVO (CRVO + BRVO)	117/202	No Assoc.	NA
Italy(21)	RVO (CRVO + BRVO)	86/71	No Assoc.	NA
Greece(11)	RVO (CRVO + BRVO)	48/53	TT genotype, no assoc. **T allele, p = 0,026**	No Assoc.
Greece(36)	RVO (CRVO + BRVO)	51/51	No Assoc.	No Assoc.
Ireland(37)	RVO (CRVO, HRVO, BRVO)	61/87	No Assoc.	NA
Ireland(38)	RVO	103/94	No Assoc.	NA
Turkey(39)	RVO	49/68	No assoc.	NA
UK(40)	CRVO	63/63	No Assoc.	NA
UK(41)	RVO (CRVO + BRVO)	40/40	No Assoc.	NA
Sweden(42)	CRVO	116/140	No Assoc.	NA
Austria(43)	BRVO	84/84	No Assoc.	NA
Austria(44)	CRVO	78/78	No Assoc.	NA
USA(23)	RVO	17/234	No Assoc.	NA
Argentina(24)	CRVO	37/144	No Assoc.	NA
Brazil(45)	RVO	55/55	No Assoc.	NA
China(46)	CRVO	64/64	No Assoc.	NA
China(26)	CRVO	68/68	No Assoc.	NA
India(47)	RVO (CRVO + BRVO)	50/50	No Assoc.	NA
Tunisia(25)	RVO	72/140	**CT genotype, p < .001** TT genotype, absent **T allele, p = .005**	**AC + CC genotypes, p = .01 C allele, p = .005**
Israel(9)	RVO (CRVO, HRVO and BRVO)	102/105	**TT genotype, p = .027**	NA
Israel(48)	RVO	59/210	**TT genotype, p = .038**	NA
Jordan(49)	RVO (CRVO + BRVO)	96/96	**TT + CT genotypes**, CRVO (n = 52), **p < .01** BRVO (n = 44), no assoc.	NA
Iran(50)	RVO	73/73	No Assoc.	NA
Iran(51)	RVO	73/73	NA	No assoc.
Spain (current study)	RVO (CRVO + BRVO)	183/176	No assoc.	No assoc.

rs1801133 or rs1801131 SNP ID number from the Single Nucleotide Polymorphism database (dbSNP); RVO, retinal vein occlusion; CRVO, central retinal vein occlusion; BRVO, branch retinal vein occlusion; HRVO, hemi-retinal vein occlusion; NA, data not available; No assoc., no association. *Not significant after multivariate analysis.

selected and the Genotypic frequencies of C677T/A1298C are shown in Table S1 (Supporting Information). According to the data, a high frequency of these variants has been observed in the IBS population (91.59%, similar to the Spanish population included in the current study, i.e., 88.07%), as well as in other Caucasian populations such as the CEU (80.81%), in Hispanic populations such as PEL (77.91%), and in Asian populations such as CHB (89.32%). Surprisingly, this trend is not maintained in African black populations, where the frequency of these variants is markedly reduced, as is the case of the YRI (42.20%) and GWD (30.97%) populations. Moreover, no TT homozygous for C677T is observed in both African black populations, while homozygous CC for A1298C has only been found in the population from Gambia. These results highlight the racial/ethnic influence on the genotype frequencies of *MTHFR*.

Nevertheless, the different genotype frequencies for the SNPs C677T and A1298C of *MTHFR* are not reflected in the RVO prevalence along the different populations. As part of the International Eye Disease Consortium(53), the pooled analysis from 68751 participants from 15 epidemiological studies from the United States, Europe, Asia and Australia reported RVO prevalence of 3.7 per 1000 (CI, 2.8–4.6) in whites (Europeans, and those of European origin), 3.9 per 1000 (CI, 1.8–6.0) in blacks (African-Americans), 5.7 per 1000 (CI, 4.5–6.8) in Asians (Chinese, Chinese-American, Malay, people of Asian origin and Europeans of Indian, Indonesian, or Asian origin), and 6.9 per 1000 (CI, 5.7–8.3) in Hispanics (Hispanic-Americans). Although there is a high prevalence in Hispanic and Asian compared to whites and blacks, the confidence intervals suggest that the differences are not statistically significant. Similarly, an additional literature review concluded that the prevalence of RVO is relatively constant in all countries(54). The lack of differences in the prevalence of RVO between races in the different ethnic groups contrasts with the lower frequency of the risk variants of *MTHFR* in African black subjects, as stated above. Association studies of *MTHFR* SNPs with RVO, along with the analysis of additional risk factors in black populations would be of interest.

The main limitation of this study, which may be extended to the case–control published studies, is the great heterogeneity within each group due to the large number of factors influencing the onset of RVO. It cannot be ruled out that the *MTHFR* polymorphisms may be a risk factor for the development of RVO in patients with other specific characteristics. In addition to other systemic conditions, the analysis of the serum levels of homocysteine, serum folate, and vitamin B12 could help to classify RVO patients in greater homogeneous subgroups. Therefore, further studies considering demographic, lifestyle, and genetic factors are required to identify specific subgroups in which the *MTHFR* polymorphisms may have an effect on the onset and progression of RVO disease.

Disclosure of interest

The authors report no conflict of interest.

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Association study of MTHFR polymorphisms with non-arteritic anterior ischemic optic neuropathy in a Spanish population (aceptado para publicación en *Biomedicine Hub*)

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Association study of MTHFR polymorphisms with non-arteritic anterior ischemic optic neuropathy in a Spanish population

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Running head: MTHFR polymorphisms and non-arteritic anterior ischemic optic neuropathy

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Keywords: non-arteritic anterior ischemic optic neuropathy; methylenetetrahydrofolate reductase (MTHFR) gene; association study; C677T; A1298C.

Abstract

Introduction: Non-arteritic anterior ischemic optic neuropathy (NAION), a painless loss of central and/or peripheral vision, is a multifactorial disease caused by insufficient blood flow through the posterior ciliary arteries to the optic nerve head. Mutations in the methylenetetrahydrofolate reductase (*MTHFR*) gene, triggering hyperhomocysteinemia as a consequence of a decreased activity of the codified enzyme, have been considered among the risk factors of NAION. **Objective:** The main aim has been to study the association of the most common MTHFR genetic polymorphisms C677T and A1298C with NAION in a Spanish population. **Methods:** In this case-control study, the association of the most common *MTHFR* genetic polymorphisms has been conducted in 94 unrelated native Spanish patients diagnosed with NAION and 204 healthy controls. Two SNPs located in the gene *MTHFR*, C677T (rs1801133) and A1298C (rs1801131) were analyzed by DNA sequencing and TaqMan assays. **Results:** The allelic and genotypic frequencies of the *MTHFR* variants obtained in the NAION group were not significantly different when compared with the control group. However, the C677T/A1298C genotype codifying the non-mutated MTHFR form was significantly more frequent in control subjects (11.27%) than in NAION patients (4.26%), suggesting a protective effect of the wild-type protein. The study of additional clinical factors including hypertension, diabetes mellitus and dyslipidemia showed no association with a higher risk of NAION. Conversely, clinical history of heart or cerebrovascular diseases was significantly higher in NAION patients comparing to controls. Over the world, risk variants of the *MTHFR* gene are highly frequent, excluding African black populations, indicating a racial influence. **Conclusions:** The MTHFR variants did not significantly increase the risk of suffering NAION. However, considering that individuals with at least one of the risk variants have the MTHFR enzyme with decreased activity, it cannot be ruled out that these mutations are relevant for the development of NAION in a subgroup of the population with other specific characteristics. These may include high plasma levels of homocysteine along with nutritional deficiencies including low folate or vitamin B12 and the combination of systemic and local risk factors.

Introduction

Ischemic optic neuropathy (ION) is a sudden loss of central and/or peripheral vision due to a decrease or interruption of blood flow to the optic nerve. It is not a single disease but a spectrum of ocular diseases characterized by the ischemic nature of the lesion in the optic nerve [1]. There are two types of ischemic optic neuropathy, depending on the region of the optic nerve affected: anterior ischemic optic neuropathy (AION), which is due to an interruption or an insufficiency in the blood flow to the optic nerve head (ONH) supplied by the posterior ciliary arteries (PCAs) [2]; and posterior ischemic optic neuropathy (PION), as a result of ischemia in the posterior part of the optic nerve [3].

Among the AION cases, the most common ischemic optic neuropathy in adults, we can distinguish between arteritic (AAION) and non-arteritic (NAION) [4]. AAION is a dangerous condition caused by inflammation of the arteries supplying blood to the optic nerve. The inflammation is mainly associated to a systemic disorder known as giant cell arteritis. NAION is the most common form of AION, where no inflammation of the arteries occurs and may result in severe visual acuity or visual field loss [5]. NAION typically presents as acute, unilateral and painless loss of central and/or peripheral vision that may progress over several hours or days. NAION mainly affects patients over 50 years of age but the neuropathy may appear at any age, and both men and women have the same rates of occurrence [6,7].

Even though the pathogenesis of NAION is unclear, is considered a multifactorial disease in which a variety of systemic and ocular risk factors play a role. Systemic risk factors include diabetes mellitus [8-15], nocturnal arterial hypotension [16,17], arterial hypertension [8,11,13,15,18], hypercholesterolemia and ischemic heart disease [10,12,13,15,18,19], blood loss [20], atherosclerosis [8], sleep apnea syndrome [21-25], cardiovascular disorders [8,15], migraine [21] and erectile dysfunction drugs such as sildenafil [26-28]. Ocular risk factors include a small and crowded disc [15,29], elevated intraocular pressure or optic disc drusen that may influence the ONH blood supply [30].

It is widely accepted that sleep-induced nocturnal arterial hypotension, sometimes aggravated by antihypertensive therapy, is an important factor in the pathogenesis of NAION. A fall in blood pressure, along with other underlying risk factors, could cause insufficient blood flow through the PCAs to the ONH. This hypothesis is based on the observation that the first

symptoms of NAION are usually noticed after nighttime sleep or after a nap, when a physiological drop in blood pressure occurs [16,17,31-34].

Great controversy exists in relation to the association of thrombophilic risk factors with NAION. While some authors point to an association between some of these factors and the disease [11,19,35-41], others have not found such a relationship [9,42]. Hyperhomocysteinemia is an important thrombophilic marker associated to an increased risk of coronary heart disease [43]. Several studies have found association between hyperhomocysteinemia and NAION [11,18,39,44]. Insufficient intake of vitamin B12, vitamin B6 and folic acid and a genetic predisposition are among the factors contributing to elevated plasma homocysteine levels. Specifically, mutations in the gene encoding the protein methylenetetrahydrofolate reductase (MTHFR) are the most commonly known genetic risk factor for hyperhomocysteinemia. The MTHFR protein is an important enzyme in the homocysteine metabolism, which catalyzes the conversion of 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate [45]. The most common *MTHFR* mutations are two single nucleotide polymorphisms (SNPs), the thermolabile *MTHFR* variant C677T and *MTHFR* A1298C. Both SNPs result in a decreased MTHFR activity, more pronounced for *MTHFR* C677T, which may leads to hyperhomocysteinemia mainly in the presence of low plasma folate levels [45-47]. Individuals with the genotype 677TT have approximately only the 30% of the MTHFR enzyme activity, whereas heterozygotes 677CT have around 65% of enzymatic activity [48].

To date, only six studies addressing the association of the *MTHFR* C677T variant with NAION have been published, obtaining controversial results. Two of them, performed in a population from Italy [40] and in a population from USA [36], observed a significant association of the 677TT genotype with the disease ($p = 0.005$ and $p = 0.004$, respectively). However, those carried out in populations from Italy [18], USA [41], Austria [11] and Israel [10] did not find evidence of higher risk of NAION in patients with this genotype. Regarding the *MTHFR* A1298C polymorphism, Felekis et al. [40] did not find a significant association with NAION in an Italian population.

Considering there is no effective treatment against NAION, the study of potential genetics risk factors could help to elucidate the pathogenesis of the disease in certain groups of patients and determine the suitability of complementary genetics test for clinical management. Through this case-control study we aim to investigate the association of the

MTHFR variants C677T and A1298C with NAION in a Spanish population of 298 cases and controls.

Materials and Methods

Study subjects

A total of 94 unrelated native Spanish patients diagnosed with NAION and 204 healthy controls recruited at the Fernández-Vega Ophthalmological Institute (IOFV, Oviedo, Principality of Asturias, Spain) were included in the present case–control study. Control subjects were selected from patients undergoing cataract surgery without a history or clinical evidence of ION. Complete ophthalmic examinations were performed for both patients and controls who were also subjected to a detailed interview aimed at collecting the presence of risk factors, including arterial hypertension, dyslipidemia and diabetes mellitus, as well as their clinical history of heart and vascular diseases.

NAION patients were diagnosis at the IOFV by visual field examination, determination of nerve fiber layer thickness by OCT, visual acuity, evoked potentials, examination of the papilla with identification of the edematous stages with or without peripapillary hemorrhages evolving to optic nerve atrophy and papillary pallor. Finally, complete neurological study including magnetic resonance imaging was carried out. Exclusion criteria for patients and controls included any history of ocular vascular disease as retinal vein occlusion (RVO) and retinal artery occlusion (RAO). Demographics characteristics of NAION patients and control subjects are showed in Table 1.

Given that the clinical factors hypertension, dyslipidemia, diabetes mellitus, heart diseases (including coronary heart disease, arrhythmias, atrial fibrillation and hypertensive heart disease) and cerebrovascular disease are influenced by sex and age, we have performed the association study using 72 patients with NAION and 72 sex- and age-matched controls. The remaining 22 NAION subjects were excluded from this specific analysis owing to lack of appropriate controls to perform the matching. When more than one control had the sex and age appropriate for matching with a specific NAION, the first one who came to the ophthalmological examination was included. No other characteristic or condition of the individuals, different from sex or age was considered for the pairing. Demographics and clinical characteristics of the sex-and age-matched NAION and control groups are reported in Table 2.

Genotyping

This study included the analysis of two SNPs located in the gene *MTHFR*, rs1801133 (C677T) and rs1801131 (A1298C). NAION patients (n=94) were genotyped by DNA sequencing. All control individuals (n=204) were genotyped by TaqMan assays. A subgroup of 25 randomly selected individuals among the 94 NAION patients was also genotyped by TaqMan assays, in order to validate the results obtained by this technology.

Sequencing

Saliva sampling and genomic DNA purification were carried out using the DANASALIVA Sample Collection Kit (DANAGEN, Barcelona, Spain). Patients did not eat, drink, smoke or chew gum at least 30 minutes before sampling. From 1 to 2 mL of saliva were collected in a tube, followed by the addition of preservative solution and finally stirred. The extraction of the genomic DNA from each primary sample of saliva was carried out according to the recommendations of the DANAGEN manufactures protocols.

Two PCR reactions designed to amplify the regions affected by the *MTHFR* variants (NM_005957) were carried out: c.677C> T (exon 4) and c.1286A> C (exon 7), using the genomic DNA from saliva. The DNA sequence was obtained through cyclic sequencing and capillary electrophoresis, using the 3130XL Genetic Analyzer (Applied Biosystems) at the Instituto de Medicina Oncológica y Molecular de Asturias (IMOMA, Oviedo, Spain). The sequence obtained was compared with that deposited in Ensembl for the *MTHFR* gene (ENSG00000177000), corresponding to the chr1 coordinates: 11846660-11866115 of the GRCh37 version of the human genome. The variants determined in each sample have been identified following international nomenclature recommendations, according to the produced effect in the nucleotide sequence (based on the reference sequence with RefSeq ID NM_005957) or protein (based on the reference sequence NP_005948).

TaqMan technology

Peripheral blood samples from 25 patients diagnosed with NAION and all the control individuals (n=204) were collected in 6 mL tubes coated with EDTA (Vacuette, Madrid, Spain). Samples were stored at −20°C until DNA isolation. Genomic DNA was obtained from blood using a commercial DNA extraction kit (FlexiGene DNA Kit; Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Allelic discrimination of the SNPs was performed with TaqMan assays, C__1202883_20 (rs1801133, C677T) and C__850486_20 (rs1801131, A1298C), provided by the

manufacturer (Applied Biosystems Inc., Foster City, CA, USA), in the 7500 Real Time PCR System (Applied Biosystems Inc., CA, USA), at the IOFV. All PCR amplifications were carried out with the thermal cycling conditions of 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min.

Statistical analysis

The SNPs were assessed for Hardy–Weinberg equilibrium (HWE) by a χ^2 test in both groups (cases and controls) with HaploView 4.0 software. The ages of the NAION patients and control subjects were compared using the Unpaired t test when the age variable was normally distributed in the compared groups, or the Mann-Whitney test when the age variable was non-normally distributed in any of the groups (GraphPad InStat 3.0, San Diego, CA, USA). The comparisons of the SNPs allelic frequencies as well as of the frequencies of the additionally considered potential risk factors, between groups, were performed using a standard χ^2 test, with a p-value of less than 0.05 considered as statistically significant. The comparison of genotypic frequencies between the NAION and control groups was performed using a χ^2 test (Pearson correction) with SPSS version 15.0 software (IBM Corporation, Armonk, NY, USA). Relative risk association was estimated by calculating odds ratios (OR) along with 95% confidence intervals (CI), using the methods described by Armitage et al. [49] and the tool PLINK (version 1.07). Additionally, we used SPSS version 15.0 software (IBM Corporation, Armonk, NY) to run a logistic regression analysis in order to control for potential confounders.

Results

The studied cohort consisted of 94 patients diagnosed with NAION and 204 control subjects of a Spanish population. Allele and genotype frequencies of the *MTHFR* genetic variants (C677T and A1298C) were determined in cases and controls and Hardy-Weinberg equilibrium for these genetic variants was confirmed in both groups. The allelic and genotypic frequencies of the *MTHFR* variants obtained in the NAION group were not significantly different when compared with those of the control group (Table 3).

Table 4 shows the nine theoretically possible genotypes resulted from the SNPs C677T and A1298C in NAION patients and control subjects. The C677T/A1298C genotype codifying the non-mutated MTHFR form (i.e., CC/AA) was significantly more frequent in control subjects (11.27%) than in NAION patients (4.26%), $p = 0.0498$, with an odd ratio of 0.38 (95% CI: 0.13-1.06). The prevalence of the genotypes responsible of MTHFR mutated forms

(i.e., CT/AC, CT/CC, CT/AA, TT/AA, TT/AC, TT/CC, CC/AC, and CC/CC) reached the 95.74% in NAION patients and 88.73% in controls, without significant differences between groups.

According to Table 1, the distribution by gender did not show significant differences between groups, while the mean age of the controls (65.48 ± 11.32 years) was significantly higher than the NOIAN patients (57.39 ± 16.54 years). In addition to those comparisons, a variety of clinical risk factors including hypertension, dyslipidemia, diabetes mellitus and history of heart and vascular diseases were assessed in a cohort of 72 patients with NAION and 72 sex- and age-matched controls. As shown in Table 2, although the prevalence of all of the studied risk factors were higher in the NAION group compared with control subjects, the differences between both groups were not statistically significant.

In contrast, the presence of any history of heart or vascular disease was found to be significantly associated with the occurrence of NAION ($p < 0.0001$). To discard possible interactions among risk factors an additional multivariate logistic regression analysis was carried out. This analysis confirmed that both history of heart disease and vascular disease were independently associated with the prevalence of NAION.

Discussion

In this study we performed the association study of the *MTHFR* C677T and A1298C polymorphisms with NAION in the Spanish population. Additional cardiovascular risk factors for this pathology were also evaluated. In our population, the risk alleles of the genetic polymorphisms C677T and A1298C are not associated with a higher prevalence of NAION disease (Table 3). There were also no significant differences between the frequencies of the genotypes responsible of MTHFR mutated forms (i.e., CT/AC, CT/CC, CT/AA, TT/AA, TT/AC, TT/CC, CC/AC, and CC/CC) when comparing NAION patients (95.74%) and control subjects (88.73%) (see Table 4).

According to previous publications, the mutant alleles of the *MTHFR* C677T and A1298C result in MTHFR protein with decreased enzymatic activity [48]. Therefore, individuals with the MTHFR mutant proteins may have high levels of plasma homocysteine, mainly if their folate levels are low [45,46]. Interestingly, although our results did not show higher prevalence of NAION among individuals with MTHFR mutant proteins, certain protective effect of wild-type protein has been observed. Thus, the genotype CC/AA codifying the non-mutated MTHFR enzyme was significant more frequent ($p=0.0498$) in control subjects

(11.27%) than in NAION patients (4.26%) (see Table 4), with odd ratio of 0.38. However, the confidence interval obtained (95% CI: 0.13-1.06) includes the value 1, and therefore it cannot be assured that the frequencies of both groups for the genotype CC/AA are significantly different.

The genetic association study was performed using a control group with an average age significantly higher than the group of NAION patients (65.48 *vs* 57.39 years, $p<0.0001$) (Table 1). However, we must stress that among controls individuals aged from 35 to 91 were included. Considering that NAION is a late-onset disorder, some misclassification among the youngest individuals in the control group might be possible. Nevertheless, this risk is minimal considering the low annual incidence rate of this disease, estimated from 2.3 to 10.3 cases per 100,000 people between persons over 50 years [50,51]. Even so, to minimize the possible misclassification of some individuals included in the control group, we reanalyzed the data excluding all individuals below 60 from the control group. We therefore obtained a new control group integrated by 144 individuals whose average age was 71.32 years and non-statistically different from NOIAN group. As expected, the results for both SNPs analyzed were similar to those obtained with the previous control group, in which the allele and genotype frequencies were not significantly different in NAION cases when compared to controls (data not shown).

Our results are consistent with those previously published in different populations around the world [10,11,18,41], but controversy still remains because several studies showed a significant association of the 677TT genotype with the NAION disease [36,40]. Discrepancy between studies could be due to the great difficulty in the proper selection of patients. NAION is a multifactorial disease and very different factors contribute to its onset or development, which produces great heterogeneity between the groups of patients under study.

The high presence of at least one of the considered risk variants in our population (88.73% in the control group and 95.74% in the NAION group) is, with small variations, a common characteristic of Caucasian, Hispanic and Asian populations. Interestingly, this frequency is much smaller in African black populations like those from Nigeria (42.20 %) or Gambia (30.97 %), in which, in addition, there is no individuals TT homozygous for C677T [52]. The racial influence on the genotypic frequencies of *MTHFR* variants could explain the higher incidence of NAION between Caucasians when compared with African black populations. However, Hispanics suffer also a lower incidence of NAION than Caucasians, while no significant differences in the *MTHFR* genotype frequencies are observed between these

ethnic groups [50,51]. On the other hand, the NAION incidence in Asians (Korean population) has been reported as similar to that of Caucasians [53]. Interracial differences in the incidence or prevalence of NAION may reflect a genetic predisposition, but *MTHFR* variants do not seem to have a significant influence.

In addition to the genetic mutations discussed, our study also included some clinical risk factors classically associated with NAION, such as hypertension, dyslipidemia, diabetes mellitus, heart diseases (i.e. coronary heart disease, arrhythmias, atrial fibrillation and hypertensive heart disease) and cerebrovascular disease (Table 2). In this particular case, to minimize the influence of gender and age, we have performed the association analysis using 72 patients with NAION and 72 sex- and age-matched controls. This pairing reduces the likelihood that any observed differences for these parameters between cases and controls result from variances in the distribution by sex and age in both groups. However, this possibility is not entirely discarded because differences in additional unconsidered factors such as lifestyle or other systemic diseases could have influence.

Although the considered clinical factors were more frequently found among NAION patients than in controls, only clinical history of heart disease ($p=0.0038$) and cerebrovascular disease ($p=0.0058$) were most prevalent in NAION patients, with statistically significant differences. The additional clinical factors evaluated (i.e. hypertension, diabetes mellitus and dyslipidemia) were not associated with a higher risk of NAION in our population (Table 2). Table 5 shows a comprehensive summary of the published case-control studies concerning the association of these clinical factors with NAION, to date. As can be observed, there is no agreement on the association of any of these clinical variables with NAION. The lack of consensus could be due to the great complexity and heterogeneity of the disease, influenced by multiple variables, hindering the selection of a homogenous group of patients and their appropriate controls. However, the fact that at least several studies have shown association of a specific genetic or clinical factor with NAION suggests that its combination with other potential risk factors may contribute to a higher predisposition to the ocular disease. The combination of systemic and local risk factors, that can be different in each patient, could be responsible for the pathology onset. Although the precise mechanism for the development of NAION is uncertain, it is widely accepted that the disease is caused by a decrease in blood flow to the ONH. One of the main factors causing insufficient blood flow through the PCAs to the ONH seems to be the nocturnal arterial hypotension during sleep in susceptible patients with the additional risk factors discussed.

The *MTHFR* mutations analyzed in this study have not a significant effect on the occurrence of NAION in our population. However, the genotype CC/AA codifying the non-mutated MTHFR enzyme showed higher frequency in control subjects than in NAION patients, which may be related with a protective effect of the wild-type protein. Interestingly, the risk variants of the *MTHFR* gene are highly prevalent in most of the populations over the world, with the exception of African black populations, suggesting a racial influence. Plasma homocysteine levels may have influenced by factors different than the variants of this gene. Nutritional deficiencies such as low levels of folate or vitamin B12 are important determinants of these levels. Thus, the analysis of the serum concentrations of homocysteine, folate and vitamin B12 could help to identify subgroups of patients with NAION in which the presence of *MTHFR* mutations could be a significant risk factor. Finally, although additional clinical factors including hypertension, diabetes mellitus and dyslipidemia were not associated with a higher risk of NAION in the Spanish population, the combination of others systemic and local risk factor, such as heart and cerebrovascular disease, may play a role in the onset of the pathology.

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Statement of Ethics

The study adheres to the tenets of the Declaration of Helsinki on Biomedical Research Involving Human Subjects, and was approved by the Clinical Research Ethics Committee at Principality of Asturias (Oviedo, Spain). All participants signed an informed consent.

Disclosure Statement

The authors have no conflicts of interest to declare

Author Contributions

Conceptualization: B.F.V., L.A., J.A.V., H.G.I.; formal analysis: B.F.V., L.A., M.G., E.A.; funding acquisition: J.A.V., H.G.I.; data collection: B.F.V., L.A.; methodology: B.F.V., L.A., J.N.; project administration: J.A.V, H.G.I., resources: M.D., J.N., H.G.I.; supervision: J.A.V., H.G.I., validation: M.G., M.D.; visualization: B.F.V., L.A., M.G.; writing-original draft: B.F.V., L.A.; writing - review & editing: J.N., J.A.V., H.G.I.

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Table 1: Demographics characteristics of the recruited NAION patients and control subjects

	NAION patients (n=94) (%)	**Controls (n=204) (%)**	***p-value***
Men/women	48 (51.06)/46 (48.94)	84 (41.18)/120(58.82)	0.1103
Age (mean years ± SD)	57.39*±16.54	65.48±11.32	**<0.0001**
Age range	18-87	35-91	

NAION, non-arteritic anterior ischemic optic neuropathy; n, number of subjects; SD, standard deviation; The asterisk (*) indicates the average age at the first event.

Table 2: Demographics and clinical characteristics of the sex- and age-matched NAION patients and controls

	NAION patients (n=72) (%)	**Controls (n=72) (%)**	***p-value***
Men/women	37 (51.39)/35 (48.61)	37 (51.39)/35 (48.61)	
Age (mean years ± SD)	61.5*±10.95	61.5±10.95	
Age range	35-82	35-82	
Hypertension, n (%)	35 (48.61)	32 (44.44)	0.6162
Dyslipidemia	40 (55.56)	30 (41.67)	0.0955
Diabetes mellitus, n (%)	17 (23.61)	10 (13.89)	0.1350
History of heart/vascular disease (Total)	25 (34.72)	4 (5.56)	**<0.0001**
Heart disease**	16 (22.22)	4 (5.56)	**0.0038**
Cerebrovascular disease	9 (12.5)	0	**0.0059**

NAION, non-arteritic anterior ischemic optic neuropathy; n, number of subjects; SD, standard deviation; *Indicates the average age at the first event; **Includes coronary heart disease and other pathologies that affect the heart such as arrhythmias, atrial fibrillation and hypertensive heart disease

Table 3: Allelic and genotypic association analysis

SNP ID		**NAION %**	**Control %**	**p-value**	**OR (95% CI)**
rs1801133 **C677T**		(n=94)	(n=204)		
Allele	C	56.91	62.50		0.91 (0.72-1.14)
	T	43.09	37.50	0.1945	1.15 (0.82-1.61)
Genotype	CC	31.91	40.69	0.6037*	0.78 (0.54-1.14)
	CT	50.00	43.63		1.15 (0.85-1.54)
	TT	18.09	15.69		1.15 (0.62-2.14)
	Total	30/47/17 (CC/CT/TT)	83/89/32 (CC/CT/TT)	0.3494	
rs1801131 **A1298C**		(n=94)	(n=204)		
Allele	A	71.28	69.61		1.02 (0.86-1.22)
	C	28.72	30.39	0.6791	0.94 (0.62-1.45)
Genotype	AA	51.06	48.53	0.8224*	1.05 (0.80-1.39)
	AC	40.43	42.16		0.96 (0.69-1.33)
	CC	8.51	9.31		0.91 (0.38-2.22)
	Total	48/38/8 (AA/AC/CC)	99/86/19 (AA/AC/CC)	0.9158	

NAION, non-arteritic anterior ischemic optic neuropathy; n, number of subjects; OR, odds ratio; CI, confidence interval. $p < 0.05$, significant. Total indicate the general test of association in the 2- by-3 table of disease-by-genotype. The asterisk (*) indicates the p-values derived from comparison of the genotypic frequencies under the recessive model (TT vs CT + CC at rs1801133, and CC vs AC + AA at rs1801131).

Table 4: Genotypic frequencies of NAION patients and control subjects.

C677T/ A1298C	NAION (n=94) (%)	CT (n=204) (%)	p-value
TT/AA	17 (18.09)	31 (15.20)	n.s.
TT/A**C**	0	0,00	n.s.
TT/**CC**	0	0,00	n.s.
C**T**/A**C**	20 (21.28)	44 (21.57)	n.s.
C**T**/**CC**	0	0,00	n.s.
C**T**/AA	27 (28.72)	45 (22.06)	n.s.
CC/A**C**	18 (19.15)	41 (20.10)	n.s.
CC/**CC**	8 (8.51)	19 (9.31)	n.s.
CC/AA	4 (4.26)	23 (11.27)	**0.0498**

NAION, non-arteritic anterior ischemic optic neuropathy; n, number of subjects. The risk allele is indicated in bold. CT, heterozygotes for C677T including CT and TC subjects; AC, heterozygotes for A1298C including CT and TC subjects. n.s., not significant.

Table 5: Association of potential clinical risk factors with NAION in case-control studies published to date

Study	Cases / Controls*	Diabetes mellitus	Systemic hypertension	Dyslipidemia/ Hypercholesterolemia	Ischemic heart disease	Cerebrovascular disease
Present study	72/72	No assoc.	No assoc.	No assoc.	**p = 0.1898** **p = 0.0038****	**p = 0.0059**
Talks et al., 1995 [54]	41/41	No assoc.	No assoc.	**p < 0.05**	No assoc.	**p < 0.001**
Jacobson et al., 1997 [9]	51/51	**p = 0.01**	No assoc.	No assoc.	No assoc.	NA
Salomon et al., 1999 [10]	61/90	**p = 0.034**	No assoc.	**p = 0.011**	**p = 0.007**	No assoc.
Weger et al., 2001 [11]	59/59	**p = 0.0263**	**p = 0.0245**	NA	No assoc.	No assoc.
Deramo et al., 2003 [12]	37/74	**p = 0.027**	No assoc.	**p = 0.036**	NA	NA
Nagy et al., 2006 [19]	37/81	No assoc.	No assoc.	**p =0.012**	No assoc.	NA
Hayreh et al., 2007 [13]	624/***	**p < 0.0001**	**p < 0.0001**	NA	**p <0.0001**	**p <0.0001**
Giambene et al, 2008 [18]	85/107	No assoc.	**p < 0.0001**	**p = 0.01**	NA	NA
Felekis et al., 2010 [40]	77/60	No assoc.	No assoc.	No assoc.	NA	NA
Kim et al., 2017 [15]	45/45	**p = 0.020**	No assoc.	**p = 0.001**	NA	NA

NA, data not available; No assoc., no association.

* Age-, gender-matched.

** Includes coronary or isquemic heart disease and other pathologies that affect the heart such as arrhythmias, atrial fibrillation and hypertensive heart disease

*** Race-, gender-, age-, and period-matched subgroup of the U.S. population

4.

Coenzyme Q10 treatment improved visual field after homonymous quadrantanopia caused by occipital lobe infarction (publicado en *American Journal of Ophthalmology Case Reports*, 2018)

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Case report

Coenzyme Q10 treatment improved visual field after homonymous quadrantanopia caused by occipital lobe infarction

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ABSTRACT

Purpose: To report the clinical findings and management of a case of occipital lobe infarction with homonymous quadrantanopia in a patient treated with vitamins and coenzyme Q10.

Observations: A currently 69-years-old patient presenting in 2007 left inferior quadrantanopia following a right occipital lobe stroke with initial visual field index of 82% and 79% in the right and left eyes, respectively. From 2007 to 2010 was treated with vitamin and antioxidant complexes, without specific signs of changes observed in the visual field (81% right eye, 79% left eye). In 2011 was treated for the first time with coenzyme Q10 (Active complex® Q10 Gold 100 mg) in addition to the vitamin and antioxidant supplementation. A promptly slight improvement of the visual field in both eyes was observed. In 2013, a remarkable improvement was noticed observing a slight scotoma where previously presented the quadrantanopia. Thereafter, in the successive one-year follow-up examinations the patient experienced an exponential improvement in the visual field with gradually fading of the scotoma. Currently the patient no longer presents any sign of quadrantanopia, with normal visual field in both eyes (99% right eye, 98% left eye).

Conclusion and importance: Spontaneous improvement more than 6 months after stroke is thought to be unlikely. However, we observed, for the first time, an amelioration of the visual field after 10 years of an occipital lobe stroke. The combination of vitamins and coenzyme Q10 (100 mg) improved the prognosis with significant recovery of the visual field, which is impossible to recover under current knowledge.

1. Introduction

Visual field defects following cerebral insults are very prevalent, with frequencies ranging from 8.3 to 25%. [1], [2] When homonymous visual field defects occurs following damage to the neural visual pathway, specifically damage posterior to the optic chiasma, the same part of the visual field is affected in both eyes. Homonymous hemianopia is a visual field defect involving either two right or the two left halves of the visual field of both eyes, while the loss of one quarter of visual field on one side in both eyes is referred to as homonymous quadrant hemianopia or quadrantanopia. Hemianopia and quadrantanopia are consequence of injuries mainly in the occipital, the parietal and the temporal lobes,[3] with high impact upon the quality of life of individuals.[4]

Approximately 60% of patients could experience spontaneous improvement in the visual field, usually within the first month after brain injury. However, the spontaneous improvement in the first 6 months decreases progressively with every successive month, while complete recovery is rare and beyond 6 months is unlikely.[5–7] Therefore, any documented improvement in the visual field of treated patients, with signs of quadrantanopia after 6 months post-injury, would be a sign of therapeutic efficacy. We report a case of occipital lobe infarction with homonymous quadrantanopia in a patient treated with vitamins and coenzyme Q10, with significant visual field recovery, 10 years after the cerebral insult.

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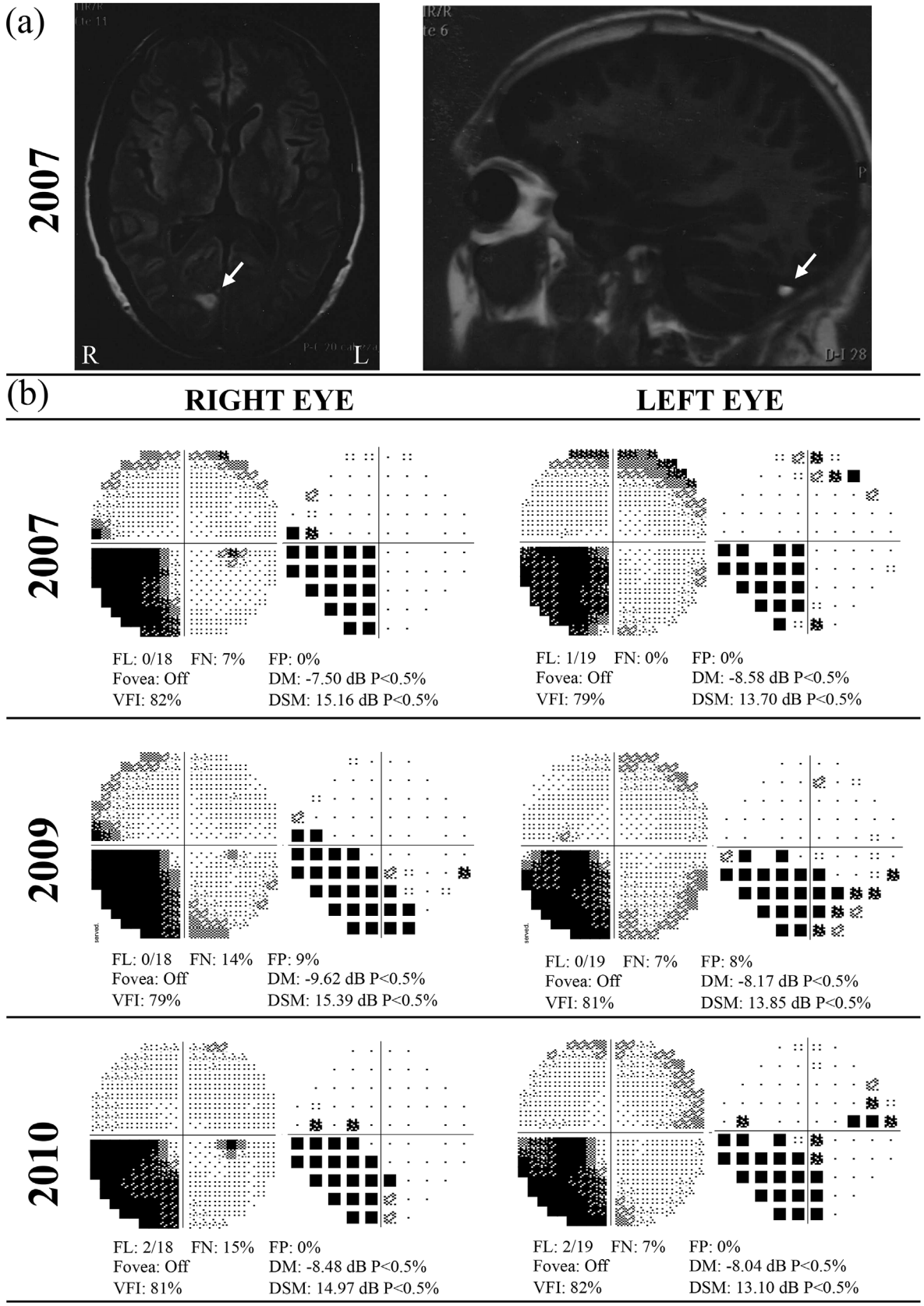

Fig. 1. (a) Brain magnetic resonance imaging (MRI) at the time of the stroke revealed an infarct in the right occipital lobe (white arrow). (b) Visual field test using the Humphrey field analyzer (HFA) and 30-2 algorithm, from 2007 to 2010. Rows represents the right and left visual fields of each respective eye. In 2007 the patient presented left inferior homonymous quadrantanopia. From 2007 to 2010 was treated with Visan, Hidroxil and Acfol, with no changes in the visual field index were observed.

2. Case report

A currently 69-years old male patient suffered in 2007 a right occipital lobe stroke with left inferior homonymous quadrantanopia (retrochiasmal lesion of the visual pathway). Magnetic resonance imaging of the brain at the time of his stroke demonstrated an infarct in the right occipital lobe (see Fig. 1, panel a). He had no significant past ocular history, nor did he report history of autoimmune disorder, or neurological disorders. Visual acuities were 20/20 in each eye. OCT analysis, retinography and fundus examination were normal. The patient did not receive restorative training, optical aids, or compensatory training. Diagnostic was carried out using the Humphrey field analyzer (HFA) instrument and 30-2 testing algorithm. The visual field index (VFI) was obtained to evaluate the percentage of the remaining visual field of the right eye (OD) and the left eye (OS), respectively.

Upon initial examination in 2007, the patient presented VFI of 82% (OD) and 79% (OS), as shown Fig. 1 (panel b). A systemic treatment consisting of Adiro 300 (Bayer, Germany), Visan (Théa laboratories, France), Hidroxil (Almirall, Spain) and Acfol (Italfarmaco, Italy) was prescribed. Every other month the patient took Hidroxil B12, B6, B1 (1 tablet each breakfast, lunch and dinner) and Acfol (1 tablet at breakfast), while in the rest month took Visan (1 tablet at breakfast), for a year. The active ingredient of Adiro 300 is acetylsalicylic acid and is used for stroke (antiplatelet). Hidroxil active ingredients include vitamins B1 (250 mg thiamin), B6 (250 mg pyridoxine) and B12 (500 mg cyanocobalamin). Acfol active ingredient is folic acid (5 mg). Visam nutritional supplement contains trace elements (e.g. zinc, copper, selenium, manganese), vitamins (A, C, E, B1−B12), lutein, zeaxanthin, glutathione, flavonoids and low levels of coenzyme Q10 (2.5 mg).

At two-year follow-up, i.e., 2009, according to Fig. 1 no changes were observed in the VFI (79% OD, 81% OS), maintaining the same treatment prescribed. We observed signs of peripheral cortical cataract (OD) and nuclear sclerosis of the lens (OI), presenting normal optic papilla, macula and retinal vascularization. In 2010 no significant changes were observed in the VFI (81% OD, 82% OS), as shown in Fig. 1 (panel b), and the same treatment was prescribed. Sign of cataracts in OD and OI was observed, while optic papilla, macula, retinal neovascularization and retinal periphery were normal.

In 2011 Active complex® Q10 Gold 100 mg (Pharma Nord, Dennmark), containing 100 mg of CoQ10 and 25 mg of vitamin C, was prescribed in alternate months (1 tablet at lunch, following manufacturer dosage guidelines), additional to Hidroxil, Acfol and Visan supplements, over a period of one year. As shown Fig. 2, the VFI in 2011 presented a promptly slight improvement of the visual field in both eyes (86% OD, 88% OS). At one-year follow up of the patient a remarkable improvement was observed in the visual field index of both eyes (91% OD, 96% OS), presenting minimal inferonasal quadrantanopia with central scotoma in the right eye and minimal inferotemporal quadrantanopia with small scotoma in the left eye. The remaining ocular parameters were normal.

In 2013 (Fig. 2), the VFI was notably improved, reaching 90% (OD) and 96% (OS). In the left eye a slight scotoma was present where the quadrantanopia was previously observed. In the right an inferonasal quadrantanopia was still present with minimum scotoma and minimal alteration in the RPE, while optic papilla, macula, retinal vascularization and periphery were normal. Medical prescription was virtually maintained, but one tablet of Active complex® Q 10 Gold 100 mg was prescribed for daily treatment along all the successive months.

In the successive one-year follow up examinations (2014–2018), the patient experimented an exponential improvement in the visual field with gradually removal of the scotoma. At present time, the patient no longer shows any sign of quadrantanopia, with normal visual field index in both eyes (99% OD, 98% OS). Summarizing, the VFI of right and left eyes are plotted in Fig. 3, from 2007 (stroke event) to 2018. The patient presented a progression rate of $+2.1 \pm 0.6\%$ per year in the OD, and $+2.2 \pm 0.8\%$ per year in the OS, at 95% confidence, with significant enhancement of the visual field maintaining the prescribed treatment, which includes daily CoQ10 supplementation.

3. Discussion

Visual field defects are common after stroke, trauma, tumor, brain surgery, and demyelinating lesions, affecting specifically up to 25% of all stroke survivors.[1,8] The impact on daily activities is significant, with poor mobility, collisions, impaired reading and driving skills, and increased dependence and disability.[9] Lesions affecting postchiasmal afferent nerve pathways generally produce homonymous visual field loss, which may be a hemianopia or quadrantanopia depending on the location of the lesion. The term hemianopia describes visual defects that occupy about one-half of an eye's visual field, while quadrantanopia describes defects confined mostly to about one-fourth of an eye's visual field.[10] The exact type of homonymous defect depends on the specific location of the injury, its extent and the presence of other lesions.[11] Our patient presented a right occipital lobe stroke with left inferior homonymous quadrantanopia, which is the most common location of the lesion (45% of cases).[6]

Spontaneous visual field improvement or restoration is common and occurs in early weeks to few months,[5] probably mediated by the removal of the cerebral edema with concomitant restitution of surrounding non-infarcted penumbral tissue, with reports ranging from 7% to 85% of clinical cases.[12,13] The recovery is variable, depending on the degree of neuronal death and stunning in the damaged visual pathways, as well as the resolution of the initial effects of the acute injury.[14] Approximately 60% of patients could experience spontaneous improvement, usually within the first month after injury, and in some case a few months later but does not extend thereafter.[7] In a recent study on the natural history of homonymous hemianopia, spontaneous improvement of the visual field defect has been observed in 38% of patients.[15] The approximate maximal period of spontaneous recovery is typically 3 months.[14] In our case, the improvement in the visual field of the patient began to occur more than 4 years after the lesion, with significant recovery 10 years later, and therefore is not likely as a result of spontaneous recovery.

Visual field improvement is usually defined as an amelioration of the field defect with significant changes in mean and pattern deviations in Humphrey visual fields. The percentage of field recovery is variable and depends upon individual.[16] No other factor including age seems to affect significantly the visual field improvement. The natural history of visual field recovery is fundamental when evaluating claims of improvement by potential rehabilitation therapy or treatments for homonymous hemianopia or quadrantanopia.[17] We observed an amelioration in the visual field, along the ten years of follow-up of our patient, from 82% (OD) and 79% (OS) in 2007 to 99% (OD) and 98% (OS) in 2018 with progression rates > 2% per year in both eyes.

Post stroke treatment includes the identification of the stroke etiology, the modification of risk factors to prevent reoccurrences, and the initiation of an early and intensive rehabilitation therapy allowing functional outcomes and improving the disability.[18,19] Interventions in patients diagnosed with homonymous quadrantanopia or hemianopia are focused on their rehabilitation through optical therapies, compensatory therapies, and visual field restitution therapies based on the hypothesis of plasticity, which has been attracted increasing attention in recent years.[2,12,20,21] The use of optical aids pursues to expand artificially the visual field, the compensatory training attempts to alleviate the resulting disability by teaching patients to make more efficient eye-movements, and the restorative therapy aims to reduce the visual field loss through prolonged training.[12] However, our patient did not receive any of those interventions, and therefore the improvement in the visual field observed was not as the result of improved neither compensation nor rehabilitation therapy.

The use of nutritional supplements in the treatment of visual field defects including quadrantanopia is widely recommended, although

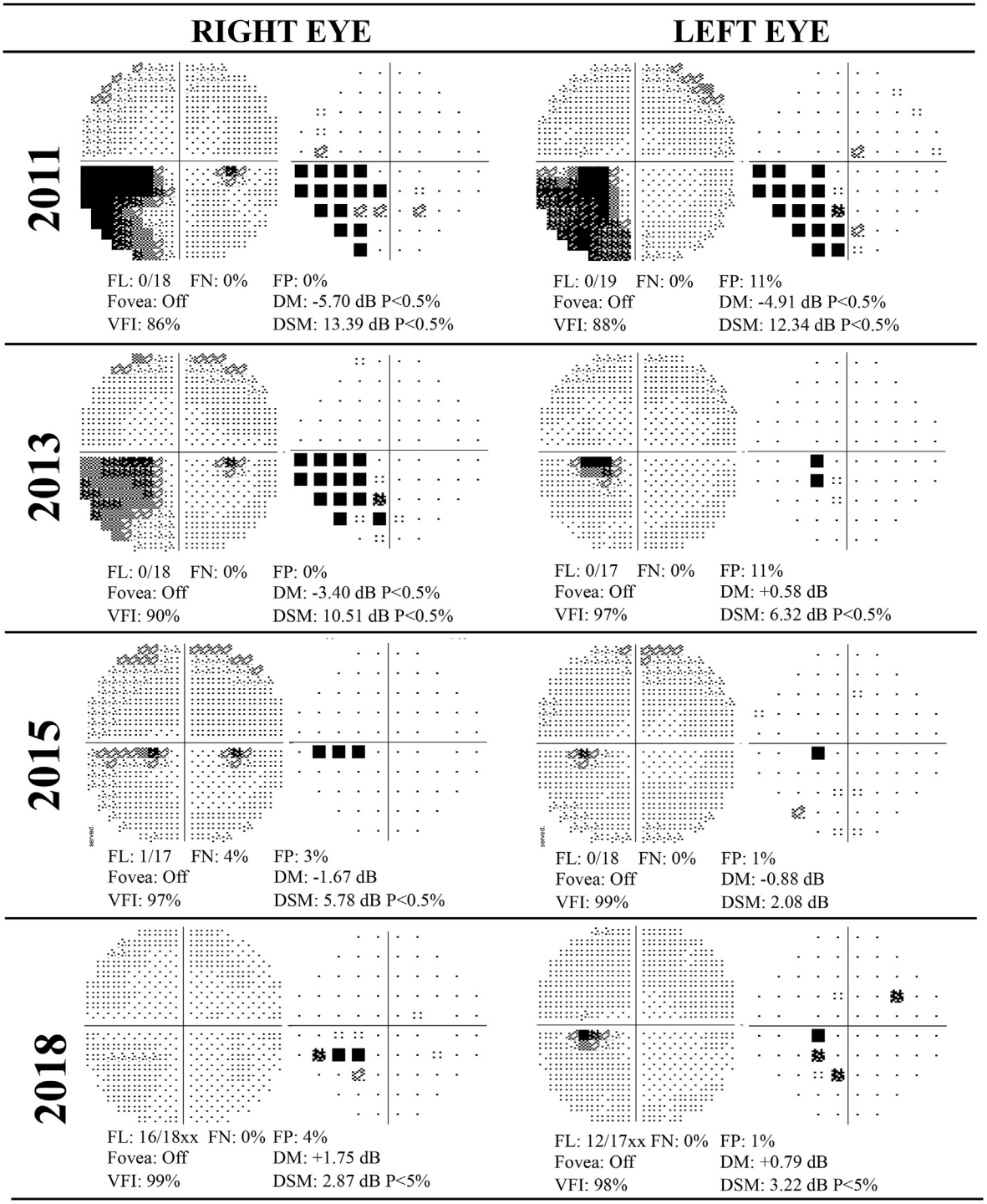

Fig. 2. Visual field test using the Humphrey field analyzer (HFA) and 30-2 algorithm, from 2011 to 2018. Rows represents the right and left visual fields of each respective eye. In 2007 the patient presented left inferior homonymous quadrantanopia. In 2011 was treated for the first time with coenzyme Q10 (Active complex® Q10 Gold 100 mg). The visual field index improved progressively until now.

there is a lack of standard pharmacological treatments or broadly accepted nutritional recommendations. Nutritional supplements including vitamins (A, C, E and B) and antioxidants are commonly used but available evidence of their effectiveness is scarce and inconsistent. Among the supplements suggested, coenzyme Q10 (CoQ10 or ubiquinone) has been extensively used to treat aging, stroke, retinal diseases, but no effects on homonymous quadrantanopia has been published to date.[22] Our patient was prescribed from 2007 to 2010 with vitamin, folic acid and antioxidant supplements, with no changes in the visual field were observed. In 2011, the treatment was complemented with Active complex® Q10 Gold 100 mg at high doses, i.e., containing 100 mg of CoQ10. While the recovery progression rate of the visual field of the patient remained constant from 2007 to 2010, we observed a great improvement of the recovery since the patient was prescribed with CoQ10 treatment in 2011 until present.

Given that spontaneous improvement has not been noted in patients with stroke after 6 months and that our patient did not receive any formal visual field training or optical aids, the improvement seen by our

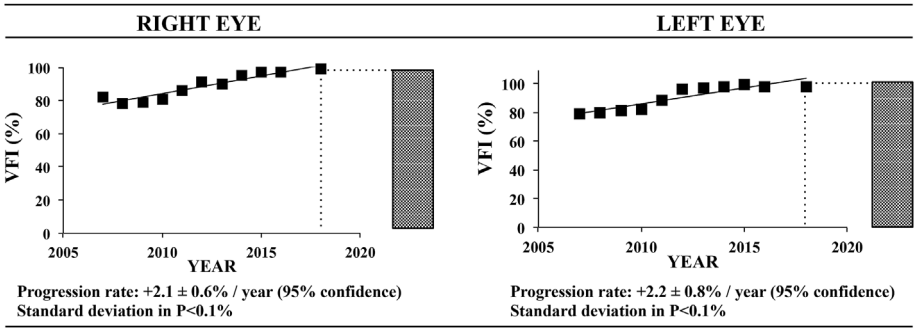

Fig. 3. The visual field index (VFI) of right and left eyes plotted from 2007 (stroke event) to 2018. Progression rates at 95% confidence are shown.

patient is most likely as result of CoQ10 treatment. CoQ10, also known as ubiquinone, ubidecarenone or coenzyme Q, is a 1,4-benzoquinone where Q refers to the quinone chemical group, and 10 refers to the number of isoprenyl chemical subunits in its tail. CoQ10, the most common coenzyme Q in humans, is an essential cofactor of the electron transport chain and acts by maintaining the mitochondrial membrane potential, supporting ATP synthesis and inhibiting reactive oxygen species generation for protecting neuronal cells against oxidative stress in neurodegenerative diseases.[23,24]

Various mechanisms for the beneficial effects of CoQ10 have been suggested including its neuroprotective role, which underlying pathways may involve a dual function, acting as a free radical scavenger and activating the mitochondrial function.[25,26] Pretreatment with CoQ10 may protect neuronal cells against oxidative stress stabilizing the mitochondrial membrane and reduced the amount of mitochondrial reactive oxidative species generation.[22,27] Moreover, the antioxidant function of CoQ10 is of great importance in the plasma membrane by reducing vitamins C and E.[28] Recent studies in animal models showed that pretreatment with CoQ10 ameliorated ischemic injury by regulating the antioxidant defense and mitochondrial function.[29,30]

Overall, CoQ10 may play a significant role in treating neurological conditions, including visual field loss following stroke, as in our case. However, since visual field recovery may involve both, improved function of injured tissue and recruitment of additional cortical structures to assume the function of the permanently damaged centers, we cannot ruled out that the plasticity in the visual system may intervene, underlying behavioral compensatory strategies.[31–33] Moreover, this case report has several limitations hinder confirming the beneficial effect of CoQ10 supplement, including discontinuation of the treatment or anatomic and physiologic evidences to observe deterioration and improvement in the visual field.

4. Conclusions

We describe a case of a patient presenting right occipital lobe stroke with left inferior homonymous quadrantanopia and more than 10 years of follow-up. We did not observe any spontaneous recovery of the visual field nor the patient received any rehabilitating therapy. After the initiation of CoQ10 treatment, the patient demonstrated improved visual field after a remote ischemic stroke, four years before, with almost complete recovery at present. Considering that spontaneous recovery is extremely unlikely 5 years after the stroke and the improvement was temporally related to the initiation of the CoQ10 supplementation, we assume that the CoQ10 treatment had beneficial effects in our patient.

Patient consent

Consent to publish the case report was not obtained. All information contained within this report has been made anonymous in compliance with institutional policies.

Conflicts of interest

The following authors have no financial disclosures: BFV, HGI, JAV, JN, AFV.

Authorship

All authors attest that they meet the current ICMJE criteria for Authorship.

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5.

The use of vitamins and coenzyme Q10 for the treatment of vascular occlusions diseases affecting the retina (en revisión en *Acta Ophthalmologica*)

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Keywords:	Vascular diseases, Retina, Visual field defects, Vitamins, Coenzyme Q10

Case Series

The use of vitamins and coenzyme Q10 for the treatment of vascular occlusion diseases affecting the retina

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ABSTRACT

Purpose: To report the clinical findings and management of a series of cases of patients presenting vascular diseases affecting the retina, treated with vitamins and coenzyme Q10 (CoQ10).

Methods: Retrospective case series of patients diagnosed with ischemic optic neuropathy, retinal artery occlusion, homonymous hemianopia or quadrantanopia following stroke and other conditions including optic nerve atrophy or retinal vascular occlusion, followed-up using the Humphrey field analyzer (HFA) instrument and 30-2 testing algorithm to determine the visual field index (VFI) and progression rates per year. Oral supplementation of CoQ10 (100mg per day) and vitamins was prescribed.

Results: Forty-eight patients (96 eyes) were included. The population-average age at diagnosis was 57±16 (range of 15-85), with averaged follow up of 43±41 months. Non-arteritic ischemic optic neuropathy patients reached VFI progression rate of +11.5±15% per year (n=18) at 95% confidence interval, after CoQ10 and vitamins supplementation. Similarly, retinal vascular occlusion and homonymous hemianopia/quadrantanopia subjects provided averaged progression rates of +22±17% (n=7) and +9.3±10.5% (n=10) per year, respectively. Patients diagnosed with others conditions affecting the retina vascularity showed averaged progression rate of +11±21% (n=13) per year. CoQ10 treatment was interrupted in one of the patients, observing and significant decrease in the VFI from 43% to 6%, which was partially recovered when the supplementation with the enzyme was restored.

Conclusion: This study supports the role of CoQ10 as therapeutic agent for vascular diseases affecting the retina. Owing to decrease visual field after the interruption of CoQ10, the beneficial effects may not be irreversible.

Keywords: Vascular diseases; Retina; Visual field defects; Vitamins; Coenzyme Q10

Introduction

Retinal dysfunctions caused by vascular disorders resulting in sudden visual loss are currently very prevalent. Vascular diseases may affect the blood flow to the optic nerve, the ophthalmic artery and the retinal vein or even produce a retrochiasmal lesion of the visual pathway following cerebral insult. Among them, ischemic optic neuropathy (ION) is a spectrum of ocular diseases affecting the optic nerve owing to the interruption of blood flow supplied by the posterior ciliary arteries (anterior ION) or the pial capillary plexus (posterior ION) (Hayreh 2009). The most common anterior ION is the non-arteritic (NAION) form, presenting as acute, unilateral and painless loss of central and/or peripheral vision that may progress over several hours or days, mainly affecting patients older than 50 years (Buono et al. 2002). On the other hand, retinal artery occlusion (RAO) is an embolic or thrombotic occlusion of either the central (CRAO) or branch (BRAO) ophthalmic artery resulting in ischemia of the retina and visual loss. The retinal artery occlusion, mainly unilateral, may be transient and last for only a few seconds or minutes if the blockage breaks up and restores, occurring mostly in male patients over 60 years (Varma et al. 2013; Limaye et al. 2018; Hayreh 1995). Besides, damage to the occipital, parietal or temporal lobes caused by cerebral insults, including stroke, trauma, tumor or brain surgery, results in severe visual field defects. Lesions affecting postchiasmal afferent nerve pathways generally produce homonymous visual field loss, which may be a hemianopia (one-half of an eye's visual field) or quadrantanopia (one-fourth of an eye's visual field) depending on the location of the lesion, with high impact upon the quality of life of patients (Goodwin 2014; Eggenberger & Pula 2014).

Vascular diseases affecting the eye are multifactorial in origin, with many risk factors contributing to their onset. Systemic risk factors include nocturnal arterial hypotension, arterial hypertension, diabetes mellitus, hyperlipidemia, ischemic heart disease, blood loss, atherosclerosis, sleep apnea syndrome, cardiovascular disorders, smoking tobacco, obesity, atrial fibrillation, hyperhomocysteinemia, varicose veins and coagulation disorders (activated protein C resistance or factor V Leiden mutation), among others (Varma et al. 2013; Hayreh et al. 1994; Hayreh 1996; Hayreh 2012; Pula & Yuen 2017). Ocular risk factors include elevated intraocular pressure, glaucoma, a small and crowded disc or optic disc drusen that may influence the optic nerve head blood supply (Hayreh 1996; Hayreh 2001).

Current therapies are unable to predictably alter the natural history of these diseases, with limited efficacy to improve vision. During the acute event, ocular massage contributes to improve retinal perfusion, while lowering intraocular pressure, parancetesis or hemodilution may increase the vascular supply. The use of vasodilators, thrombolytics or high concentration of oxygen, are also recommended. The approach is mainly based in clinical evaluation and reduction as many risk factors as possible to diminish the risk of any further episode. However,

no definitive standard treatment options exist due to insufficient high-quality evidence based research (Hayreh 2009; Varma et al. 2013; Pambakian 2015).

Prescription of nutritional supplements, including vitamins, antioxidants and omega 3 fatty acids, is widely recommended in the treatment of visual field defects of vascular origin (Brown et al. 1998; Demmig-Adams & Adams 2013). However, there is a lack of standard pharmacological treatments or broadly accepted nutritional recommendations owing to absence of effectiveness evidence. One of the nutritional supplements of potential interest for the treatment of retinal dystrophies is the coenzyme Q10 (CoQ10) (Salama et al. 2013; Zhang et al. 2017a,b; Martucci & Nucci 2019; Yang et al. 2016). CoQ10, a vitamin-like compound, is an physiological component of the electron transport chain, which maintains the mitochondrial membrane potential, supports ATP synthesis and functions as antioxidant protecting neurosensorial cells (Somayajulu et al. 2005).

In a seminal case report (Fernández-Vega et al. 2018), we documented the clinical findings and management of a case of occipital lobe infarction with homonymous quadrantanopia in a patient treated with vitamins and coenzyme Q10, with significant visual field recovery, 10 years after the cerebral insult. This documented improvement in the visual field of the treated patient, after more than 6 months post-injury, may be a sign of therapeutic efficacy. To address this observation, in the current work we present a retrospective case series study of vascular occlusion affecting the retina, including NAION, RAO, homonymous hemianopia or quadrantanopia and optic nerve dystrophies, inter alia, treated with vitamins and CoQ10.

Patients and Methods

Patients suffering vascular diseases affecting the retina and treated with vitamins and CoQ10 supplements were retrospectively reviewed. Specifically, 18 patients diagnosed with NAION, 10 patients with homonymous hemianopia (or quadrantanopia) caused by cerebral stroke, 7 patients with RAO, and 13 patients with other conditions including optic nerve atrophy, cones dystrophy, retinitis pigmentosa and retinal vascular occlusion were recruited at the Ophthalmological Institute Fernández-Vega. Patients were diagnosed by indirect ophthalmoscopy, visual field analysis, determination of nerve fiber layer thickness and macula by optical coherence tomography (OCT), visual acuity, evoked potentials, examination of the papilla and anterior segment, fluorescein angiography (FA), best-corrected visual field acuity, and neurological study including magnetic resonance imaging, when needed..

None of the patients received restorative training, optical aids, or compensatory training. Patient follow up was carried out using the Humphrey field analyzer (HFA) instrument and 30-2 testing algorithm. The visual field index (VFI) reflecting retinal ganglion cell loss and function, and the

Mean Deviation (MD) in decibels (dB) representing the retina sensibility were obtained to evaluate the percentage of the remaining visual field of the right eye and the left eye, respectively, providing the progression rate along the follow-up of each patient. No subjects involved in this study presented with other relevant ocular pathologies.

The study adheres to the tenets of the Declaration of Helsinki on Biomedical Research Involving Human Subjects, and was approved by the Clinical Research Ethics Committee at Principality of Asturias de Asturias (Oviedo, Spain). Demographics and clinical characteristics of patients are reported in Tables 1 and S1 of Supplemental Information, in which the disease, sex, affected eye, age at diagnosis and at initiation of CoQ10 and vitamins supplementation treatments were indicated, along relevant ocular and systemic diseases. A group of other diseases was included to simplify the study, which included optic nerve atrophy, retinitis pigmentosa, retinal vascular occlusion, etc.

Once each of the patients was diagnosed, a systemic treatment with vitamins and antioxidants was prescribed, mainly consisting of: Active complex® Q10 Gold 100 mg (Pharma Nord, Denmark) (1 tablet at lunch all the months, following manufacturer dosage guidelines); Hidroxil B12, B6, B1 (Almirall, Spain, 1 tablet at breakfast, lunch and dinner in alternate months); Acfol (Italfarmaco, Italy, 1 tablet at breakfast in alternate months); Visan (Théa laboratories, France, 1 tablet at breakfast in the resting month); and Adiro 300 (Bayer, Germany). Specific treatment and dosages have been included in Supplemental Table S1, for each patient. Active complex® Q10 Gold 100 mg contains 100 mg of CoQ10 and 25 mg of vitamin C, while the active ingredient of Adiro 300 is acetylsalicylic acid and is used for stroke (antiplatelet). Hidroxil active ingredients include vitamins B1 (250 mg thiamin), B6 (250 mg pyridoxine) and B12 (500 mg cyanocobalamin). Acfol active ingredient is folic acid (5 mg). Visan nutritional supplement contains trace elements (e.g. zinc, copper, selenium, manganese), vitamins (A, C, E, B1–B12), lutein, zeaxanthin, glutathione, flavonoids and low levels of coenzyme Q10 (2.5 mg).

Patient follow-up and examination has been highly variable, depending on the severity of the disease. Upon initial examination and following treatment prescription, the patient was initially revised from 2-6 months to every 1-year follow-up. Clinical history of patients included in this study covers a range from 5 to 203 months, with averaged follow up of 43±41 months.

Results

The studied cohort consisted of 48 patients diagnosed with NAION (18), homonymous visual field loss (hemianopia or quadrantanopia) following stroke (10), RAO (7), or other conditions (13), from a Spanish population (Table 1). The population-average age reached at diagnosis was 57±16, with a range age of 15-85. The 46% of patients were females, but with highly variability

depending on the disease. The range age of NAION and RAO were 50-81 and 48-83, respectively, indicating both are late-onset disorders.

Insert Table 1 here

Follow-up of each condition was depicted by VFI and MD parameters of each eye, obtaining the VFI progression rate per year in the right and left eye, at 95% of confidence. Table 2 shows the NAION cases, including the number of months of follow up, the initial (first examination) and final (last examination) VFI and MD in % and dB, respectively, and the VFI progression rate in % per year of both eyes, although only one of the eyes was affected in each case. The different superscripts show the date of CoQ10 treatment initiation, i.e., at month 0 upon initial examination or at the corresponding month. VFI progression rates were highly variable, with no significant changes in non-affected eyes. However, the affected eyes experimented progression rates of +0.2±0.6% per year (NAION18 left eye, no significant differences), +2.1±1.9% per year (NAION01 left eye), or of +23.2±14.5% (NAION13 left eye). Three patients presented upon initial examination VFI of 0% (NAION02), 4% (NAION14) and 3% (NAION17), significantly improving to 21%, 20% and 73%, respectively, to date. The average of progression rate reached +11.5±15% per year considering all the patients. Most of the patients experimented significant enhancement of the visual field at 95% of confidence after the prescription of vitamins and CoQ10 treatments.

Insert Table 2 here

Characteristics of cases diagnosed with homonymous hemianopia or quadrantanopia following stroke has been summarized in Table 3 (labeled as *Stroke*). Both eyes were affected in all patients and the VFI progression rate was highly heterogeneous depending on the case, which an average of progression rate reaching +9.3±10.5% per year, considering all the patients in this subgroup. Follow-up of individual patients ranges from 6 (Stroke02) to 203 (Stroke03) months, with progression rates of +0.6±0.9% (right eye of Stroke01 patient) to +33.3±10.7% (right eye of Stroke10 patient). Once again, most of the patients experimented significant enhancement of the visual field at 95% of confidence after the prescription of vitamins and CoQ10 treatments. Stroke07 case must be highlighted considering that upon initial examination did not present specific signs of visual pathway lesions, while at 3rd month a cerebral ictus with peripheral alteration was observed with VFI of 88% (right) and 80% (left) and almost total recovery after CoQ10 and vitamins treatment, with progression rates of +3.3±3.8% and +4.9±4.6% in right and left eyes, respectively (95% confidence level).

Insert Table 3 here

Figure 1 exemplifies one of the selected cases (i.e., Stroke06) of a patient diagnosed with right homonymous incomplete hemianopia after left occipital lobe stroke, showing the Visual field

test using the (HFA) and 30-2 algorithm, from February 2017 to May 2019. In 2017 the patient presented right homonymous hemianopia with VFI of 72% and 58% in the right and left eyes, respectively. At that time, the patient was prescribed with Active complex® Q10 Gold 100 mg, containing 100 mg of CoQ10 and 25 mg of vitamin C, in alternate months (1 tablet at lunch, following manufacturer dosage guidelines), additional to Hidroxil, Acfol and Visan supplements. At two-year follow up, i.e., 2019, the VFI of the right eye presented a promptly slight improvement of the visual field (76%) and a remarkable improvement was observed in the visual field index of left eye (71%), with progression rates of +6.9±8.6 and +5.6±1.9 per year, respectively.

Insert Figure 1 here

Additionally, Figure 2 depicts a very interesting case of a young patient diagnosed with right hemianopia of unknown origin (Stroke04), affecting the right eye with initial VFI of 51%. The patient also presented RPE alteration and myopia with growth hormones prescription 2012 to 2015. During the first four months of follow up the VFI remained almost constant, up to 58%. At fourth month, oral treatments of vitamins and CoQ10 were prescribed, observing a significant progression of VFI, with a rate of +30.3±6.4% for the affected right eye and 99% of the visual field at the 20th month of follow-up.

Insert Figure 2 here

RAO patients treated with CoQ10 and vitamins from the first month following the acute event presented the most dramatic vision improvement, as shown Table 3. The average of progression rate accounted for +22±17% per year considering all the patients. Follow-up of individual patients ranges from 4 (RAO03) to 53 (RAO07) months, with progression rates of +3.0±5.3 (left eye of RAO07 patient) to +49.4±42.9 (right eye of RAO02 patient). Two patients presented upon initial examination VFI of 0% (RAO01 and RAO02), significantly improving to 75% and 46%, respectively.

Insert Table 4 here

Specifically, VFI evolution of the RAO01 case, i.e., patient diagnosed with CRAO, has been summarized in Figure 3, from October 2017 to June 2019. In 2017 the patient presented a VFI of 98% in the non-affected left eye, while this index reached 0% in the affected right eye without any sign of vision. The patient was hence treated with CoQ10 and vitamin supplementation, and after 9 months of follow-up the patient experimented a pronounced improvement of VFI to 75% in the right eye. The VFI progression rates per year reached +39.9±25.6 and +1.8±4.0 in the right and left eye, respectively.

Insert Figure 3 here

Finally, patients with different conditions affecting the vascularity of the retina (n=13) are collected in Table 4. These include optic neuritis, optic nerve atrophy, neuroretinitis, cones dystrophy, retinitis pigmentosa, retinal vascular occlusion and two unknown etiology. The follow-up covers a period of 14 to 110 months, with an average of progression rate reaching +11±21% per year considering all the patients. Most of the patients presented both eyes affected, with minimum VFI detected of 0% and 2% (right and left eye of OC04), 5% and 11% (right and left eye of OC10), or 12% and 19% (right and left eye of OC09). Specifically, the OC04 patient experiment almost a complete recovery in the VFI of left eye, with an increase from 2% to 91% (95% of confidence). It must be stressed the OC09 case, initially non-treated with C0Q10 and constant VFI from the first month (12% left and 9% right eye) to the 47th (12% left and 9% right eye), with progression rates of +0.0±3.7 and +0.4±2.7, for the right and left eye. After the 47th month, the patient was prescribed with CoQ10 and vitamin supplementation, experimenting a significant recovery of VFI to 49% in both eyes.

Discussion

This case series study shows clinical outcome of patients with retinal diseases of vascular origin treated with CoQ10 and vitamins between different periods from 2009 to 2019. Visual field defects are common after vascular occlusions, stroke, trauma, tumor, brain surgery, and demyelinating lesions, with high impact on daily activities, with poor mobility, collisions, impaired reading and driving skills, and increased dependence and disability (Prem Senthil et al. 2019; Kerkhoff 1999). Prognosis of retinal diseases of vascular origin its uncertain and adverse with current knowledge. For example, RAO symptoms will remain stable or worse over the time unless patient has a cilioretinal artery, which lessen the chances of damage (Augsburger & Magargal 1980), while NAION clinical course generally remains stable, with most cases showing no significant improvement or deterioration over time (Miller & Arnold 2015). Moreover, stroke following homonymous hemianopia (or quadrantanopia) has poor prognosis with unlikely spontaneous recovery (Pambakian & Kennard 1997).

Usually, visual field defects of vascular origin present recovery in the first days after the insult, probably mediated by the removal of the edema and concomitant restitution of surrounding non-infarcted penumbral tissue, with uncertain further amelioration of the disease. In NAION or RAO, spontaneous improvement of visual acuity is not unusual during first weeks following the event, although significant improvement in visual field seems to occur less commonly than does improvement in acuity (Scherer RW et al. 2008). Besides, approximately 60% of patients diagnosed with hemianopia could experience spontaneous improvement in the visual field, usually in the first 10 days after brain injury, decreasing progressively with every successive month, with less than 10% of patients recovering their full field (Frolov et al. 2017). Therefore, the recovery is variable, depending on the degree of neuronal death and the removal of the

initial effects of the acute injury (Pambakian 2015; Mirshahi et al. 2008). In our cases series, the improvement in the visual field of the patients began to occur in most of the case more than 2 months after the lesion, with significant recovery in the follow-up, and therefore is not likely as a result of spontaneous recovery.

Visual field improvement implies significant changes in mean and pattern deviations in Humphrey VFI, with variable recovery depending upon individual. In this cases series we have obtained the natural history of visual field recovery of 48 patients at different stages of the disease and upon variable treatment periods. Vascular diseases affecting the retina are initially addressed to the identification of the vascular etiology, the modification of risk factors to prevent reoccurrences, improve retinal perfusion, lowering intraocular pressure, increase the vascular supply using vasodilators and initiation of an early and intensive rehabilitation therapy allowing clinical outcomes and improving the disability (Pambakian 2015; Han et al. 2002). There is an absence of consensus about gold-standard-treatment due to inadequate evidenced based research. Currently, vitamins and antioxidants supplements are widely used, without broadly accepted nutritional recommendations owing to absence of effectiveness evidence. Recently, CoQ10 supplement has been proposed for the treatment of retinal dystrophies (Yang et al. 2016). In line with this, in most of the cases included in this study we have observed amelioration in the visual field following CoQ10 and vitamins supplementation, along each respective follow-up, with averaged progression rate of +13±16% per year (Tables 2, 3, 4 and 5).

Among the studied cases, the patients diagnosed with RAO (n=7) experimented the highest increase of VFI progression rate. All of them were immediately treated with vitamins and CoQ10 following the diagnosis, although recovery of vision were experimented over more than 4 months periods, with significant enhancement of the visual field maintaining the prescribed treatment, which includes daily CoQ10 supplementation, and therefore the improvement in the visual field observed was not as the result of neither compensation nor rehabilitation therapy. Both embolic and thrombotic occlusions of the ophthalmic artery resulting in ischemia are accompanied by severe prognosis, even if the blockage is removed. Therefore, the observed VFI progression rates may be related with the proposed supplementation treatment. Regarding homonymous hemianopia or quadrantanopia patients following stroke (n=10), the VFI progression rates were significant higher in both eyes. None of these patients received restorative therapies including optical aids or compensatory training, and therefore the improvement in the visual field observed was not as the result of rehabilitation therapy. Other conditions affecting the vascularity of the retina (n=13) showed similar VFI progression rates, with one experiencing a full recovery of visual field (OC04), while OC09 showed an increase

on VFI only when CoQ10 and vitamin supplementation was prescribed, at 47th month of follow-up.

Moreover, NAION cases (n=18) follow-up showed significant increased progression rates, similar to those of RAO cases, following supplementation treatment. Very interesting was the NAION09 patient, which VFI of left eye remained almost constant during 64 months of follow-up and without treatment of CoQ10. However, in the 64th month was treated with CoQ10 showing at the 106 month a VFI of 55%, with an averaged progression rate of +2.3±2, which may imply the effectiveness of this coenzyme in vision restoration. Interestingly, Figure 4 shows the visual field test using the HFA and 30-2 algorithm, from 2017 to 2019 of the NAION02 case. The patient received CoQ10 treatment after initial examination (month 0), later stopped at 3rd month and restarted at month 10. Rows represents the right and left visual fields of each respective eye, showing the left eye dramatically affected. Upon initial treatment with CoQ10, the patient experimented a recovery of the VFI from 0% to 43% in the left eye (March 2017). However, after treatment interruption it was observed deterioration in the visual field in November 2017 to 6%. Finally, following treatment restoration an improvement of the VFI was observed to current 21% (June 2019).

Insert Figure 4 here

This observation is crucial since the natural history of visual field recovery is fundamental when evaluating claims of improvement by potential treatments for vascular diseases affecting the retina (Kedar et al. 2011). We demonstrate that discontinuing the treatment with worsening in the VFI and rechallenge it with significant improvement in the visual field supports the role of CoQ10 as therapeutic agent for vascular diseases affecting the retina. Moreover, since the interruption of CoQ10 decreases visual field may suggests that the beneficial effects are not irreversible, but this observation needs further confirmation.

CoQ10, also known as ubiquinone, ubidecarenone or coenzyme Q, is a 1,4-benzoquinone and the most common coenzyme Q in humans. CoQ10 is an essential component in mitochondrial bioenergetics acting as intracellular antioxidant and protecting neuronal cells against oxidative stress in neurodegenerative diseases (Somayajulu et al. 2005; Littarru & Tiano 2007; Lee 2014). It has been hypothesized that CoQ10 may inhibits microglia cell activation maintaining its mitochondrial function and prevent the glutamate-induced cytotoxicity that may contribute to neural degeneration (Lu et al. 2017). The levels of CoQ10 may be depleted during an acute event, leading to alterations in mitochondrial energy production and increased free radical damage due to the reduced scavenging capacity (Yang et al. 2016).

Oral administration of CoQ10 showed neuroprotective effects in neurodegenerative diseases, including Parkinson's disease and age-related macular degeneration (Zhang et al. 2017b; Beal

2004), as well as in cardiovascular diseases in which might ameliorate endothelial dysfunction (Molyneux et al. 2008). The dual role of this enzyme may contribute to restrains extracellular glutamate accumulation and excitotoxicity reducing the harmful effect of ischemia/reperfusion on mitochondrial energy metabolism (Nucci et al. 2007). Overall, CoQ10 has been proposed as neurotherapeutic agent, but additional multicenter studies on the potential usefulness its supplementation with conventional therapy in neurological diseases are mandatory (Russo et al. 2008; Hernández-Camacho et al. 2018).

In conclusion, a case series study of patients diagnosed with retinal dysfunctions caused by vascular disorders demonstrated the therapeutic potential of CoQ10 in combination with vitamins with significant improvement in the visual field. However, oral CoQ10 treatment must be evaluated in randomized, double-masked, controlled, prospective clinical studies to support these findings, in which dosing accuracy and the duration of therapy should be addressed. The use of CoQ10 in clinical practice with traditional treatments can lead to improved patient outcomes.

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Tables

Table 1. Demographic characteristics of patients

Study population (n)	Age at diagnosis (mean ± SD)	Current age (mean ± SD)	Age at CoQ10 treatment initiation (mean ± SD)	Age Range	Gender (female/male)
NAION (18)	61 ± 8	65 ± 7	61 ± 7	50-81	8(44%)/10
Stroke (10)	56 ± 21	63 ± 18	63 ± 18	19-71	4(40%)/6
RAO (7)	65 ± 11	67 ± 11	65 ± 11	48-83	2(29%)/5
OC (13)	47 ± 23	54 ± 22	51 ± 22	15-85	8(61%)/5
Population-averaged (48)	57 ± 8	62 ± 6	60 ± 6	15-85	22(46%)/26

Table 2. Clinical characteristics of NAION cases, VFI and MD follow-up and progression rate.

Case	Disease	Follow-up months	Eye	Initial VFI (%)	Final VFI (%)	Initial MD (dB)	Final MD (dB)	VFI Progression Rate (%/year)[Ŧ]
NAION01	NAION (with secondary optic atrophy)	91*	Right	96	100	-3.91	1.03	-0.2±0.2
			Left	49	67	-17.91	-10.68	+2.0±1.9
NAION02	NAION	29**	Right	92	98	-6.37	-0.27	+2.6±2.6
			Left	0	21	-30.82	-23.18	+5.0±14.8
NAION03	NAION (with homonymous hemianopia)	37*	Right	97	98	-2.4	-1.81	+1.1±8.2
			Left	81	88	-6.79	-2.35	+2.4±8.6
NAION04	NAION	5*	Right	97	99	-3.72	-1.52	+1.0±1.1
			Left	14	35	-27.39	-21.64	+58.1±10.5
NAION05	NAION	10*	Right	88	93	-5.7	-3	+2.8±5.0
			Left	36	45	-19.22	-15.67	+11.8±17.6
NAION06	NAION (with inferior hemianopia)	64*	*Right*	63	88	-16.43	-6.62	+5.9±2.6
			Left	97	100	-1.24	0.67	-0.1±0.3
NAION07	NAION	31*	*Right*	52	67	-17.88	-12.38	+6.0±14.4
			Left	98	99	-1.48	-0.63	+0.60±0.6
NAION08	NAION	14*	*Right*	46	79	-16.91	-6.68	+19.7±13.9
			Left	93	99	-4.07	-0.13	+2.1±2.6
NAION09	NAION	106***	*Right*	20	55	-23.96	-15.03	+2.3±2.1
			Left	98	99	-2.38	-0.02	+0.0±0.1
NAION10	NAION	76*	Right	99	99	-1.33	-0.28	0.0±0.3
			Left	46	46	-17.9	-17.47	+0.7±4.0
NAION11	NAION	12*	Right	97	99	-0.34	0.39	+0.7±1.1
			Left	14	27	-27.15	-23.73	+24.5±6.7
NAION12	NAION	42*	Right	78	94	-7.76	-2.15	+1.4±6.8
			Left	17	25	-28.56	-26.27	+2.0±4.1
NAION13	NAION	41*	Right	98	98	-0.53	1.69	+0.0±0.0
			Left	56	85	-13.3	-5.26	+23.2±14.5
NAION14	NAION	18*	*Right*	4	20	-29.64	-25.05	+14.2±10.98
			Left	100	97	-2.26	-1.61	+0.1±6.3
NAION15	NAION	89*	*Right*	67	80	-11.05	-5.96	+0.7±1.2
			Left	100	99	-0.77	-0.39	-0.1±0.2
NAION16	NAION	5*	Right	98	99	-0.27	1.3	+0.50±0.1
			Left	78	91	-10.03	-5.02	+8.0±6.6
NAION17	NAION	22*	Right	89	95	-8.69	-5.45	+1.6±0.3
			Left	3	73	-30.68	-10.16	+26.6±16.6
NAION18	NAION	102*	*Right*	52	62	-17.03	-12.1	-0.7±2.0
			Left	100	99	-2.11	-1.34	+0.2±0.6

Right or left in italics indicate the affected eye; * initiation of CoQ10 treatment in month 0; **initiation of CoQ10 treatment in month 0, stopped in month 3 and re-initiated in month 10; *** initiation of CoQ10 in month 64; [Ŧ]95% of confidence; [#]CoQ10 from alternate to all the months treatment.

Table 3. Clinical characteristics of homonymous hemianopia or quadrantanopia cases following stroke, VFI and MD follow-up and progression rate.

Case	Disease	Follow-up months	Eye	Initial VFI (%)	Final VFI (%)	Initial MD (dB)	Final MD (dB)	VFI Progression Rate (%/year)[Ŧ]
Stroke01	Inferior homonymous Hemianopia following stroke	203[¥]	Right	55	73	-16.8	-11.43	+0.6±0.9
			Left	56	78	-16.31	-9	+0.8±0.8
Stroke02	Left homonymous Hemianopia following right retrochiasmal lesion	6*	Right	60	73	-14.01	-10.47	+22.2±51.1
			Left	71	66	-8.65	-9.31	-10.5±5.1
Stroke03	Right Superior homonymous Hemianopia following lef inferior retrochiasmal lesion	19[¢]	Right	84	84	-7.72	-7.61	-0.1±10.1
			Left	78	80	-10.01	-10.54	+0.7±18.1
Stroke04	Right Hemianopia (unknown origin)	20[£]	Right	51	99	-17.21	-1.18	+30.3±6.4
			Left	99	100	-2.37	-0.58	+2.2±4.2
Stroke05	Right inferior homonymous quadrantanopia (left superior retrochiasmal lesion)	13*	Right	77	81	-11.21	-9.94	+2.6±15.7
			Left	62	90	-14.01	-4.28	+20.5±14.4
Stroke06	Right homonymous incomplete hemianopia (left occipital lobe stroke)	27*	Right	72	76	-12.57	-6.92	+6.9±8.6
			Left	58	71	-16.09	-12.77	+5.6±1.9
Stroke07	Stroke (cerebrovascular ictus) with peripheral alteration	22**	Right	96	94	-3.34	-2.53	+3.3±3.8
			Left	90	88	-5.97	-4.31	+4.9±4.6
Stroke08	Left inferior homonymous quadrantanopia (right superior retrochiasmal lesion)	90[Δ]	Right	88	91	-5.62	-1.55	+2.3±3.1
			Left	89	90	-5.36	-5.24	+3.1±3.8
Stroke09	Right homonymous hemianopia (left retrochiasmal lesion)	12*	Right	56	65	-14.34	-10.2	+7.7±5.2
			Left	50	57	-16.84	-15.31	+3.8±23.8
Stroke10	Left homonymous hemianopia (right retrochiasmal lesion)	12*	Right	27	48	-22.53	-18.96	+33.3±10.7
			Left	26	35	-21.28	-17.62	+16.0±4.5

*initiation of CoQ10 treatment in month 0; **Stroke on 3rd month and CoQ10 in15th month; [¥]intiation of CoQ10 treatment in month 195; [¢]intiation of CoQ10 treatment in month 10; [£]intiation of CoQ10 treatment oin month 4; [Δ]intiation of CoQ10 treatment in month 68; [Ŧ]95% of confidence.

Table 4. Clinical characteristics of RAO patients, VFI and MD follow-up and progression rate.

Case	Disease	Follow-up months	Eye	Initial VFI (%)	Final VFI (%)	Initial MD (dB)	Final MD (dB)	VFI Progression Rate (%/year)[Ŧ]
RAO01	CRAO	20*	*Right*	0	75	-31.24	-10.19	+39.9±25.6
			Left	98	99	-1.27	0.94	+1.8±4.0
RAO02	CRAO	9*	*Right*	0	46	-31.88	-17.3	+49.4±42.9
			Left	96	94	-3.15	-2.19	+1.0±10.1
RAO03	RAO (temporal inferior with nasal superior quadrantanopia)	4*	Right	100	96	0.9	-2.37	-2.0±1.9
			Left	48	78	-18.55	-8.06	+27.5±12.7
RAO04	RAO (temporal inferior with optic nerve atrophy)	7*	Right	98	100	-0.57	0.7	+1.0±0.8
			Left	62	78	-10.79	-7.04	+12.2.5±10.6
RAO05	RAO (temporal inferior with superior hemianopia)	15*	*Right*	47	87	-15.45	-5.71	+13.1±16.9
			Left	98	99	-3.17	-1.99	+0.2±0.2
RAO06	RAO (temporal superior)	5*	*Right*	56	61	-14.44	-11.09	+8.9±3.5
			Left	98	98	-2.27	-1.54	+0.0±0.0
RAO07	RAO (temporal superior)	53*	Right	95	98	-2.5	-0.39	+0.2±1.1
			Left	51	61	-18.3	-12.68	+3.0±5.3

Right or left in italics indicate the affected eye; *initiation of CoQ10 treatment in month 0; [Ŧ]95% of confidence

Table 5. Clinical characteristics of patients with other conditions, VFI and MD follow-up and progression rate.

Case	Disease	Follow-up months	Eye	Initial VFI (%)	Final VFI (%)	Initial MD (dB)	Final MD (dB)	VFI Progression Rate (%/year)[T]
OC01	Optic Neuritis	92*	*Right*	87	97	-9.54	-2.61	+0.9±0.5
			Left	71	94	-9.89	-3.19	-2.4±0.6
OC02	Optic Neuritis causing optic nerve atrophy	97*	Right	99	98	-1.44	-2.22	-0.4±0.4
			Left	70	91	-11.12	-7.06	+0.8±3.5
OC03	Bilateral Optic Neuritis	16*	*Right*	48	92	-20.89	-5.65	+24.2±28.2
			Left	22	93	-27.32	-3.94	+61.7±32.9
OC04	Optic Nerve Atrophy (post-meningitis)	63*	*Right*	0	11	-31.02	-25.79	+76.2±12.3
			Left	2	91	-30.22	-2.4	+257.0±42.30
OC05	Optic Nerve Atrophy (intracranial hypertension)	110*	*Right*	89	85	-0.87	-7.12	+1.3±3.8
			Left	89	88	1.15	-6.72	+0.6±2.4
OC06	Optic Nerve Atrophy (drug toxicity, etambutol)	22*	*Right*	86	99	-4.58	-0.28	+4.2±2.4
			Left	87	99	-5.67	-0.1	+7.1±3.0
OC07	Neuroretinitis	26*	*Right*	70	88	-11.47	-2.31	+6.8±5.4
			Left	100	100	4.06	4.18	+0.1±0.4
OC08	Tapetoretinal dystrophy (with superior hemianopia)	34*	*Right*	31	40	-26.17	-20.8	+2.2±2.9
			Left	22	48	-27.64	-18.42	+2.5±3.9
OC09	Retinitis pigmentosa	53[¥]	*Right*	12	49	-27.92	-19.46	+0.0±3.7
			Left	19	49	-26.88	-19.85	+0.4±2.7
OC10	Unknown	14*	*Right*	5	80	-28.75	-7.54	+15.5±4.4
			Left	11	88	-27.76	-8.5	+10.0±4.9
OC11	Unknown (optic nerve injury)	68[¢]	*Right*	95	98	-5.13	-1.9	+4.1±24.6
			Left	90	99	-6.4	-0.34	+4.6±22.0
OC12	Cones Dystrophy	103[£]	*Right*	66	70	-6.99	-9.96	+0.1±1.7
			Left	71	70	-7.39	-10	-0.6±1.1
OC13	Retinal Vascular Occlusion	89[Δ]	Right	99	99	-0.22	-0.51	+0.6±1.7
			Left	31	58	-24.44	-17.84	+5.2±2.9

Right and/or left in italics indicate the affected eye; *initiation of CoQ10 treatment in month 0; [¥]intiation of CoQ10 treatment in month 47; [¢]intiation of CoQ10 treatment in month 40; [£]intiation of CoQ10 treatment in month 93; [Δ] intiation of CoQ10 treatment in month 57; [T] 95% of confidence

Figure Legends

Figure 1. Visual field test using the Humphrey field analyzer and 30-2 algorithm, from February 2017 to May 2019 of case of right homonymous incomplete hemianopia after left occipital lobe stroke (Stroke06 case). In 2017 was treated with CoQ10 and vitamins to May 2019, with a progressive improved of the visual field index in both eyes. Progression rates at 95% confidence are shown.

Figure 2. Visual field test using the Humphrey field analyzer and 30-2 algorithm obtained from March 2017 to November 2018 of a case of right hemianopia of unknown origin (Stroke 04). After four month of the acute event was treated with CoQ10 and vitamins to date, with a progressive improved of the visual field index in right affected eye. Progression rates at 95% confidence are shown.

Figure 3. Summary of visual field test the Humphrey field analyzer and 30-2 algorithm, from October 2017 to June 2019 of a patient diagnosed with CRAO affecting the right eye (RAO01). From 2017 to date was treated with CoQ10 and vitamins, with a progressive improved of the visual field index in both eyes. Progression rates at 95% confidence are shown.

Figure 4. Visual field test using the Humphrey field analyzer and 30-2 algorithm, from 2017 to 2019 of a patient diagnosed with NAION (case NAION02). Rows represents the right and left visual fields of each respective eye. In January 2017 the patient presented NAION in left eye, receiving CoQ10 treatment. In March 2017 the CoQ10 treatment was interrupted with deterioration in the visual field in November 2017, restarting the treatment with an observed improve in the visual field index in June 2019.

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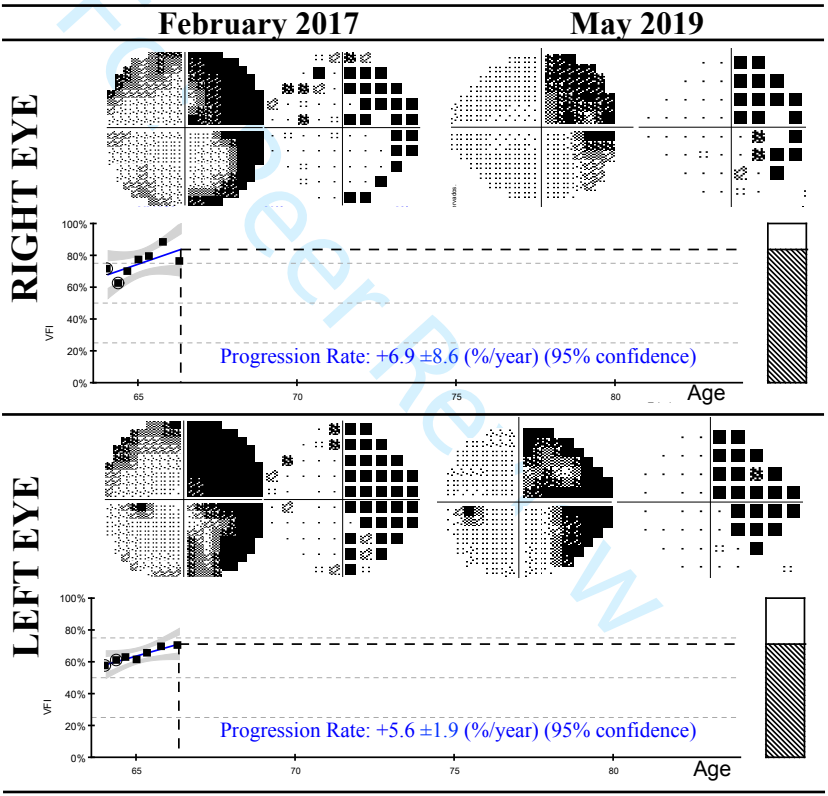
Figure 1



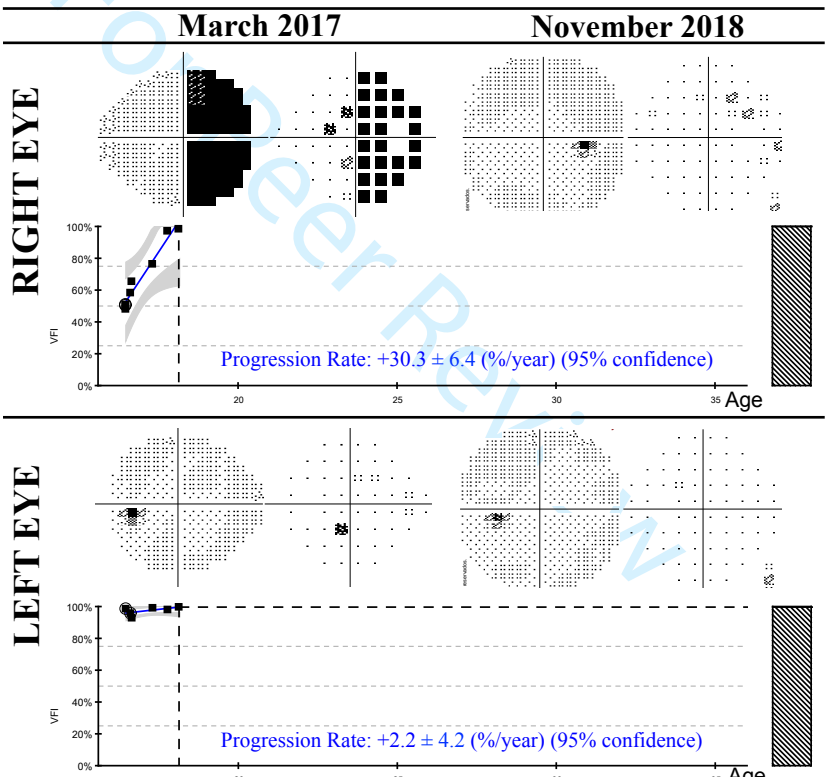
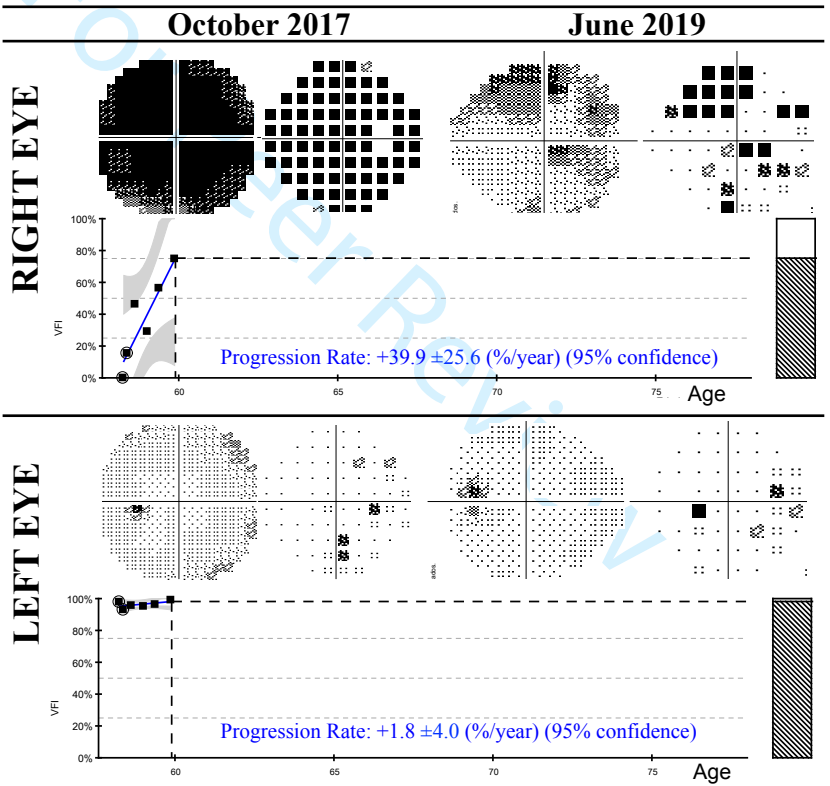
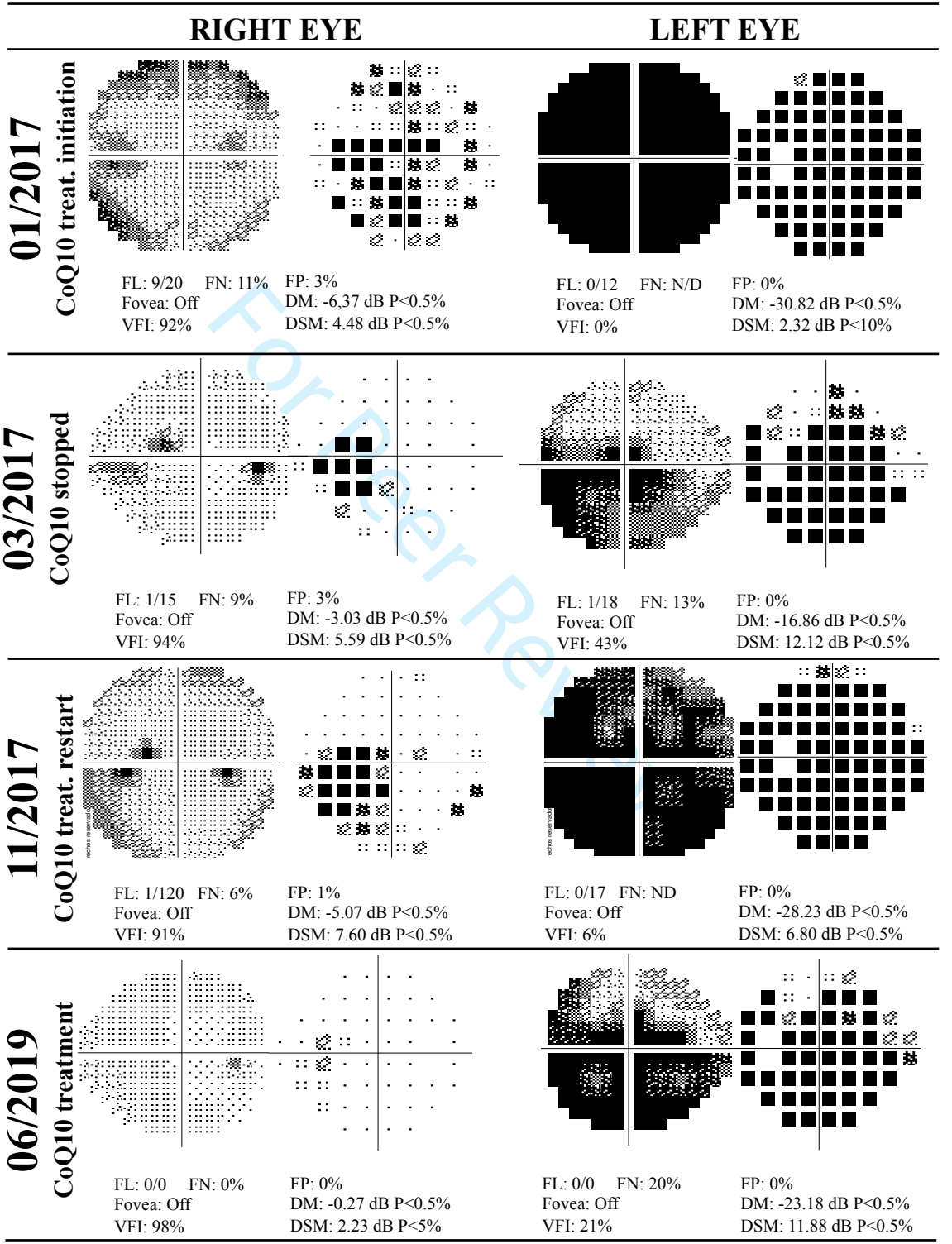


Figure 3





6.

The association study of lipid metabolism genes polymorphisms with AMD identifies a protective role for APOE-E2 allele in the wet form of Northern Spanish patients (publicado en Acta Ophthalmologica, 2019)

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The association study of lipid metabolism gene polymorphisms with AMD identifies a protective role for APOE-E2 allele in the wet form in a Northern Spanish population

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ABSTRACT.

Purpose: **To elucidate the potential role of eleven single nucleotide polymorphisms (SNPs) in the most relevant lipid metabolism genes in Northern Spanish patients with age-related macular degeneration (AMD).**

Methods: **A case-control study of 228 unrelated native Northern Spanish patients diagnosed with AMD (73 dry and 155 wet) and 95 healthy controls was performed. DNA was isolated from peripheral blood and genotyped for the SNPs *APOE* rs429358 and rs7412; *CTEP* rs3764261; *LIPC* rs10468017 and rs493258; *LPL* rs12678919; *ABCA1* rs1883025; *ABCA4* rs76157638, rs3112831 and rs1800555; and *SCARB1* rs5888, using TaqMan probes. An additional association study of ε2, ε3 and ε4 major isoforms of *APOE* gene with AMD has been carried out.**

Results: **The allele and genotype frequencies for each of the eleven sequence variants in the lipid metabolism genes did not show significant differences when comparing AMD cases and controls. Statistical analysis revealed that *APOE*-ε2 carrier genotypes were less frequently observed in patients with wet AMD compared to controls (5.8% versus 13.7%, respectively: $p = 3.28 \times 10^{-2}$; OR = 0.42, 95% CI: 0.19–0.95). The frequency of the allele T of rs10468017 (*LIPC* gene) was lower in dry AMD cases compared to controls (15.8 versus 27.9%, respectively: $p = 8.4 \times 10^{-3}$ OR = 0.57, 95% CI: 0.33–0.98).**

Conclusions: **Our results suggest a protective role for APOE-ε2 allele to wet AMD in the Northern Spanish population.**

Key words: age-related macular degeneration – genetic association – lipid metabolism genes – Northern Spanish population – single nucleotide polymorphism

*Both authors contributed equally to this work.

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Introduction

Age-related macular degeneration (AMD), a neurodegenerative disease affecting 200 million patients, is the third cause of irreversible blindness worldwide (Resnikoff & Keys 2012) and the leading cause for people over the age of 60 in developed countries (Klein et al. 2004; Pascolini et al. 2004). The number of AMD cases is expected to increase to 288 million in 2040 (Wong et al. 2014).

Age-related macular degeneration (AMD) is characterized by a progressive loss of central vision with multifactorial etiology. Pigmentary abnormalities in the retinal pigmented epithelium (RPE), accumulation of lipids in Bruch's membrane (BrM) and sub-RPE deposits (drusen) formation appear in the early stages of AMD, which may progress to an advanced stage distinguishing two forms: late dry AMD, characterized by geography atrophy (GA), or wet AMD (exudative or neovascular AMD) characterized by the development of choroidal neovascularization (CNV) and finally disciform scar. Dry AMD accounts for 85–90% of all cases of AMD and approximately 10–20% of them may progress to wet AMD if untreated (Hyman & Neborsky 2002).

The pathogenesis of AMD remains poorly understood, involving an interplay of diverse type of factors: age, smoking (Khan et al. 2006; Cackett et al. 2011; Kabasawa et al. 2011), obesity and dietary fat consumption (Seddon et al. 2003a,b, 2006) and single nucleotide polymorphisms (SNPs) (Katta et al. 2009; Chen et al. 2010a; Liu et al. 2012a; Fritsche et al. 2013). Genetic studies have implicated genes coding for complement factors (i.e. *CFH*, *CFI* and *CFB*), complement components (i.e. *C2* and *C3*), extracellular matrix remodelling (i.e. *COL10A1*), angiogenesis (i.e. *VEGFA* and *TGFBR1*) and lipid metabolism (i.e. *LIPC* and *APOE*) in the pathophysiology of intermediate and advanced AMD (Klein et al. 2005; Sobrin et al. 2012; Fritsche et al. 2013).

Altered lipid metabolism contributes to significant accumulation of lipoprotein-like particles and other debris in the elastic and inner collagenous layers (ICL) of the BrM, evolving to a "lipid wall" formation between the ICL and the basal lamina of the RPE (Ruberti et al. 2003; Huang et al. 2007; Sarks 1976; Pauleikhoff et al. 1990; Bird & Marshall 1986; : van der Schaft et al. 1992; Curcio & Millican 1999), blocking the physiologic transport (Hogan & Alvarado 1967; Killingsworth 1987; Curcio & Millican 1999). Moreover, additional ageing effects decrease the hydraulic conductivity, the permeability to macromolecular transport, and the thickness and the elasticity of BrM, disturbing the metabolism of the RPE and photoreceptor cells (Moore et al. 1995; Starita et al. 1996; Hussain et al. 2002; Ugarte et al. 2006).

Up to date, there are limited studies that have explored the association between lipid metabolism genes and the distinct clinical forms of AMD (i.e. dry and neovascular), in the Spanish population. Previous studies have reported the genetic association between *APOE* and *ABCA4* (rs3112831) with AMD, respectively (Asensio-Sanchez et al. (2006) and Brion et al. (2011)). The present study examined the association of the most relevant SNPs described in lipid metabolism genes, including lipase C, hepatic type (*LIPC*) rs10468017 and rs493258, lipoprotein lipase (*LPL*) rs12678919, ATP binding cassette subfamily A member 1 (*ABCA1*) rs1883025, ATP binding cassette subfamily A member 4 (*ABCA4*) rs1800555, rs76157638 and rs3112831, cholesteryl ester transfer protein (*CETP*) rs3764261, scavenger receptor class B member 1 (*SCARB1*) rs5888, and apolipoprotein E (*APOE*) rs429358 and rs7412, with AMD cases (both wet and dry forms), in a Northern Spanish population.

Material and methods

Study subjects

The present case-control study involved 228 unrelated native Northern Spanish patients diagnosed with AMD (73 dry and 155 wet forms) and 95 healthy controls, recruited at the Instituto Oftalmológico Fernández-Vega (Asturias, Spain). The population origin analysed in the present study was Caucasian and mostly from the northernmost regions of Spain (43.34% Asturias, 9.29% Galicia, 7.74% Cantabria, 4.95% País Vasco, 1.24% La Rioja and 0.93% Navarra) and from other regions located in the Northern half of the country (22.60% Castilla y León, 0.62% Cataluña, 1.24% Aragón and 8.05% Comunidad de Madrid). Complete ophthalmic examinations were performed for patients and controls, including slit lamp biomicroscopy and funduscopy in both eyes. Age-related macular degeneration-diagnosed patients were further examined by fluorescence fundus angiography or optical coherence tomography. Individuals were classified as follows: wet AMD with evidence of CNV in any eye and dry AMD with evidence of geographic atrophy (GA) in any eye and absence of CNV. Control subjects were selected from patients undergoing cataract surgery and absence of AMD or glaucoma. Subjects with other relevant ocular pathologies, such as retinopathies or maculopathies, were excluded from this study. To avoid possible misclassification, considering that AMD is a late-onset disorder, only people over 60 were recruited as controls.

The study adheres to the tenets of the Declaration of Helsinki on Biomedical Research Involving Human Subjects and was approved by the Clinical Research Ethics Committee at Principality of Asturias (Oviedo, Spain). All participants signed an informed consent.

Genotyping

Peripheral blood was collected in 6-ml tubes coated with EDTA (Vacuette, Madrid, Spain), which blocks the coagulation cascade. Samples were stored at −20°C until use. Genomic DNA was obtained from blood using a commercial DNA extraction kit (FlexiGene DNA Kit; Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Allelic discrimination was performed with TaqMan probes *APOE* rs429358 (C___3084793_20) and rs7412 (C____904973_10); *CTEP* rs3764261 (C__27513218_10); *LIPC* rs10468017 (C__29910029_10) and rs493258 (C___1929355_10); *LPL* rs12678919 (C___9639494_10); *ABCA1* rs1883025 (C___2959486_10); *ABCA4* rs76157638 (C__27860830_20) and rs1800555 (C____868772_20) and *SCARB1* rs5888 (C___7497008_1_), and a Custom TaqMan (R) SNP Genotyping Assay for *ABCA4* rs3112831 (ANH493H), provided by the manufacturer (Applied Biosystems Inc., Foster City, CA, USA) in the 7500 Real Time PCR System (Applied Biosystems Inc., Foster City, CA, USA). All PCR amplifications were performed with the thermal cycling conditions of 95°C for 10 min, followed by 50 cycles of 92°C for 15 second and 60°C for 1 min. The genotyping results were confirmed in a random subgroup of our samples using direct DNA sequencing.

Statistical analysis

All the SNPs were assessed for Hardy–Weinberg equilibrium by a χ^2 test in both groups with HaploView 4.0 software (Daly Lab, Broad Institute, Cambridge, MA). The ages of the AMD patients (AMD, dry, wet forms) and control subjects were compared using the Mann–Whitney test (GraphPad InStat 3.0, San Diego, CA, USA). The comparison of the SNPs allelic frequencies between the AMD and control groups was performed using a standard χ^2 test, with a p-value of less than 4.54×10^{-3} (0.05/11) considered as statistically different (Bonferroni method was used for the adjustment of multiple comparisons). Additionally, we used SPSS version 15.0 (IBM Corp., Armonk, NY, USA) to run a logistic regression analysis in order to control for potential confounders. The comparison of genotypic frequencies between the AMD, including disease subgroups, and controls were performed using a χ^2 test (Pearson correction) with SPSS version 15.0 (IBM Corp., Armonk, NY).

Relative risk association was estimated by calculating odds ratios (OR) along with 95% confidence intervals (CI) using the methods described by Armitage et al. (2002) and PLINK (v1.07) as described by Purcell et al. 2007;. Linkage disequilibrium (LD) plot was generated with HaploView 4.0 software (Daly Lab, Broad Institute, Cambridge, MA), and blocks were defined by Gabriel et al. (2002) algorithm. Individual haplotypes and their estimated population frequencies were performed using HaploView 4.0 software (Daly Lab, Broad Institute, Cambridge, MA) with all of the parameters set at the default values.

Results

Demographic characteristics of the recruited 228 AMD Northern Spanish patients (including dry and wet forms) and 95 healthy controls are shown in Table 1. Statistical significant differences were obtained for age, when compared controls with AMD (all cases, dry or wet forms) ($p < 0.05$) and for sex when compared all AMD or dry AMD cases with controls ($p < 0.05$).

Association study

In the present study, the allelic and genotypic frequencies for each of the eleven sequence variants in the lipid metabolism genes (*LIPC*: rs10468017 and rs493258; *LPL*: rs12678919; *ABCA1*: rs1883025; *ABCA4*: rs1800555, rs76157638 and rs3112831; *CETP*: rs3764261; *SCARB1*: rs5888; and *APOE*: rs429358 and rs7412;) were first analysed in all AMD cases ($n = 228$) and controls ($n = 95$) (Table 2), which were in Hardy–Weinberg equilibrium ($p > 0.01$) for the studied genetic variants. Overall, comparison of allele and genotype frequencies between AMD cases and controls did not show significant differences (Table 2). Similarly, the additional association study of the SNPs with each of the different AMD forms separately (dry or wet) revealed not significant differences, as shown in Table S1. Specifically and considering each individual gene, the following observations must be highlighted:

LIPC and *LPL* polymorphisms

No association was found between the *LIPC* SNPs analysed (rs10468017 and rs493258) and AMD. Significant higher frequency of the allele C ($p = 8.4 \times 10^{-3}$; rs10468017) and of the allele T ($p = 4.27 \times 10^{-2}$; rs493258) was detected in patients with dry and wet forms of AMD, respectively (Table S1). However, although the frequency of the genotype CC (rs10468017) showed significant differences between AMD dry cases and controls under recessive model ($p = 2.51 \times 10^{-2}$, CC versus CT + TT), these not remained significant after Bonferroni correction for multiple testing ($p < 4.5 \times 10^{-3}$).

Similarly, no significant differences were found in the allelic frequencies of *LPL* rs12678919 when compared controls and AMD cases. The analysis of dry AMD cases showed higher genotypic frequency (AG) in controls, under a recessive model ($p = 4.61 \times 10^{-2}$; GG versus AG + AA), but these differences were not significant after the stringent Bonferroni correction ($p < 4.5 \times 10^{-3}$) (Table S1).

ABCA1, *ABCA4*, *CETP* and *SCARB1* polymorphisms

Allele and genotype frequencies of the polymorphisms located in the genes of the ABC transporters, *ABCA1* (rs1883025) and *ABCA4* (rs1800555, rs76157638 and rs3112831), did not significantly differ in AMD cases (including dry or wet forms) when compared to controls (Tables 2 and S1). Likewise, no association was found in the *CETP* polymorphism studied (rs376461) with AMD (Table 2). Furthermore, the comparison between AMD cases (AMD, dry, wet forms) and controls in the allele and genotype frequencies of the SNP rs5888, placed in the *SCARB1* gene, showed no significant differences (Tables 2 and S1).

APOE polymorphisms

The allelic and genotypic frequencies of the genetic variants rs429358 and rs7412 located in the *APOE* gene were not significantly different when compared AMD patients (AMD, dry or wet forms) with controls (Tables 2 and S1). Considering that the polymorphic *APOE* gene results in the ε2, ε3 and ε4 major isoforms, an additional association study with AMD has been carried out. Allele frequencies of ε2, ε3 and ε4 were similar in both controls and AMD cases (AMD, dry or wet forms) (Table 3). We investigated whether the ε2, ε3 and ε4 allele carriers were associated with AMD. No statistical differences were found with the ε3 and ε4 allele carriers when compared controls with AMD cases (AMD, dry or wet forms). In the case of the ε2 allele carriers, an association with protective effect for the wet form of AMD was detected ($p = 3.28 \times 10^{-2}$; OR = 0.42, 95% CI: 0.19–0.95).

Comparison with the Iberian Population in Spain

The frequency of the variants obtained in Northern Spanish patients were additionally compared with the Iberian Population in Spain (IBS) included in the 1000 Genomes Project Consortium Phase 3 (Auton et al. 2015). The allelic and genotypic frequencies of rs429358 (*APOE*) in our control subjects and in the IBS population revealed significant differences ($p = 0.0239$ and $p = 5.3 \times 10^{-3}$, respectively) (Table S1). The association study with AMD using the IBS populations as the control group (Table S1) showed the allele T of the SNP rs429358 (*APOE*) as a genetic risk variant for AMD ($p = 2.58 \times 10^{-2}$; OR = 1.11, 95% CI: 1.01–1.21), and also for dry and wet forms separately ($p = 1.47 \times 10^{-2}$; OR = 1.11, 95% CI: 1.02–1.22 and $p = 3.46 \times 10^{-2}$; OR = 1.10, 95% CI: 1.01–1.21, respectively). Moreover, the frequency of the genotypes TT was significantly higher in AMD with respect to the

Table 1. Demographic characteristics of AMD patients and controls

Study population (*n*)	Age (mean ± SD)	Age range	Gender (female/male)
Controls (95)	73.2 ± 8.06	60–92	52 (54.7%)/43
AMD (228)	78.0 ± 7.99†	52–99	154 (67.5%)/74*
Dry AMD (73)	76.2 ± 8.72†	52–99	55 (75.3%)/18*
Wet AMD (155)	78.9 ± 7.51†,‡	63–94	99 (63.9%)/56

n = number of subjects; SD = standard deviation.
*Indicates a $p < 0.05$ when compared to controls (χ^2 test).
† Indicates a $p < 0.05$ when compared to controls (Mann–Whitney test);
‡ Indicates a $p < 0.05$ when compared to dry AMD cases (Mann–Whitney test).

Table 2. Allelic and genotypic association analysis of AMD patients and control subjects

					CT Vs AMD	
	SNP ID		Control %	AMD %	p-value	OR (95% CI)
LIPC	*rs10468017*		(*n* = 95)	(*n* = 228)		
	Allele	C	72.1	76.3	0.2596	1.06 (0.90–1.11)
		T	27.9	23.7		0.85 (0.53–1.36)
	Genotype	CC	55.79	60.53		1.08 (0.86–1.37)
		CT	32.63	31.58	0.4299*	0.97 (0.65–1.45)
		TT	11.58	7.89		0.68 (0.29–1.61)
		Total	53/31/11 (CC/CT/TT)	118/72/18 (CC/CT/TT)	0.5241	
	rs493258		(*n* = 95)	(*n* = 228)		
	Allele	T	44.2	48	0.3759	1.09 (0.80–1.47)
		C	55.8	52		0.93 (0.72–1.20)
	Genotype	CC	30.53	28.51		0.93 (0.61–1.43)
		CT	50.53	46.93	0.2739*	0.93 (0.70–1.23)
		TT	18.94	24.56		1.30 (0.76–2.21)
		Total	29/48/18 (CC/CT/TT)	65/107/56 (CC/CT/TT)	0.5496	
LPL	*rs12678919*		(*n* = 95)	(*n* = 228)		
	Allele	G	12.6	13.8	0.6879	1.09 (0.54–2.23)
		A	87.4	86.2		0.99 (0.88–1.10)
	Genotype	AA	74.74	74.56		1.0 (0.85–1.17)
		AG	25.26	23.25	0.1457*	0.92 (0.56–1.50)
		GG	0	2.19		NA
		Total	71/24/0 (AA/AG/GG)	170/53/5 (AA/AG/GG)	0.3332	
ABCA1	*rs1883025*		(*n* = 95)	(*n* = 228)		
	Allele	C	73.2	73.2	0.9817	1.00 (0.85–1.18)
		T	26.8	26.8		1.00 (0.63–1.58)
	Genotype	TT	9.47	6.14		0.65 (0.24–1.72)
		TC	34.74	41.23	0.604*	1.19 (0.83–1.69)
		CC	55.79	52.63		0.94 (0.73–1.22)
		Total	9/33/53 (TT/TC/CC)	14/94/120 (TT/TC/CC)	0.3886	
ABCA4	*rs1800555*		(*n* = 95)	(*n* = 228)		
	Allele	C	99.5	99.8	0.5222	1.00 (0.99–1.02)
		T	0.5	0.2		0.40 (0.01–70.94)
	Genotype	CC	98.95	99.56		1.01 (0.98–1.03)
		CT	1.05	0.44	0.5214*	0.73 (0.04–11.80)
		TT	0	0		NA
		Total	94/1/0 (CC/CT/TT)	227/1/0 (CC/CT/TT)	0.8142	
	rs76157638		(*n* = 95)	(*n* = 228)		
	Allele	C	99.5	99.8	0.5222	1.00 (0.99–1.02)
		G	0.5	0.2		0.40 (0.01–70.94)
	Genotype	CC	98.95	99.56		1.01 (0.98–1.03)
		CG	1.05	0.44	0.5214*	0.42 (0.01–14.00)
		GG	0	0		NA
		Total	94/1/0 (CC/CG/GG)	227/1/0 (CC/CG/GG)	0.8142	
	rs3112831		(*n* = 95)	(*n* = 228)		
	Allele	A	61.1	62.5	0.7297	1.02 (0.82–1.28)
		G	38.9	37.5		0.96 (0.68–1.38)
	Genotype	AA	38.95	39.47		1.01 (0.72–1.43)
		AG	44.21	46.05	0.9287*	1.04 (0.77–1.41)
		GG	16.84	14.47		0.86 (0.45–1.64)
		Total	37/42/16 (AA/AG/GG)	90/105/33 (AA/AG/GG)	0.8594	
CETP	*rs3764261*		(*n* = 95)	(*n* = 228)		
	Allele	C	74.2	73.7	0.8445	1.00 (0.84–1.17)
		A	25.8	26.5		1.03 (0.64–1.64)
	Genotype	AA	5.26	10.53		2.00 (0.73–5.49)
		AC	41.05	32.03	0.1316*	0.78 (0.54–1.13)
		CC	53.68	57.46		1.07 (0.83–1.40)
		Total	5/39/51 (AA/AC/CC)	24/73/131 (AA/AC/CC)	0.1483	
SCARB1	*rs5888*		(*n* = 95)	(*n* = 228)		
	Allele	G	51.1	58.1	0.0994	1.14 (0.88–1.47)
		A	48.9	41.9		0.86 (0.63–1.16)
	Genotype	AA	24.21	18.42		0.76 (0.44–1.30)
		AG	49.47	46.93	0.1441*	0.95 (0.71–1.26)
		GG	26.32	34.65		1.32 (0.86–2.01)

Table 2. (Continued)

	SNP ID		Control %	AMD %	CT Vs AMD p-value	CT Vs AMD OR (95% CI)
		Total	23/47/25 (AA/AG/GG)	42/107/79 (AA/AG/GG)	0.2652	
APOE	*rs429358*		(n = 95)	(n = 228)		
	Allele	C	4.7	4.8	0.9621	1.02 (0.30–3.53)
		T	95.3	95.2		1.0 (0.94–1.07)
	Genotype	CC	0	0		NA
		CT	9.47	9.65	0.9643*	1.02 (0.43–2.40)
		TT	90.53	90.35		
		Total	0/9/86 (CC/CT/TT)	0/22/206 (CC/CT/TT)	0.999	
	rs7412		(n = 95)	(n = 228)		
	Allele	C	93.2	95.4	0.246	1.02 (0.96–1.10)
		T	6.8	4.6		0.68 (0.21–2.14)
	Genotype	CC	86.32	91.23		1.06 (0.96–1.17)
		CT	13.69	8.33	0.1841*	0.61 (0.27–1.37)
		TT	0	0.44		NA
		Total	82/13/0 (CC/CT/TT)	208/19/1 (CC/CT/TT)	0.2815	

The Bonferroni-corrected significance level for the allelic frequencies comparisons was 4.54×10^{-3} (0.05/11). Total indicate the general test of association in the 2- by-3 table of disease-by-genotype. The asterisk symbol (*) indicates the OR values and p-values derived from comparison of the genotypic frequencies under the recessive model (CC versus CT + TT at rs10468017, TT versus CT + CC at rs493258, GG versus AG + AA at rs12678919, CC versus CT + TT at rs1883025, CC versus CT + TT at rs1800555 and CC versus CG + GG at rs76157638, AA versus AG + GG at rs3112831, AA versus AC + CC at rs3764261, GG versus AG + AA at rs5888, TT versus CT + CC at rs429358 and CC versus CT + TT at rs7412). NA, the odds ratio was not available where the number of individuals with two copies of the risk allele was zero.
CI = confidence interval, n = number of subjects; OR = odds ratio.

IBS population ($p = 3.38 \times 10^{-5}$; OR = 1.24, 95% CI: 1.05–1.42), conferring 1.24-fold increased risk for AMD cases (recessive association model: TT versus CT + CC), being similar for each forms (dry AMD: $p = 1.67 \times 10^{-3}$; OR = 1.22, 95% CI: 1.06–1.40 and wet AMD ($p = 4 \times 10^{-4}$; OR = 1.23, 95% CI: 1.07–1.41).

The rest of the SNPs analysed in this study did not show significant differences between the IBS population and the AMD cases or Northern Spanish controls (Table S1). Regarding AMD forms, the allele frequencies of rs10468017 (*LIPC* gene) were significantly different in dry AMD cases as compared with IBS population ($p = 5.3 \times 10^{-3}$), with increased disease susceptibility of 1.25-fold (OR = 1.25, 95% CI: 1.06–1.47) for the allele C, losing the statistical significance after Bonferroni correction for multiple testing ($p < 4.5 \times 10^{-3}$). The frequency of the genotype CC was significantly higher in dry AMD than in the IBS population under recessive association model ($p = 8.8 \times 10^{-4}$; CC versus CT + TT), conferring this genotype approximately 1.52-fold increased risk for dry AMD (OR = 1.52, 95% CI: 1.20–1.93), whereas the genotypes CT and TT may protect from the disease (OR = 0.59, 95% CI: 0.38–0.91 and OR = 0.31, 95% CI: 0.11–0.92, respectively).

The logistic regression multivariate analysis (multivariate linear regression analysis and backwards stepwise regression analysis) confirmed that the SNP rs10468017 (at *LIPC* gene) was independently associated with the prevalence of AMD (data not shown). The eleven SNPs studied are not completely independent among themselves, since they are part of the same gene or they are located in nearby genes; thus, the logistic regression analysis detects collinearity among them, providing redundant information in the model.

Haplotype analysis and Linkage Disequilibrium

Pairwise linkage disequilibrium (LD) analysis of the studied SNPs identified only one linkage block, which included two SNPs (rs10468017 and rs493258) located at *LIPC* gene, being in strong LD (D‘ = 0.89). The haplotype analysis not inferred any haplotypes associated with AMD or its clinical forms (dry or wet) (data not shown).

Discussion

The present study examined the association of eleven SNPs located in genes related with the metabolism of lipids (*LIPC*: rs10468017 and rs493258; *LPL*: rs12678919; *ABCA1*: rs1883025; *ABCA4*: rs1800555, rs76157638 and rs3112831; *CETP*: rs3764261 and *SCARB1*: rs5888; and *APOE*: rs429358 and rs7412) in a Northern Spanish population with AMD, distinguishing dry and wet forms. Genome-wide association studies have identified lipid metabolism pathway genes associated with AMD (Chen et al. 2010b; Neale et al. 2010 and Colak et al. 2011), with few studies analysing them in the Spanish population. Although Asensio-Sanchez et al. (2006) and Brion et al. (2011) reported the genetic association of *APOE* and *ABCA4* with AMD in the Spanish population, the candidate genes *LIPC, LPL, ABCA1, CETP* and *SCARB1* remain unexplored.

Most of the subjects included in this study were from the northernmost regions of Spain (67.49%) and from other regions of the Northern half of the country (32.51%). The covariates age and sex were statistically different when comparing the recruited Northern Spanish AMD patients with controls (Table 1). Although age is a well-recognized risk factor for AMD, data on gender differences are conflicting (Rudnicka et al. 2012). Several studies have shown association of female gender with increased risk for AMD development (Age-Related Eye Disease

Table 3. *APOE* allele and genotype frequency distribution in the major isoforms ε2, ε3 and ε4

					CT Vs AMD		CT Vs Dry		CT Vs Wet		Dry Vs Wet	
APOE	Control %	AMD %	Dry AMD %	Wet AMD %	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)
Allele	(n = 95)	(n = 228)	(n = 73)	(n = 155)								
ε2	6.8	4.6	7.5	3.2	0.7973$^{\bullet}$	0.68 (0.21–2.14)	0.9632$^{\bullet}$	1.10 (0.41–3.00)	0.5021$^{\bullet}$	0.47 (0.13–1.73)	0.3838$^{\bullet}$	0.43 (0.12–1.53)
ε3	88.4	90.6	88.4	91.6		1.02 (0.93–1.13)		1.00 (0.90–1.11)		1.04 (0.94–1.14)		1.04 (0.94–1.14)
ε4	4.7	4.8	4.1	5.2		1.02 (0.29–3.53)		0.87 (0.24–3.18)		1.11 (0.33–3.73)		1.27 (0.36–4.49)
Genotype												
ε2ε2	0	0.44	0	0.65	0.1841*	0.64 (0.33–1.23)*	0.7987*	1.10 (0.52–2.31)*	0.0328*	0.42 (0.19–0.95)*	0.0211*	0.38 (0.17–0.89)*
ε2ε3	13.68	8.33	15.07	5.16								
ε2ε4	0	0	0	0	0.5178†	NA†	1†	NA†	0.4329†	NA†	0.4916†	NA†
ε3ε3	76.84	81.58	76.71	83.87								
ε3ε4	9.47	9.65	8.22	10.32	0.9643‡	1.02 (0.49–2.13)‡	0.267‡	1.59 (0.70–3.63)‡	0.8284‡	1.09 (0.50–2.37)‡	0.3007‡	0.68 (0.33–1.40)‡
ε4ε4	0	0	0	0								

The black point ($^{\bullet}$) indicates the general test of association in the 2- by-3 table of disease-by-allele. The symbols (*, † and ‡) indicate the p-values and OR values derived from comparison of the genotypic frequencies (ε2, ε3 or ε4 carrier, respectively) under a dominant model. NA, the odds ratio was not available where the number of individuals with two copies of the risk allele was zero.
CI = confidence interval; n = number of subjects; OR = odds ratio.

Study Research Group, 2000), while others have not observed gender differences (Smith et al. 2001). Specifically, in the studied Northern Spanish population, 67.5% of AMD patients and 54.7% of controls were females, showing female gender association with increased risk of the disease. Different risk factors may contribute to higher prevalence of AMD in women, including longer life expectancy, body mass index and hormone estrogen decline in postmenopausal women (Freeman et al. 2005; Snow et al. 2002), but these studies still require further evidences (Defay et al. 2004).

We studied the two genetic variants located along the *LIPC* gene, encoding hepatic triglyceride lipase that catalyses the hydrolysis of triglycerides and regulates HDL-cholesterol levels (Hasham & Pillarisetti 2006). The covariates age and sex were statistically different when comparing the recruited Northern Spanish AMD patients with controls, and to discard possible confounders effects among these demographic conditions and the studied SNPs, an additional multivariate logistic regression analysis was carried out. This analysis confirmed that the SNP rs10468017 was independently associated with the prevalence of AMD. The SNP rs10468017, placed in the promoter region, has been previously reported to be associated with protection of late AMD (allele T) in different Caucasian populations (Reynolds et al. 2010; Neale et al. 2010; Seddon et al. 2010; Yu et al. 2011a,b; Peter et al. 2011; Cipriani et al. 2012; Yu et al. 2012; and Liutkeviciene et al. 2019), but not associated in European ancestry (Sobrin et al. 2011), Chinese (Tian et al. 2012 and Zhang et al. 2013) or Indian populations (Rajendran et al. 2018). Although our results were consistent with the protection of the allele T in the dry form of AMD, the association was not significant after Bonferroni correction. Regarding *LIPC* rs493258, a protective effect of AMD of the allele T had been reported in Caucasians (Chen et al. 2010b; Neale et al. 2010; Peter et al. 2011 and Cipriani et al. 2012), but not in our North Spanish populations nor in Chinese individuals (Zhang et al. 2013). *LIPC* has been related to the accumulation of drusen and progression to advanced AMD (Yu et al. 2012), probably through the modulation of the lipid homeostasis (Lee et al. 2013).

The variant rs3764261 of *CETP* gene was not associated with AMD (either dry or wet AMD cases) in our Northern Spanish population. Conflicting results were reported to date, with several studies showing association (Chen et al. 2010b; Neale et al. 2010; Yu et al. 2011a, 2012 and Liu et al. 2014) or not (Fauser et al. 2011; Peter et al. 2011; Yu et al. 2011b; Cipriani et al. 2012; Zhang et al. 2013 and Meng et al. 2015) in Caucasians and Asian populations. The *CETP* gene encodes the cholesteryl ester transfer protein, involved in the transfer of triglycerides from very low-density lipoproteins (VLDL) to HDL, resulting in relatively triglyceride-enriched HDL and low-density lipoprotein species (LDL) (Chapman et al. 2010). Cholesteryl ester transfer protein (CETP) is involved in transport of oxidized lipids from the outer segments of the photoreceptors and other membranes to HDL-like protein particles, which then are internalized by the RPE and excreted into the circulation via ABCG1 transporters through BrM (Rodriguez & Larrayoz 2010). The oxidation products from the accumulation of oxidized lipids in the retina, due to a possible dysfunction of CETP and ABCG1, may initiate inflammation (Hollyfield et al. 2008) and abnormal angiogenesis (Shaw et al. 2012) contributing to the development of neovascular AMD.

The *LPL* gene, which encodes a protein involved in HDL metabolism with dual functions of triglyceride hydrolase and ligand/bridging factor for receptor-mediated lipoprotein uptake, has been widely studied with controversial and inconsistent results. The association between *LPL* rs12678919 polymorphism and AMD varies among different ethnic groups (Chen et al. 2010a; Neale et al. 2010; Fauser et al. 2011; Peter et al. 2011; Tian et al. 2012; Merle et al. 2013; Zhang et al. 2013 and Liutkeviciene et al. 2019), while in our Northern Spanish cohort we did not find significant differences when comparing AMD (including dry and wet) cases with controls. Moreover, we analysed the association of another traditional gene related with lipid metabolism, the *SCARB1* encoding a multi-ligand cell surface receptor that mediates selective cholesterol uptake and efflux (Acton et al. 1996 and Ji et al. 1997). The variant rs5888 of the *SCARB1* gene was reported to be associated with AMD in Caucasian populations (Zerbib et al. 2009 and Stanislovaitiene et al. 2017), but in the present work we found a lack of association in the Northern Spanish population.

We additionally studied two members of the family of (ATP)-binding cassette (ABC). The *ABCA1* gene encodes the ABC transporter A1 (ABCA1), a cholesterol efflux pump in the cellular lipid removal pathway (Cavelier et al. 2006; Liu & Tang 2012b) expressed in human retina and RPE (Tserentsoodol et al. 2006; Duncan et al. 2009; Zheng et al. 2012 and Storti et al. 2017). Although many authors have found an association between *ABCA1* rs1883025 and AMD (Chen et al. 2010b; Wang et al. 2016 and Rajendran et al. 2018), in the present study no statistical differences were found in the Northern Spanish cohort, in agreement with other reports in Caucasian (Neale et al. 2010; Fauser et al. 2011 and Peter et al. 2011) or Asian populations (Tian et al. 2012; Zhang et al. 2013 and Li et al. 2014). On the other hand, the *ABCA4* encodes a protein implicated in the clearance of all-trans-retinal aldehyde from photoreceptors completing the photo-cycle (Papermaster et al. 1982; Azarian & Travis 1997 and Molday et al. 2000), in which mutations are responsible for Stargardt macular dystrophy, related retinal degenerative diseases and may predispose to AMD (Allikmets 2000; Beharry et al. 2004; Ratnapriya & Chew 2013). Regarding the Spanish population, although Brion et al. (2011) reported an association between *ABCA4* rs3113831 and AMD, the analysis of this and other two genetic variants located along the *ABCA4* gene (rs1800555, rs76157638) in our Northern Spanish patients showed no association with AMD either dry or wet AMD cases.

Finally, the *APOE* gene have three common alleles (ε2, ε3 and ε4), which results from two polymorphisms (rs429358 and rs7412) and encodes the isoforms E2 (Cys112, Cys158), E3 (Cys112, Arg158) and E4 (Arg112, Arg158) with different biochemical and functional properties (Mahley & Rall 2000). The polymorphic APOE protein acts as a ligand for low-density lipoprotein transporting triglycerides to peripheral tissues and modulating cholesterol and other lipids homeostasis (Corbo & Scacchi 1999; Mahley & Rall 2000). Furthermore, APOE, the major apolipoprotein of the central nervous system and component of plasma and cerebrospinal fluid, has been shown to be a component of drusen and accumulated in the cytoplasm of overlaying RPE cells (Anderson et al. 2001). Former studies have analysed the association of *APOE* gene polymorphisms and AMD (Klaver et al. 1998; Souied et al. 1998; Pang et al. 2000; Schmidt et al. 2000, 2002; Simonelli et al. 2001; : Schultz et al. 2003; Baird et al. 2004; Zareparsi et al. 2004; Gotoh et al. 2004; Tikellis et al. 2007; Losonczy et al. 2011; Sun et al. 2011; Yu et al. 2011b; Adams et al. 2012 and Shen et al. 2015), highlighting that the ε4 allele decreased AMD risk in Caucasian populations (Klaver et al. 1998; Souied et al. 1998; Schmidt et al. 2000, 2002; Simonelli et al. 2001; Baird et al. 2004; Zareparsi et al. 2004; Tikellis et al. 2007 and Shen et al. 2015), with the exception of Schultz et al. 2003 work, while there was a lack of association in Asian cohorts (Pang et al. 2000; Gotoh et al. 2004 and Sun et al. 2011). A previous study in a Spanish cohort (Asensio-Sanchez et al. 2006) reported significant differences, being the ε4 allele associated with an increased risk of AMD. However, in the current study, we showed no association in the North Spanish population when comparing control and AMD cases (Table 3).

Interestingly, we found that the frequencies of ε2 carriers were significantly higher in controls than in wet AMD cases in the Northern Spanish cohort, describing, for the first time to our knowledge, a protective role of ε2 for wet AMD. The logistic regression multivariate analysis confirmed that the ε2 allele carriers show a decreased risk of AMD in our population. While carriers of the ε4 allele of the APOE gene have higher total and VLDL cholesterol levels than non-carriers (Rasmussen 2016), the systemic effects of the ε2 allele are not clear. The ε2 major isoform has less than 2% of normal LDL receptor binding activity, which may increase plasma levels of cholesterol and triglycerides (Weisgraber et al. 1982; Leduc et al. 2011). Considering that wet AMD cases have significant lower frequencies of ε2

carriers in our population, the systemic levels of lipoproteins may be lower compared to controls, contributing with additional factors to reduce the risk of macular disease. However, to confirm and understand the precise role of ε2 in AMD, further studies with larger samples are required, considering collectively systemic levels of proteins related to the lipid metabolism.

We additionally compared the frequency of the variants analysed in the present study with the IBS population included in the 1000 Genomes Project Consortium Phase 3 (Auton et al. 2015), commonly used as control in association studies (Table S1). The comparison with our control reported significant differences only in the SNP rs429358 located in the *APOE* gene indicating genetic singularities in our studied population, which reflect the importance of selecting a control group with the same ethnic origin. Finally, this study has several limitations, including the relatively small sample size and the low or null frequencies of some homozygous variants in subgroups, which reduced the statistical power limiting the evaluation of the effects in stratified analysis, specifically in the different forms of AMD. In addition, the potential for residual confounding effects from unknown or unmeasured parameters is not adjusted in this study, and therefore, replication in large AMD sample cohorts distinguishing for geographic origin within our country is warranted. Another important underlying limitation when studying genetics of the dry and wet forms of AMD, which is a challenge for any genotype study tackling with this disease, deals with the likelihood of an individual may onset wet AMD hereafter. To minimize this risk, the dry AMD patients included in the present study were classified considering whether geographic atrophy (i.e. the hallmark of late dry AMD) was present in any eye, absence of any signal of choroidal neovascularization was observed, and all recruited patients were periodically followed-up to monitor their clinical evolution until data analysis.

In conclusion, the association of eleven SNPs located in genes related with the metabolism of lipids with AMD showed the *APOE*-ε2 allele being protective to the wet form and also determined a slight trend towards protecting the allele T of rs10468017 (*LIPC* gene) in dry AMD cases. Overall, our results suggest that these variants represent a genetic protective factor for wet (*APOE*) or dry (*LIPC*) AMD in the Northern Spanish population.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Allelic and genotypic association analysis of dry and wet AMD patients and control subjects in Northern Spanish and IBS populations.

7. DISCUSIÓN

El presente trabajo de tesis doctoral aborda el estudio de factores de riesgo tradicionalmente ligados a trastornos cardiovasculares en patologías vasculares retinianas y el efecto del CoQ10 como agente terapéutico para el tratamiento de este tipo de patologías. Las enfermedades vasculares que afectan a la retina y/o al fascículo óptico son relativamente muy prevalentes, e.g. 0.5 a 2.0 % en OVRs (Laouri et al., 2011) y 0.1 % en NOIA (Lee et al., 2011), produciendo una importante pérdida visual en la mayor parte de los casos. Las OVRs y las NOIAs son la segunda causa de pérdida de visión de origen vascular, tras la retinopatía diabética, mientras que la DMAE es una de las principales causas de ceguera central irreversible en personas mayores de 60 años. Su origen es, en todos los casos, multifactorial, destacando la asociación de factores de riesgo vascular con relación directa en el desarrollo de estas patologías retinianas y/o del nervio óptico. En este trabajo se han realizado estudios de asociación génica en pacientes diagnosticados con OVR, NOIA o DMAE y controles sanos, y se ha llevado a cabo la evaluación del efecto neuroprotector del CoQ10 como agente terapéutico en el tratamiento de patologías retinianas y/o del nervio óptico causadas por trastornos vasculares. En los siguientes apartados se discuten los resultados más relevantes del presente trabajo de investigación.

7.1. Estudio de asociación de las variantes de alta frecuencia del gen *MTHFR* con OVR, en una población española

En el primer trabajo que forma parte de esta tesis doctoral se ha realizado un estudio de asociación de las variantes más comunes del gen *MTHFR*, C677T y A1298C, con OVR en una población española. La OVR es una enfermedad multifactorial que ocurre con mayor frecuencia en pacientes de edad avanzada. Existen dos tipos principales de OVR: la OVCR central y la obstrucción de rama venosa, aunque excepcionalmente la oclusión puede ser de hemirretina superior o inferior, comportándose como una OVCR (Sivaprasad et al., 2015).

Diversos factores de riesgo contribuyen a la patogénesis de la OVR, entre los que se incluyen el glaucoma de ángulo abierto, la hipertensión arterial, la diabetes, la hiperlipidemia, el tabaquismo y la aterosclerosis (Martinez et al., 2014). Sin embargo, los estudios de asociación llevados a cabo hasta la fecha de otros factores de riesgo como la trombofilia, la resistencia a la proteína C activada, la apena del sueño o la hiperhomocisteinemia, han proporcionado resultados contradictorios (Janssen et al., 2005). Las anomalías en la cascada de coagulación pueden tener un componente genético, identificándose varios polimorfismos en genes que codifican proteínas implicadas en el sistema de coagulación, como el Factor V Leiden, el Factor II (protrombina) o en el gen *MTHFR*, cuyas mutaciones son consideradas factores de riesgo protrombóticos (McGimpsey et al., 2009).

Los polimorfismos de un solo nucleótido (SNP) más comunes en el gen *MTHFR*, i.e., C677T y A1298C, afectan a la actividad del enzima MTHFR responsable de catalizar la conversión de 5,10-metilenetetrahidrofolato en 5-metiltetrahidrofolato, parte crítica del proceso de remetilación de la homocisteína en metionina. El SNP C677T, ubicado en el dominio catalítico del enzima MTHFR, da como resultado una proteína termolábil con actividad enzimática disminuida, lo que puede conducir a altos niveles de homocisteína en plasma principalmente en presencia de bajos niveles de folato (Frosst et al., 1995). Por otro lado, el polimorfismo A1298C también se ha asociado con la reducción de la actividad del enzima MTHFR, pero en menor medida que la mutación C677T (Weisberg et al., 1998).

En esta investigación, en la que se ha llevado a cabo un estudio de asociación de los polimorfismos C677T y A1298C del gen *MTHFR* con OVR en 358 casos y controles de una población española, se ha demostrado que la prevalencia de estos SNPs no es significativamente diferente al comparar ambos grupos. Sin embargo, el alelo de riesgo T del polimorfismo C677T se ha observado en el 60.65% de los pacientes con OVR y en el 59.10% de los sujetos control, mientras que el alelo de riesgo C del polimorfismo A1298C se ha encontrado en el 46.45% de los pacientes con OVR y en el 51.14% de los controles, responsables en ambos casos de la síntesis de enzimas MTHFR termolábiles. De este modo, debe destacarse que la prevalencia de los genotipos responsables de las isoformas termolábiles de MTHFR alcanza el 85.25% en los pacientes con OVR y el

88.07% en los sujetos control. Por tanto, más del 85% de la población analizada posee una actividad enzimática de MTHFR disminuida en comparación con la forma nativa, pudiendo contribuir a altos niveles de homocisteína en plasma. Sin embargo, los niveles de homocisteína en plasma no solo dependen de la actividad del enzima MTHFR, sino también de los niveles de folato, vitamina B12 y creatinina, entre otros (D'Angelo et al., 2000).

Por otro lado, los factores de riesgo clásicamente asociados con OVR, como la hipertensión, dislipidemia, diabetes mellitus, glaucoma y enfermedad renal (Hayreh et al., 2001), se encontraron con mayor frecuencia entre los pacientes con OVR que en los controles, aunque solo la hipertensión fue un factor de riesgo estadísticamente significativo. La diabetes se asoció significativamente con OVR central, aunque debido al bajo número de sujetos en este subgrupo resulta imprescindible confirmar esta observación en una población mayor.

Como se ha señalado, las variantes del gen *MTHFR* pueden desencadenar una disminución en la actividad del enzima sintetizado, contribuyendo, junto con factores de riesgo adicionales, al desarrollo de OVR (Frosst et al., 1995). Además de en la población analizada en este estudio, las variantes responsables de la forma termolábil de MTHFR son altamente frecuentes en poblaciones como la ibérica en España (91,59%), la caucásica de residentes de Utah con ascendencia del norte de Europa CEU (80,81%), poblaciones hispanas (77,91% en la peruana) y asiáticas (89,32% en la China). Sorprendentemente, esta tendencia no se mantiene en las poblaciones africanas donde la frecuencia de estas variantes se reduce notablemente, como es el caso de las poblaciones nigerianas (42,20%) y gambianas (30,97%), destacando la influencia racial o étnica en las frecuencias de genotipos del gen *MTHFR* (The 1000 Genomes Project, 2015). En este sentido, es probable que estas diferencias sean debidas a una ventaja selectiva en los embriones humanos en poblaciones con fortificación y suplementación con ácido fólico, frente a aquellos con deficiencia de folato. Sin embargo, por lo que respecta a este punto, no se han realizado suficientes estudios genético-poblacionales que aborden este aspecto (Thögersen et al., 2001).

Por tanto, no se puede descartar que los polimorfismos del gen *MTHFR* puedan ser un factor de riesgo para el desarrollo de OVR en pacientes con otras características específicas y/o factores de riesgo clínicos. Además de otras condiciones sistémicas, el análisis de los niveles séricos de homocisteína, folato o vitamina B12 podría contribuir a clasificar a los pacientes con OVR en subgrupos específicos en los que los polimorfismos del gen *MTHFR* pudieran tener un efecto sobre el inicio y la progresión de la enfermedad. Así mismo, considerando la importancia del *MTHFR* en el fenómeno de metilación a través del ciclo del folato y la metionina, podría postularse que este gen puede modular otros genes potenciando o disminuyendo la expresión de los mismos, e interviniendo en procesos tan importantes como la síntesis y reparación del ADN y ARN y la metilación de biomoléculas a través de la metionina, interviniendo en el control de la homeostasis celular.

7.2. Estudio de asociación de los polimorfismos del gen *MTHFR* con neuropatía óptica isquémica anterior no arterítica en una población española

En el segundo trabajo de esta tesis se ha estudiado la asociación de los polimorfismos C667T y A1298C del gen MTHFR con la NOIA no arterítica. La NOIA es debida a una interrupción o insuficiencia en el flujo sanguíneo que irriga la cabeza del nervio óptico. Su forma clínica más común es la no arterítica, presentándose como una pérdida aguda de visión central y/o periférica, unilateral e indolora y afectando por lo general a pacientes mayores de 50 años (Buono et al., 2002). Se trata de una enfermedad multifactorial a cuya patogénesis contribuyen factores de riesgo sistémicos como la diabetes, la hipotensión o hipertensión arterial, la hipercolesterolemia o trastornos cardiovasculares (Hayreh et al., 1994), así como factores de riesgo locales como la presión intraocular elevada o drusas en el disco óptico (Hayreh et al., 2001b).

Sin embargo, la asociación de factores de riesgo trombofílicos con NOIA no está exenta de controversia, ya que los resultados obtenidos hasta la fecha son contradictorios (Felekis et al., 2010). Diversos estudios han descrito una asociación entre la hiperhomocisteinemia y la prevalencia de NOIA, en los que una ingesta insuficiente de vitamina B12, vitamina B6 y ácido fólico, así como una predisposición genética,

contribuyen a niveles elevados de homocisteína en plasma (Biousse et al., 2000). Como se ha mencionado en el estudio anterior, las mutaciones en el gen *MTHFR* constituyen el principal factor de riesgo genético para la hiperhomocisteinemia.

En esta investigación se realizó el estudio de asociación de los polimorfismos C677T y A1298C del gen *MTHFR* con NOIA en una población española formada por 298 casos y controles, así como el análisis adicional de factores de riesgo cardiovascular. De todos los factores de riesgo clínicos analizados, solamente el historial de enfermedad cardíaca y cerebrovascular mostraron diferencias estadísticamente significativas entre pacientes y controles, lo que indica una mayor predisposición a la enfermedad ocular. Por el contrario, los alelos de riesgo de los polimorfismos del gen MTHFR no están asociados con una mayor prevalencia de NOIA, sin diferencias significativas entre las frecuencias de los genotipos responsables de las formas mutadas de *MTHFR* (88.73% en sujetos control y 95.74% en pacientes con NOIA). Estos resultados son consistentes con los publicados previamente en diferentes poblaciones de todo el mundo, aunque se han observado ciertas discrepancias en otras poblaciones, probablemente debidas a la gran dificultad en la selección adecuada de pacientes, ya al tratarse de una enfermedad multifactorial, factores muy diversos contribuyen a su aparición o desarrollo, lo que produce una gran heterogeneidad entre los grupos de pacientes seleccionados. (Giambene et al., 2009). Como se comentó anteriormente, la alta prevalencia de al menos una de las variantes de riesgo de *MTHFR* es una característica común de las poblaciones caucásicas, hispanas y asiáticas, al contrario de lo que se observa en poblaciones africanas.

Debe destacarse que, en la población española analizada, se ha identificado cierto efecto protector de la proteína de tipo salvaje, ya que el genotipo CC/AA que codifica el enzima MTHFR no mutado fue significativamente más frecuente en sujetos control (11.27%) que en pacientes con NOIA (4.26%), aunque el intervalo de confianza obtenido sugiere que estos resultados deben confirmarse en una población adicional. Por tanto, el estudio de factores de riesgo genético podría ayudar a dilucidar la patogénesis de la enfermedad en ciertos grupos de pacientes y determinar la idoneidad de la prueba genética complementaria para el manejo clínico, en combinación con el análisis de las concentraciones séricas de folato, vitamina B12 y otros aminoácidos.

7.3. Mejoría del campo visual de un caso de cuadrantanopsia homónima causada por un infarto del lóbulo occipital tras tratamiento con CoQ10

En el tercer trabajo de investigación se abordó un estudio retrospectivo de la evolución del campo visual de un paciente diagnosticado con cuadrantanopsia homónima causada por un accidente cerebrovascular y tratado con CoQ10 y vitaminas. Los defectos del campo visual son comunes después de un accidente cerebrovascular, con un impacto muy significativo sobre las actividades diarias y consecuente mayor dependencia y discapacidad (Gilhotra et al., 2002). Las lesiones que afectan a las vías nerviosas aferentes retroquiasmáticas generalmente producen una pérdida de campo visual homónima, que puede ser una hemianopsia o cuadrantanopsia según la ubicación de la lesión.

En este estudio se evaluó el campo visual de un paciente varón de 69 años de edad que sufrió en el año 2007 un accidente cerebrovascular en el lóbulo occipital derecho con la consecuente cuadrantanopsia homónima inferior izquierda, siendo esta la ubicación más común de la lesión (Zhang et al., 2006). El índice de campo visual tras el accidente cerebrovascular fue del 82% y 79% en los ojos derecho e izquierdo, respectivamente, por lo que fue prescrito con un tratamiento constituido por complejos vitamínicos y antioxidantes durante el periodo 2007 a 2010, sin observarse cambios o mejoría en el campo visual. A partir del año 2011 se trató por primera vez con CoQ10 (Active complex® Q10 Gold 100 mg), además de la suplementación con vitaminas y antioxidantes, observándose una inmediata y ligera mejoría del campo visual en ambos ojos. Posteriormente, en los sucesivos exámenes de seguimiento anuales el paciente experimentó una mejora exponencial en el campo visual, con tasas de progresión superiores al 2% anual, hasta la actualidad en la que el paciente ya no presenta ningún signo de cuadrantanopsia, con campo visual normal en ambos ojos (99% en el ojo derecho, 98% en el ojo izquierdo).

La mejora o restauración espontánea del campo visual puede ocurrir en las primeras semanas hasta unos pocos meses tras el accidente cerebrovascular, probablemente mediada por la eliminación del edema cerebral con la restitución concomitante del tejido no infartado circundante (Lane et al., 2008). La recuperación es variable,

dependiendo del grado de muerte neuronal así como la resolución de los efectos iniciales de la lesión aguda, estimándose en un 60% los pacientes que podrían experimentar una mejora espontánea, generalmente dentro del primer mes después de la lesión, con un período máximo de recuperación espontánea de 3 meses tras el infarto (Frolov et al., 2017). En nuestro caso, la mejora en el campo visual del paciente comenzó 4 años después de producirse la lesión, con una recuperación significativa a los 10 años del evento, siendo improbable que haya sido debido a una recuperación espontánea del campo visual.

El tratamiento posterior al accidente cerebrovascular incluye la identificación de la etiología del accidente cerebrovascular, la modificación de los factores de riesgo para prevenir las recurrencias y el inicio de una terapia de rehabilitación temprana e intensiva que permita resultados funcionales y mejore la discapacidad visual. Se recomienda ampliamente el empleo de suplementos nutricionales para el tratamiento de los defectos del campo visual, incluida la cuadrantanopsia, aunque hay una ausencia notable de tratamientos farmacológicos estándar o recomendaciones nutricionales ampliamente aceptadas. Los suplementos nutricionales que incluyen vitaminas (A, C, E y B) y antioxidantes se usan comúnmente, aunque las evidencias sobre su efectividad son escasas (Demmig-Adams y Adams, 2013). La CoQ10 se ha utilizado previamente para tratar los accidentes cerebrovasculares y algunas enfermedades que afectan a la retina, como DMAE o retinopatía diabética (Zhang et al., 2017), pero hasta la fecha no se han publicado sus efectos sobre la cuadrantanopsia homónima. El paciente estudiado fue tratado de 2007 a 2010 con suplementos vitamínicos, ácido fólico y antioxidantes, sin que se observaran cambios en el campo visual. Sin embargo, el tratamiento complementado con CoQ10 en dosis altas (100 mg) a partir de 2011 evidenció una recuperación del campo visual.

La CoQ10 es un cofactor esencial de la cadena transportadora de electrones que actúa manteniendo el potencial de membrana mitocondrial, contribuyendo a la síntesis de ATP, inhibiendo la generación de especies reactivas de oxígeno y protegiendo las células neuronales frente el estrés oxidativo (Littarru et al., 2010). Por tanto, la CoQ10 puede desempeñar un papel fundamental en el tratamiento de afecciones neurológicas, incluida la pérdida del campo visual después del accidente cerebrovascular. Sin

embargo, para confirmar el efecto beneficioso de la suplementación con coenzima Q10 resulta imprescindible realizar una interrupción del tratamiento como prueba de concepto que permita evaluar la reversibilidad de la recuperación del campo visual.

7.4. Vitaminas y CoQ10 para el tratamiento de oclusiones vascular que afectan a la retina y/o al nervio óptico

En el cuarto trabajo de esta tesis doctoral se describen los hallazgos clínicos y el manejo de una serie de casos de pacientes que presentan enfermedades vasculares que afectan a la retina y/o al nervio óptico, tratados con vitaminas y CoQ10, en diferentes periodos entre 2009 y 20019. De los 48 casos retrospectivos analizados se han incluido pacientes diagnosticados con NOIA, oclusión de la arteria retiniana (OAR), hemianopsia o cuadrantanopsia homónimas después de un accidente cerebrovascular y otras afecciones, incluida la atrofia del nervio óptico o la OVR, determinándose en todos ellos las tasas de progresión de campo visual tras la prescripción de una suplementación oral de CoQ10 (100 mg por día) y vitaminas.

Los defectos del campo visual son comunes después de oclusiones vasculares, derrames cerebrales, traumatismos, tumores, neurocirugías y enfermedades desmielinizantes, con efectos devastadores sobre la visión y consecuente dependencia y discapacidad. El pronóstico de estas enfermedades de origen vascular que afectan a la retina y/o al nervio óptico es adverso con una recuperación visual limitada (Prem Senthil et al., 2019). Los pacientes diagnosticados con NOIA o OAR pueden experimentar una mejoría espontánea de la agudeza visual durante las primeras semanas después del evento, aunque la mejora significativa en el campo visual parece ocurrir con menos frecuencia que la agudeza (Scherer et al., 2008). Como se ha mencionado en el trabajo anterior, los pacientes diagnosticados con hemianopsia podrían experimentar una mejora espontánea en el campo visual en los primeros 10 días después de la lesión cerebral, disminuyendo progresivamente con cada mes sucesivo, con menos del 10% de los pacientes recuperando completamente su campo visual (Frolov et al., 2017). Por lo tanto, la recuperación es variable, dependiendo del grado de muerte neuronal y la eliminación de los efectos iniciales de la lesión aguda (Pambakian et al., 2015). En nuestra serie de casos, tras la suplementación oral con CoQ10 y el tratamiento

combinado con vitaminas, la mejora en el campo visual de los pacientes comenzó a ocurrir en la mayoría de los casos más de 2 meses después de la lesión, con una recuperación significativa durante el seguimiento clínico, resultando improbable una recuperación espontánea de la visión.

En la mayoría de los 48 casos incluidos en este estudio se ha observado una mejoría significativa en el campo visual después del tratamiento, con una tasa de progresión promedio de +13±16 % por año. De todos ellos, los pacientes diagnosticados con OAR (n=7) experimentaron el mayor aumento de la tasa de progresión del índice de campo visual. Los pacientes fueron tratados con vitaminas y CoQ10 inmediatamente después del diagnóstico, aunque la recuperación de la visión se percibió durante períodos superiores a 4 meses, con una mejora significativa del campo visual manteniendo el tratamiento prescrito. Por otro lado, los pacientes diagnosticados con hemianopsia o cuadrantanopsia homónimas después de un accidente cerebrovascular (n=10) presentaron un incremento significativo del índice de campo visual en ambos ojos tras el tratamiento con el CoQ10 y vitaminas, no recibiendo terapias restauradoras, por lo que la mejoría en el campo visual observado no fue el resultado de la terapia de rehabilitación. Respecto a los casos que presentaron otras afecciones que afectan a la vascularización de la retina (n=13) se obtuvieron tasas de progresión del índice de campo visual promedio de +11±21%, con una recuperación total del campo visual en uno del los casos.

Los pacientes diagnosticados con NOIA (n=18) y tratados con CoQ10 mostraron tasas de progresión elevadas, similares a las de los casos de OAR. Uno de los casos (NAION09) presentó un índice de campo visual en el ojo izquierdo constante durante los 64 meses de seguimiento sin tratamiento, observándose que tras el tratamiento con CoQ10 a partir del mes 64 se alcanzó una tasa de progresión promedio de +2.3±2 % anual del campo visual, indicando una elevada efectividad de esta coenzima en la restauración de la visión. Muy llamativas han sido las observaciones realizadas en el caso NAION02, tratado con CoQ10 después del examen inicial (mes 0), posteriormente interrumpido al tercer mes y reiniciado el tratamiento al mes 10. Tras el tratamiento inicial, el paciente experimentó una recuperación del campo visual del 0% al 43% en el ojo izquierdo. Sin embargo, después de la interrupción del tratamiento se observó un deterioro en el

campo visual al 6%, mientras que tras la restauración del tratamiento con CoQ10 se recuperó en parte hasta alcanzar el 21% actual. Se ha demostrado, por tanto, que la suspensión del tratamiento de CoQ10 se traduce en un empeoramiento del campo visual, mientras que su prescripción implica una mejora significativa en el campo visual. Estas evidencias sustentan el papel del CoQ10 como agente terapéutico para el tratamiento de las enfermedades vasculares que afectan la retina y/o al nervio óptico. Además, dado que la interrupción del tratamiento con CoQ10 disminuye el campo visual, es necesario llevar a cabo estudios adicionales que permitan evaluar la reversibilidad o irreversibilidad de sus efectos.

7.5. El estudio de asociación de polimorfismos en genes del metabolismo de lípidos con la DMAE identifica un papel protector del alelo APOE-E2 para la forma húmeda en la población española

En el quinto de los trabajos de esta tesis se llevó a cabo un estudio de asociación de once polimorfismos en siete genes del metabolismo lipídico con la DMAE, en una población de pacientes del norte de España. La DMAE es la tercera causa de ceguera central irreversible en todo el mundo y la principal causa en personas mayores de 60 años en países desarrollados (Wong et al., 2014). Se distinguen dos formas clínicas de DMAE en sus etapas avanzadas, la forma seca caracterizada por una atrofia geográfica (85-90% de los casos) y la forma húmeda caracterizada por el desarrollo de neovascularización coroidea y cicatriz disciforme (10-15% de los casos) (Hyman y Neborsky, 2002). La DMAE es una enfermedad multifactorial, contribuyendo a su desarrollo factores de riesgo como la edad, el tabaquismo, la obesidad, el consumo de grasas y la predisposición genética. Se han identificado diversos genes asociados con la fisiopatología de la DMAE, incluidos los genes que codifican factores de complemento, la remodelación de la matriz extracelular y el metabolismo de lípidos (Katta et al., 2009).

Un metabolismo lipídico alterado contribuye a la acumulación de lipoproteínas sobre la membrana de Bruch, formando parte de la composición de las drusas y bloqueando el transporte fisiológico a las células del epitelio pigmentario de la retina y de los fotorreceptores, con la consecuente degeneración y muerte celular (Huang et al., 2007). En este trabajo de investigación se llevó a cabo el estudio de asociación génica

de once polimorfismos en genes relacionados con el metabolismo de lípidos, incluidos la apolipoproteína E (*APOE*), la lipasa de triacilglicerol hepática (*LIPC*), la lipoproteinlipasa (*LPL*), el gen codificante de una proteína transportadora ABC miembro 1 (*ABCA1*) y miembro 4 (*ABCA4*), el receptor scavenger clase B, tipo 1 (*SCARB1*), y la proteína de transferencia de éster de colesterol (*CETP*), mediante un estudio caso-control realizado en una población española formada por 95 sujetos control y 228 pacientes con DMAE (distinguiendo las formas seca y húmeda).

La mayoría de los sujetos incluidos en este estudio provienen de las regiones más al norte de España (67,49%) y de otras regiones de la mitad norte del país (32,51%). En esta población, las covariables edad y sexo fueron estadísticamente diferentes cuando se compararon los pacientes reclutados con DMAE con los controles. La edad es un factor de riesgo bien reconocido, mientras que las diferencias de género han sido sujeto de controversia por lo contradictorio de los resultados publicados (Rudnika et al., 2012). En la población estudiada existe una asociación de género femenino con un mayor riesgo de desarrollar DMAE, en línea con el estudio AREDS (Age-Related Eye Disease Study Research Group, 2000). Diferentes factores de riesgo pueden contribuir a una mayor prevalencia de la DMAE en las mujeres, incluida una mayor esperanza de vida, el índice de masa corporal y la disminución de estrógenos hormonales en mujeres posmenopáusicas (Freeman et al., 2005).

La comparación de las frecuencias de alelos y genotipos entre los pacientes con DMAE y los controles no proporcionó diferencias significativas para ninguno de los once polimorfismos estudiados. Del mismo modo, el estudio de asociación de los SNPs con cada una de las formas de DMAE, i.e., seca o húmeda, tampoco proporcionó diferencias significativas entre grupos para la mayoría de los polimorfismos. Sin embargo, el alelo T del SNP rs10468017 del gen *LIPC*, responsable de codificar la lipasa hepática que cataliza la hidrólisis de los triglicéridos y regula los niveles de colesterol, mostró frecuencias significativamente más bajas entre los pacientes con DMAE seca que en los sujetos control. Estudios previos han mostrado que este SNP se encuentra asociado con la protección frente a la DMAE tardía en diferentes poblaciones caucásicas (Liutkeviciene et al., 2019), pero no así en diversas poblaciones con ascendencia europea (Sobrin et al., 2011) o asiáticas (Zhang et al., 2013), entre otras. Este efecto

protector para la forma seca de la DMAE perdió la significancia estadística después de la corrección de Bonferroni para comparaciones múltiples, siendo necesario confirmar estos resultados en otras poblaciones con mayor número de individuos.

Mención especial recibe el gen *APOE*, cuyas frecuencias alélicas y genotípicas de las variantes rs429358 y rs7412 no fueron significativamente diferentes al comparar pacientes con DMAE frente a los sujetos control. No obstante, teniendo en cuenta que estos dos polimorfismos del gen *APOE* dan como resultado los tres alelos comunes ε2, ε3 y ε4 que codifican las isoformas E2 (Cys112, Cys158), E3 (Cys112, Arg158) y E4 (Arg112, Arg158) con diferentes propiedades bioquímicas y funcionales (Mahley & Rall, 2000), se llevó a cabo un estudio de asociación adicional. Este análisis detectó que la frecuencia de los portadores de ε2 era significativamente más alta en los controles que en los casos con DMAE húmeda en la cohorte del norte de España, describiendo por primera vez un efecto protector de ε2 para la forma neovascular de la DMAE. Los efectos sistémicos en los portadores del alelo ε2 no están claros, describiéndose que la isoforma principal ε2 tiene menos del 2% de la actividad normal de unión al receptor de las lipoproteínas de baja densidad (LDL), lo que puede aumentar los niveles plasmáticos de colesterol y triglicéridos (Leduc et al., 2011). Este hecho parece ser contradictorio considerando que durante la patogénesis de la DMAE se observa una acumulación de lipoproteínas en la membrana de Bruch. Teniendo en cuenta que la frecuencia de los portadores ε2 entre los pacientes con DMAE húmeda es significativamente más baja que los controles en nuestra población, los niveles sistémicos de lipoproteínas podrían también ser más bajos en comparación con el grupo control, por lo que factores adicionales pueden contribuir a reducir el riesgo de enfermedad macular en la población del norte de España.

8. CONCLUSIONES

El trabajo realizado en la presente Tesis Doctoral ha dado lugar, tras el análisis de los resultados, a una serie de conclusiones que pueden resumirse en los siguientes puntos:

1.- Las variantes T de C677T y C de A1298C del gen *MTHFR* no aumentaron significativamente el riesgo de sufrir oclusión venosa retiniana (OVR) en la población española estudiada y, por lo tanto, factores de riesgo adicionales probablemente modulados por el propio *MTHFR* están contribuyendo al inicio de la enfermedad.

2.- Ninguna variante de los polimorfismos de un solo nucleótido C677T y A1298C de *MTHFR* está asociados con una mayor prevalencia de la neuropatía óptica isquémica anterior (NOIA) en la población española estudiada.

3.- El genotipo CC / AA que codifica el enzima MTHFR 100% funcional mostró una frecuencia más alta en sujetos control que en pacientes con NOIA, indicando un posible efecto protector de la proteína de tipo silvestre.

4.- El análisis de las concentraciones séricas de homocisteína, ácido fólico y vitamina B12 podría ayudar a identificar subgrupos de pacientes con OVR o NOIA en los que la presencia de las variantes T de C677T y C de A1298C en *MTHFR* podrían ser un factor de riesgo significativo.

5.- La hipertensión se asoció con un mayor riesgo de padecer OVR y la historia clínica de enfermedades cardíacas o cerebrovasculares fue significativamente mayor en pacientes con NOIA, mientras que factores clínicos adicionales como diabetes mellitus, dislipidemia o glaucoma no se asociaron con un mayor riesgo de padecer OVR o NOIA en la población española estudiada.

6.- La población española estudiada (tanto controles como pacientes con OVR o NOIA), así como poblaciones caucásicas (CEU), hispanas (PEL) o asiáticas, presentan una alta frecuencia de las variantes T de C677T y C de A1298C del gen *MTHFR* responsables de una disminución de la actividad del enzima sintetizado, mientras que poblaciones negras africanas muestran una baja frecuencia de estas variantes, lo que demuestra la influencia racial/étnica en las frecuencias genotípicas de este gen.

7.- La falta de diferencias en la prevalencia de OVR entre las distintas razas contrasta con la menor frecuencia de las variantes de *MTHFR* observada en poblaciones negras africanas, en las que el estudio de estos polimorfismos junto con el de otros posibles factores de riesgo podría ser especialmente interesante.

8.- El tratamiento con coenzima Q10 (100 mg de CoQ10), combinado con vitaminas, mejoró significativamente el campo visual y el pronóstico de un paciente con accidente cerebrovascular del lóbulo occipital superior derecho y consecuente cuadrantanopsia homónima inferior izquierda, diagnosticado hace más de 10 años, hasta una recuperación casi completa en la actualidad.

9.- El tratamiento con CoQ10 ha demostrado su potencial como agente terapéutico con una mejoría significativa del campo visual en una serie de casos con distintos tipos de afectación de campo visual, causadas por trastornos vasculares a nivel de retina o nervio óptico con pérdida teórica irreversible del campo visual.

10.- La interrupción del tratamiento con CoQ10 en un paciente diagnosticado con NOIA produjo un empeoramiento en el campo visual, que se recuperó tras la restauración del tratamiento, cobrando más fuerza los efectos beneficiosos de este enzima.

11.- El estudio de asociación de polimorfismos de un solo nucleótido en genes del metabolismo de lípidos con la degeneración macular asociada a la edad (DMAE)

demostró un papel protector del alelo APOE-ε2 para la forma neovascular en la población española.

12.- La frecuencia significativamente más baja del alelo T del polimorfismo de un solo nucleótido rs10468017 del gen *LIPC* en los casos con DMAE seca con respecto a los controles sugiere un papel protector frente al desarrollo de esta forma de la enfermedad, en la población española.

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Anexo 1.

Curriculum vitae

Nombre **Beatriz Fernández-Vega Sanz**

Actualmente trabajo en el Instituto Oftalmológico Fernández-Vega como retinóloga neurooftalmóloga y subinvestigadora en diversos ensayos clínicos.

Títulos académicos:

1986	Licenciada en Medicina y Cirugía por la Universidad de Oviedo, España
1993	Especialidad Oftalmología por la Universidad de Catania , Italia.
	Número de colegiado: 333306616

Formación post- grado:

1993	Neovascularización subretiniana macular con tratamiento Interferon Alfa 2ª (Universidad de Catania, Italia).
1991-1993	Programa de doctorado de investigación en el departamento de Biología y Morfología celular de la Universidad de Oviedo: bases morfológicas y funcionales de la neurología clínica.
Miembro de :	Sociedad española de oftalmología.
	Sociedad de vítreo y retina.

Experiencia en investigación clínica:

- Phase III. Lucentis in AMD. Sustain (2005- 2006)- Sub Investigator
- Phase III. Posurdex RVO trial (2005- 2007) - Sub Investigator
- Phase III. FAME Study (2006- 2009) - Sub Investigator
- Phase III. Vegf- Trap Eye study 3111523 (View2) (2006- 2010) - Principal Investigator
- Phase III. Posurdex DME trial (2007- 2011) -Sub-Investigator
- Phase II. FOVEA (2011- 2012) - Sub- Investigator
- Phase III. Vegf- Trap Eye study VIVID - Principal Investigator
- Phase III. Ozurdex vs. Lucentis AGN 206207- 204 DME (2012- 2014) - Sub Investigator
- Phase IV Bayer. Ran Polaris Sub-investigador.

- Phase III. MAF-AGN- OPH-RET-004: Allergan COMO (2012-2015)- Sub-Investigator
- Phase III. Novartis OCTAVE- CRFB002A2405 (2013- 2015)- Sub- Investigator
- Phase III. Novartis TREND- CRFB002A411 (2013 – open) – Sub-Investigator
- Phase III. In-Eye (SERV) – CRFB002AES03T (2013- Sub- Investigator
- Phase III OPH1004 . Ophthotech – "Estudio de fase III, randomized, doble-masked, controlled trial to establish the safety and efficacy and intravitreous administration of FOVISTA administered in combination with either Avastin or Eylea compared to Avastin or Eylea monotherapy in subjects with subfoveal neovascular age-related macular degeneration Su- Investigator)
- Phase III . Alcon . Harrier .RTH258-C002 . (Sub- Investigator)

- Phase III Bayer. Aries . Managing neovascular age-related macular degeneration (nAMD) over 2 years with a treat and extend regimen of 2 mg intravitreal (IVT) aflibercept- a randomized, open-label, active-controlled, parallel-group phase IV/IIIb study. (Sub-Investigator)
- Phase IV. Bayer. Centera. A multicenter, single-arm, interventional phase 4 study to evaluate a treat and extend regimen of intravitreal aflibercept for treatment of macular edema secondary to central retinal vein occlusion. (Sub-Investigator)
- El ciclo redox Zinc- metalotioneína como diana terapéutica en el tratamiento de la DMAE. 1-01-2015 al 31-12-2015
- Myopred. Influence of posterior vitreous detachment on retinal detachment after lens surgery in myopic eyes. VIROS –Hanusch Hospital (ESCRS) (Sub-investigator
- Phase IV. Bayer. Centera. A multicenter, single-arm, interventional pahse 4 study to evaluate a treat and extend regimen of intravitreal aflibercept for treatment of macular edema secondary to central retinal vein occlusion
- A randomized, double-masked, Sham-controlled Phase 4 Estudy on the Efficacy, safety and tolerability of intravitreal Aflibercept Monotherapy compared to Aflibercept with Adjunctive Photodynamic Therapy in patients with Polypoidal Choroidal Vasculopathy
- OPT 302-1002 A dose-ranging study of intravitreal OPT-302 in combination with ranibizumab, compared with ranibizumab alone, in participants with neovascular age-related macular degeneration (wet AMD)
- SAPPHIRE. A randomized, masked controlled trial to study the safety and efficacy of suprachoroidal CLSTA in conjunction with intravitreal aflibercept in subjects with retinal vein occlusion.
- A multicenter, double-masked, randomized dose-ranging trial to evaluate the efficacy and safety of conbercept intravitreal injection in subjects with neovascular age related macular degeneration. Protocol nº: KHB-1801. Promotor: Chengdu Kanghong Biotechnology Co, Ltd
- RHINE. A phase 3, multicenter, randomized, double-masked, active comparator-controlled study to evaluate the efficacy and safety of RO6867461 in patients with Diabetic Macular Edema. Eudract nº: 2017- 005105-12. Promotor Hoffmann-La Roche Ltd
- A phase 3 multicenter, randomized double-masked, active comparator-controlled study to evaluate the efficacy and safety of faricimab in patients with neovascular age-related macular degeneration (Lucerne) Eudract nº 2018-004042-42.

Publicaciones Científicas

• B. Fernández-Vega, H. González-Iglesias, J.A. Vega, J. Nicieza, A. Fernández-Vega. (2019) Coenzyme Q10 treatment improved visual field after homonymous quadrantanopia caused by occipital lobe infarction. American Journal of Ophthalmology, Case Reports., 13: 70-75

• B. Fernández-Vega, L. Álvarez, M. García, E. Artime, A. González Fernández, C. Fernández-Vega, J. Nicieza, J.A. Vega, H. González-Iglesias H. (2019) Association study of high-frequency variants of MTHFR gene with retinal vein occlusion in a Spanish population. Ophthalmic Genet., 16:1-8.

• B. Fernández-Vega, M. García, L. Olivares, L. Álvarez, A. González-Fernández, E.

Artime, A. Fernández-Vega Cueto, T. Cobo, M. Coca-Prados, J.A. Vega, H. González Iglesias. The assocation study of lipid metabolism genes polymorphisms with AMD identifies a protective role for APO-E2 allele in the wet form of Spanish patients. Acta Ophthalmologica, 2019, doi: 10.1111/aos.14280

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• B. Fernandez-Vega, L. Álvarez, M. García, E. Artime, M. Diñeiro, J. Nicieza, J.A. Vega, H. Gonzalez-Iglesias. Association study of MTHFR polymorphisms with non-arteritic anterior ischemic optic neuropathy in a Spanish population. Biomedicine Hub, 2019, Aceptado

• M. García, L. Álvarez, Á. Fernández, H. González-Iglesias, J. Escribano, B. Fernández-Vega, E. Villota, L. Fernández-Vega Cueto, A. Fernández-Vega and M. Coca-Prados. (2017) Metallothionein polymorphisms in a Northern Spanish population with neovascular and dry forms of age-related macular degeneration. Ophthalmic Genetics. 38(5):451-458.

• B. Fernández-Vega, Á. Fernández-Vega, CM Rangel, J. Nicieza, E. Villota, JA Vega, R. Sanchez- Avila. (2016) Blockade of Tumor Necrosis Factor Alpha: A Role for Adalimumab in Neovascular Age‐Related Macular Degeneration Refractory to Anti-Angiogenesis Therapy? Case Rep Ophthalmol. 17;7(1):154‐62

• M. García, L. Álvarez, A. M Nogacka, H. González-Iglesias, J. Escribano, B. Fernández-Vega, A. Fernández-Vega, L. Fernández-Vega, M. Coca-Prados. (2015) CFH polymorphisms in a Northern Spanish population with neovascular and dry forms of Age-Related Macular Degeneration. Acta Ophthalmologica. 93 (8):e658 – e666. doi:10.1111/aos.12790

Congresos:

1988

Retinopatía multifocal en el Congreso de la Sociedad Española de Oftalmología.

1989

Ponencia sobre Dipripazol en el tratamiento del Glaucoma en el Congreso de la Sociedad Española de Oftalmología.

Ponencia sobre retinopatía del prematuro en el Congreso de la Sociedad Española de Oftalmología. Publicado en el Archivo de la Sociedad Española de Oftalmología en 1990, 58:529-532.

Extracción de cuerpos intraoculares mostrado a través de dispositivos audiovisuales en el Congreso de la Sociedad Española de Oftalmología.

Ponencia en Taormina, Italia, sobre nuestra experiencia clínica en el tratamiento del agujero macular .

Ponencia en la Universidad de Medicina de la Universidad de Oviedo sobre complicaciones y resultados de las lentes IOGEL usadas para pacientes diabéticos y pacientes con glaucoma.

1990

Publicación en los " anales de la Sociedad Ergoftalmológica española", 1990 19:279-276.

Nuestra experiencia para ROP , III stadio B Plus, en el congreso de la Sociedad Oftalmológica de Schepens.

1992

Ponencia en la Sociedad española de oftalmología sobre Hemorragia Coroidea Masiva después de una hernia de iris .

Sulla posibilita di modificare una bozza filtrante. Publicado en el Bolletino di Ocularistica. Anno 71, supp1º 1992, 259-270

Interferon: Nueva perspectiva en el tratamiento de la neovascularización subretiniana, primera experiencia.
Ponencia en la Sociedad Oftalmológica de Tosco-Umbro- Emiliano- Marchigiana.

1993

Ponencia en la Sociedad Oftalmológica de Sicilia sobre nuestra experiencia en el tratamiento en la neovascularización subretiniana.

2016

M. García, L. Álvarez, A. Fernández, H. González-Iglesias, J. Escribano, B. Fernández-Vega, A. Fernández-Vega, E. Villota, L. Fernández-Vega, M. Coca-Prados. Metallothionein polymorphisms in a Northern Spanish population with neovascular and dry forms of Age-Related Macular Degeneration (AMD). Poster Communication, EVER Congress, Niza (France), (October 2016). Abstract #T073

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Montserrat Garcia; Lydia Álvarez; Alicja Maria Nogacka; Hector Gonzalez-Iglesias; Julio Escribano; Beatriz Fernández-Vega; Alvaro Fernández-Vega; Luis Fernandez-Vega; Miguel Coca-Prados. Complement factor H polymorphisms associated with exudative or dry AMD in the Spanish population. Poster Communication, ARVO, Denver (CO, USA), (May 2015). Abstract # Invest. Ophthalmol. Vis. Sci.. 2015; 56(7):783

2019

Poster II Congreso Interdisciplinar de Genética Humana en Madrid . Coautora.
C2083: elevado rendimiento diagnóstico y utilidad clínica de un panel NGS para el diagnóstico de las distrofias retinianas hereditarias.

Anexo 2.

Publicaciones relacionadas con la tesis

García M, Álvarez L, Nogacka AM, González-Iglesias H, Escribano J, **Fernández-Vega B**, Fernández-Vega Á, Fernández-Vega L, Coca-Prados M. CFH polymorphisms in a Northern Spanish population with neovascular and dry forms of age-related macular degeneration.
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Fernández-Vega B, Fernández-Vega Á, Rangel CM2, Nicieza J, Villota-Deleu E, Vega JA, Sanchez-Avila RM.
Blockade of Tumor Necrosis Factor-Alpha: A Role for Adalimumab in Neovascular Age-Related Macular Degeneration Refractory to Anti-Angiogenesis Therapy?
Case Rep Ophthalmol. 2016; 7:154-62.
doi: 10.1159/000445102.

García M, Álvarez L, Fernández Á, González-Iglesias H, Escribano J, **Fernández-Vega B**, Villota E, Fernández-Vega Cueto L, Fernández-Vega Á, Coca-Prados M.
Metallothionein polymorphisms in a Northern Spanish population with neovascular and dry forms of age-related macular degeneration.
Ophthalmic Genet. 2017; 38:451-458.
doi: 10.1080/13816810.2017.1288825.

Acta Ophthalmologica

CFH polymorphisms in a Northern Spanish population with neovascular and dry forms of age-related macular degeneration

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ABSTRACT.

Purpose: **To elucidate the potential role of single-nucleotide polymorphisms (SNPs) in complement factor H (*CFH*) gene in Northern Spanish patients with age-related macular degeneration (AMD).**

Methods: **A case–control study of 130 unrelated native Northern Spanish diagnosed with AMD (46 dry, 35 neovascular and 49 mixed) and 96 healthy controls matched by age and ethnicity were enrolled. DNA was isolated from peripheral blood and genotyped for AMD-associated SNPs (rs3753394, rs529825, rs800292, rs3766404, rs203674, rs10671170, rs3753396 and rs1065489) using TaqMan probes and restriction fragment length polymorphism (RFLP). The association study was performed using the HaploView 4.0 software.**

Results: **The allelic frequency analysis revealed that rs529825, rs800292, rs203674 and rs10671170 were significantly associated with an increased risk for AMD. The haplotypes CGG (rs3753394, rs529825 and rs800292) and GCAG (rs203674, rs1061170, rs3753396 and rs1065489) were significantly associated with AMD while the haplotypes CAA (rs3753394, rs529825 and rs800292) and TTAG (rs203674, rs1061170, rs3753396 and rs1065489) were found to be protective. Small differences in allelic frequencies were found between dry and neovascular cases; however, these differences were not significant and did not distinguish one form the other.**

Conclusions: **This study found significant association of SNPs rs529825, rs800292, rs203674 and rs1061170 in the *CFH* gene with susceptibility to AMD. We identified haplotypes that confer protection or increased risk of AMD but not specific genetic variants in *CFH* capable to distinguish the different clinical forms of AMD in this cohort. Collectively, our results confirmed that *CFH* represents a strong genetic risk factor for this disease in the Northern Spanish population.**

Key words: age-related macular degeneration – *CFH* gene – dry AMD – genetic association – haplotypes – Neovascular – Northern Spanish population – single-nucleotide polymorphism

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Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in elderly population in developed countries (Klein et al. 2004; Pascolini et al. 2004), affecting 50 million individuals worldwide. AMD is a neurodegenerative disease characterized by a progressive loss of central vision with a multifactorial aetiology. Patients in early or intermediate stages of the disease do not lose central vision but instead have other impairments, such as limited vision at night, reduced light perception and difficulty in reading (Bhutto & Lutty 2012). Early AMD is characterized by the development of drusens between the retinal pigmented epithelium (RPE) and Bruch's membrane, and pigmentary abnormalities in the RPE (dry AMD). With the progression to an advanced stage, AMD is manifested by geography atrophy (GA) (late dry AMD) or the development of choroidal neovascularization (CNV) and subretinal neovascular fibrous tissue called disciform scar (wet, exudative or neovascular AMD).

Numerous population-based studies have been conducted and have provided information on the incidence and prevalence of AMD. For example, the Beaver Dam Eye Study (DNES), based on the population in Wisconsin (USA), found that the incidence of early AMD, increased from 3.9% in

individuals aged 43–54 years to 22.8% in persons 75 years and older. In this later group, the incidence and prevalence rates of late AMD (CNV or GA) were 5.4% and 7.1%, respectively (Klein et al. 1992). In the Australian population, the incidence of AMD is 6.3% in people at 80 years and older (Mukesh et al. 2004). The Blue Mountain Eyes Study (BMES) has revealed that in the Australian population, end-stage AMD was present in 1.9% of the Caucasian population, increasing from 0% among people younger than 55 years of age to 18.5% among those 85 years of age or older (Mitchell et al. 1995) being similar the prevalence of early and late AMD in Asian Malay population (Kawasaki et al. 2008).

In the US population (The National Health and Nutrition Examination Survey), the total prevalence of any AMD in people age 40 years or older was 6.5% (7.2 million people), and more than 800 000 patients were estimated to have the late stage of AMD (GA or CNV) (Klein et al. 2011). The Baltimore Eye Survey revealed that bilateral blindness was higher in whites (30%) than in African Americans (0%) (Sommer et al. 1991). Other studies have found that the incidence of AMD among the African American population is less frequent than among the Caucasian population (Schachat et al. 1995; Leske et al. 2004). The Los Angeles Latino Eye Study found that Hispanics have a lower prevalence of advanced AMD than non-Hispanics but a relatively high rate of early AMD (Varma et al. 2004).

Nowadays, the pathogenesis of AMD remains poorly understood. It is generally accepted that AMD is a multifactorial disease where age, smoking (Khan et al. 2006; Cackett et al. 2011; Kabasawa et al. 2011), obesity and dietary fat consumption (Seddon et al. 2003a,b, 2006) and the presence of rare single-nucleotide polymorphisms (SNPs) may contribute to the development of the disease (Katta et al. 2009; Chen et al. 2010; Liu et al. 2012; Fritsche et al. 2013). Studies on SNPs in genes coding for complement factors (i.e. *CFH*, *CFI*, *CFB*) and complement components (i.e. *C2* and *C3*) had implicated the complement system in the pathophysiology of intermediate and advanced AMD examined and demonstrated that a common variant in the complement factor H (*CFH*) gene was strongly associated with AMD (Klein et al. 2005; Sobrin et al. 2012). The *CFH* variant rs1061170 (Y402H) and the risk of developing AMD have been extensively studied in populations worldwide, including Asia (Kondo et al. 2011), North India (Sharma et al. 2013), Tunis (Yücel et al. 2012), Central Europe (Wegscheider et al. 2007), France (Zerbib et al. 2013), Hungary (Losonczy et al. 2011), Poland (Teper et al. 2012), Brazil (Teixeira et al. 2010), Mexico (Buentello-Volante et al. 2012) and Africa (Ziskind et al. 2008) confirming that the C allele of the *CFH* Y402H variant has been strongly associated with this pathology with the notable exception of the Japanese people (Gotoh et al. 2006; Okamoto et al. 2006). Among the most studied SNPs of the *CFH* gene associated with AMD are rs3753394, rs529825, rs800292, rs3766404, rs203674, rs3753396 and rs1065489. These SNPs are located in important functional domains of the *CFH* gene including coding region. Hageman et al. (2005) determined the frequencies of these polymorphisms in AMD patients of European American descent and identified several protective and risk haplotypes for AMD.

Single-nucleotide polymorphisms of the *CFH* gene have been examined in AMD patients in many populations around the world including Spain (Brión et al. 2011; Martínez-Barricarte et al. 2012; Cruz-González et al. 2013). This study was designed (i) to investigate the association of the most relevant SNPs described to date in the *CFH* gene (rs3753394, rs529825, rs800292, rs3766404, rs203674, rs1061170, rs3753396 and rs1065489) with AMD cases with either, the dry form, the wet form or both, from the Northern region of Spain; (ii) to identify risk and protective haplotypes among these AMD cases; and (iii) to compare our results with earlier studies carried out with a Spanish multicentre group of AMD cases (Brión et al. 2011).

Materials and Methods

Study subjects

This case–control study included 130 unrelated native Spanish patients diagnosed with AMD and 96 healthy controls who were recruited at the Instituto Oftalmológico Fernández-Vega (Asturias, Spain). Complete ophthalmic examinations were performed for both patients and controls, including slitlamp biomicroscopy and funduscopy in both eyes. AMD-diagnosed patients were further examined by fluorescence fundus angiography, indocyanine green angiography or optical coherence tomography. Individuals were identified as follows: dry AMD with evidence of GA in any eye; wet AMD with evidence of CNV in any eye, mixed AMD subjects with CNV and GA in any eye (in the same eye or in the contralateral one). Control subjects were selected from patients undergoing cataract surgery and the absence of AMD or glaucoma. Subjects with other relevant ocular pathologies such as retinopathies or maculopathies were excluded from this study. To avoid possible misclassification, considering that AMD is a late-onset disorder, only people aged 60 or above were recruited as controls. The number of subjects, gender and the mean and range of ages in each group (AMD with dry form, AMD with wet form, AMD with dry and wet forms and controls) is shown in Table 1.

The study adheres to the tenets of the Declaration of Helsinki on Biomedical Research Involving Human Subjects, and was approved by the Clinical Research Ethics Committee at the Hospital Universitario Central de Asturias (Oviedo, Spain). All participants signed an informed consent.

Table 1. Demographic characteristics of AMD patients and controls.

Study population (*n*)	Age (mean ± SD)	Age Range	Gender (female/male)
Controls (96)	73.3 ± 8.03	60–92	53 (55.2%)/43
AMD (130)	77.7 ± 7.84	52–99	79 (60.8%)/51
Dry AMD (46)	77.0 ± 9.20	52–99	28 (60.9%)/18
Wet AMD (35)	77.3 ± 6.55	65–91	24 (68.6%)/11
Mixed AMD (49)	78.7 ± 7.33	64–93	27 (55.1%)/22

n = number of subjects; SD = standard deviation; AMD, age-related macular degeneration.

Genotyping

Peripheral blood was collected in 6-mL K2E K2EDTA tubes coated with EDTA, which blocks the coagulation cascade (Vacuette, Madrid, Spain). Tubes were stored at −20°C until use. Genomic DNA was obtained from the blood samples of all studied subjects using a commercial DNA extraction kit (FlexiGene DNA Kit; Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Allelic discrimination was performed with TaqMan probes provided by the manufacturer with ABI assays (Applied Biosystems Inc., Foster City, CA, USA) (C_2530387_10 [rs3753394], C_2250476_10 [rs529825], C_2530382_10 [rs800292], C_11890065_10 [rs3766404], C_2530311_10 [rs203674], C_2530296_10 [rs3753396] and C_2530274_1_ [rs1065489]) and in the 7500 real-time PCR system (Applied Biosystems Inc.). All PCR amplifications were performed with the thermal cycling conditions of 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min.

Genotyping of the SNP rs10611170 was performed by generating restriction fragment length polymorphism (RPFL). First of all, an amplicon of 497 pb long (forward: 5′-AGAGTTG TTCAAGCAAAGTGACC-3′, reverse: 5′-GGGAGTAGGAGACCAGCCAT-3′) containing the SNP was generated by PCR amplification consisting on an initial denaturation at 94°C/4 min, followed by 35 cycles (94°C/30 s, 62°C/30 s and 72°C/60 s), and a final extension at 72°C/10 min. The PCR products were separated by agarose gel electrophoresis. The enzyme NIaIII (Thermo Fisher Scientific Inc, Waltham, MA, USA) was used for digestion of the 497-bp amplicon containing the intronic SNP rs1061170. Digestion of DNA fragments with the allele T at this position resulted two fragments of 469 and 28 bp, whereas fragments containing the allele C resulted three fragments of 384, 85 and 28 bp. When both alleles, T and C, were present, digestion of DNA fragments resulted four fragments (469, 384, 85 and 28 bp). Fragments resulted from each digestion reaction were separated on 2% agarose gels and stained with ethidium bromide. The genotyping results were confirmed in a random subgroup of our samples using direct DNA sequencing.

Statistical analysis

All the SNPs were assessed for Hardy–Weinberg equilibrium (HWE) by a χ^2 test in both groups with HaploView 4.0 software (Daly Lab, Broad Institute, Cambridge, MA, USA). The comparison of the SNPs allelic frequencies between the AMD and control groups was performed using a standard χ^2 test, with a p-value of $<6.25 \times 10^{-3}$ (0.05/8) being considered as statistically different (Bonferroni method was used for the adjustment of multiple comparisons). Additionally, we used SIGMAPLOT v11 software (http://www.sigmaplot.com/) to run a logistic regression analysis to control for potential confounders.

The comparison of genotypic frequencies between the AMD and control groups was performed using a χ^2 test (Pearson correction) with SPSS version 15.0 (IBM Copr., Armonk, NY, USA). Relative risk association was estimated by calculating odds ratios (OR) along with 95% confidence intervals (CI) using the methods described in Armitage et al. (2002) and PLINK (v1.07) as described by Purcell et al. (2007). Linkage disequilibrium (LD) plot was generated with HAPLOVIEW 4.0 software (Daly Lab, Broad Institute), and blocks were defined by Gabriel et al. (2002) algorithm. Individual haplotypes and their estimated population frequencies were performed using HAPLOVIEW 4.0 software (Daly Lab, Broad Institute) with all of the parameters set at the default values. Haplotype association analysis were performed using a standard χ^2 test with a p-value < 0.05 considered statistically significant with HAPLOVIEW 4.0 software (Daly Lab, Broad Institute).

Results

The demographic and clinical characteristics of the three AMD subgroups (i.e. dry, wet and mixed) totalling 130 Spanish AMD cases and 96 healthy control subjects, recruited in this study are shown in Table 1.

Association study of *CFH* SNPs

The allelic frequencies for each of the eight sequence variants analysed (rs3753394, rs529825, rs800292, rs3766404, rs203674, s1061170, rs3753396 and rs1065489) in all the AMD cases ($n = 130$) and controls ($n = 96$) are shown in Table 2.

Allele frequencies of rs529825, rs800292, rs203674 and rs1061170 were significantly different in AMD cases when compared to controls. Increased disease susceptibility ranged from approximately 1.19 times for the alleles G of SNPs rs800292 and rs529825 to 1.65 times for the allele C of SNP rs1061170

The allele C of SNP rs1061170 (Y402H) showed the most significant association with AMD ($p = 2.07 \times 10^{-6}$; OR = 1.65, 95% CI: 1.21–2.26). This association was similar in both forms of AMD (dry: $p = 4.00 \times 10^{-4}$; OR = 1.64, 95% CI: 1.13–2.39; wet: $p = 6.70 \times 10^{-3}$; OR = 1.54, 95% CI: 1.01–2.33; see Supporting Information).

The G allele of SNP rs203674 (IVS10) was detected in a statistically significant higher frequency in patients with AMD than in controls ($p = 1.96 \times 10^{-6}$), with AMD patients being 1.61-fold more likely to have a G allele than a T allele (OR = 1.61, 95% CI: 1.20–2.17).

The G allele of SNP rs529825 (IVS1) and the G allele of rs800292 (I62V) also showed a strong association with AMD ($p = 1.00 \times 10^{-4}$; OR = 1.19, 95% CI: 1.04–1.37; $p = 2.00 \times 10^{-4}$; OR = 1.19, 95% CI: 1.04–1.36, respectively). All these associations remained significant after the Bonferroni correction for multiple testing ($p < 6.25 \times 10^{-3}$).

The allelic frequencies of the SNPs rs3766404, rs3753394, rs3753396 and rs1065489 in AMD cases were not significantly different from controls ($p = 1.2 \times 10^{-2}$, $p = 0.9678$; $p = 0.6771$ and $p = 0.8275$). The G allele of the SNP rs203674 showed association with the dry form of AMD ($p = 3.00 \times 10^{-4}$; OR = 1.61, 95% CI: 1.14–2.30; Supporting Information) conferring 1.61-fold increased risk for dry AMD, but not with the exudative form ($p = 6.60 \times 10^{-3}$; OR = 1.50, 95% CI: 1.01–2.23; Supporting Information).

The significant differences found in the allelic frequencies of the SNPs analyzed between dry AMD and wet AMD cases when compared to controls, could not distinguish between the two clinical forms of AMD (dry or wet) (Supporting Information).

The observed genotype frequencies of the eight *CFH* SNPs were in HWE

Table 2. Allelic and genotypic association analysis.

SNP ID		AMD % (n = 130)	Control % (n = 96)	p-value	OR (95% CI)
rs3753394					
Allele	C	69.6	69.8		1.00 (0.84–1.19)
	T	30.4	30.2	0.9678	1.01 (0.67–1.50)
Genotype	CC	49.23	48.96	0.9643	1.00 (0.77–1.31)*
	CT	40.77	41.67		0.98 (0.71–1.34)
	TT	10	9.37		1.07 (0.48–2.39)
	Total	64/53/13 (CC/CT/TT)	47/40/9 (CC/CT/TT)	0.9831	
rs529825					
Allele	G	87.7	73.4	1.00×10^{-4}	1.19 (1.04–1.37)
	A	12.3	26.6		0.46 (0.26–0.81)
Genotype	GG	76.92	54.17	3.14×10^{-4}	1.42 (1.15–1.77)*
	GA	21.54	38.54		0.56 (0.37–0.84)
	AA	1.54	7.29		0.21 (0.04–0.99)
	Total	100/28/2 (GG/GA/AA)	52/37/7 (GG/GA/AA)	7.49×10^{-4}	
rs800292					
Allele	G	87.3	73.4	2.00×10^{-4}	1.19 (1.04–1.36)
	A	12.7	26.6		0.48 (0.27–0.84)
Genotype	GG	76.15	55.21	9.11×10^{-4}	1.38 (1.12–1.70)*
	GA	22.31	36.46		0.61 (0.40–0.93)
	AA	1.54	8.33		0.18 (0.04–0.85)
	Total	99/29/2 (GG/GA/AA)	53/35/8 (GG/GA/AA)	1.31×10^{-3}	
rs3766404					
Allele	T	93.8	87	1.2×10^{-2}	1.08 (0.99–1.18)
	C	6.2	13		0.48 (0.20–1.11)
Genotype	TT	87.7	75	1.35×10^{-2}	1.17 (1.03–1.33)*
	TC	12.3	23.96		0.51 (0.29–0.92)
	CC	0	1.04		NA
	Total	114/16/0 (TT/TC/CC)	72/23/1 (TT/TC/CC)	3.37×10^{-2}	
rs203674					
Allele	G	59.6	37	1.96×10^{-6}	1.61 (1.20–2.17)
	T	40.4	63		0.64 (0.49–0.83)
Genotype	GG	33.08	14.58	1.55×10^{-3}	2.27 (1.32–3.90)*
	GT	53.08	44.79		1.18 (0.90–1.56)
	TT	13.84	40.63		0.34 (0.21–0.56)
	Total	43/69/18 (GG/GT/TT)	14/43/39 (GG/GT/TT)	6.29×10^{-6}	
rs1061170					
Allele	C	56.9	34.4	2.07×10^{-6}	1.65 (1.21–2.26)
	T	43.1	65.6		0.66 (0.51–0.84)
Genotype	CC	31.54	11.46	3.92×10^{-4}	2.75 (1.49–5.07)*
	CT	50.77	45.83		1.11 (0.84–1.46)
	TT	17.69	42.71		0.41 (0.27–0.64)
	Total	41/66/23 (CC/CT/TT)	11/44/41 (CC/CT/TT)	1.54×10^{-5}	
rs3753396					
Allele	A	79.2	77.6	0.6771	1.02 (0.89–1.17)
	G	20.8	22.4		0.93 (0.56–1.53)
Genotype	AA	60	62.5	0.7034	0.96 (0.78–1.18)*
	AG	38.46	30.21		1.27 (0.88–1.85)
	GG	1.54	7.29		0.21 (0.04–0.99)
	Total	78/50/2 (AA/AG/GG)	60/29/7 (AA/AG/GG)	5.72×10^{-2}	
rs1065489					
Allele	G	78.5	77.6	0.8275	1.01 (0.88–1.16)
	T	21.5	22.4		0.96 (0.58–1.58)
Genotype	GG	58.46	62.5	0.5397	0.93 (0.76–1.16)*
	GT	40	30.21		1.32 (0.91–1.92)
	TT	1.54	7.29		0.21 (0.04–0.99)
	Total	76/52/2 (GG/GT/TT)	60/29/7 (GG/GT/TT)	4.47×10^{-2}	

n = number of subjects; OR = odds ratio; CI = confidence interval; GA = geography atrophy; SNP = single-nucleotide polymorphisms; AMD, age-related macular degeneration.

The Bonferroni-corrected significance level for the allelic frequencies comparisons was 6.25×10^{-3} (0.05/8). Total indicates that the significance of the association was determined by a 2-by-3 table (disease-by genotype) using the χ^2 test (Pearson correction). The asterisk indicate the OR values and p values derived from comparison of the genotypic frequencies under the recessive model (CC versus CT+TT at rs3753394, GG versus GA+AA at rs529825, GG versus GA+AA at rs800292, TT versus TC+CC at rs3766404, TT versus GT+GG at rs203674, TT versus CT+CC at rs1061170, AA versus AG+GG at rs3753396 and GG versus GT+TT at rs1065489). NA, the odds ratio was not available where the number of individuals with two copies of the risk allele was zero.

($p > 0.01$) in both AMD and control groups (see Table 2). Statistically significant differences were observed between AMD subjects and controls when the genotypic frequencies for each of the five SNPs with significantly increased allelic frequency in cases (rs529825, rs800292, rs203674 and rs1061170) were compared.

The frequency of genotype GG at SNP rs203674 was significantly higher in AMD than in control under recessive association model ($p = 1.55 \times 10^{-3}$; GG versus GT+TT) conferring approximately twofold increased risk for AMD (OR = 2.27, 95% CI: 1.32–3.90) whereas the genotype TT could protect from the disease (OR = 0.34, 95% CI: 0.21–0.56). Similar results were obtained under dominant association model ($p = 4.60 \times 10^{-6}$; GG+GT versus TT).

Genotype CC at SNP rs1061170 was strongly associated with AMD ($p = 3.92 \times 10^{-4}$, CC versus CT+TT) conferring more that twofold increased risk (OR = 2.75, 95% CI: 1.49–5.07), whereas the genotype TT appeared to be protective for AMD (OR = 0.41, 95% CI: 0.27–0.64). These significant differences had been observed in the dry form of AMD ($p = 5.46 \times 10^{-3}$, CC versus CT+TT) conferring more than twofold increased risk (OR = 2.66, 95% CI: 1.31–5.39) for the CC genotype while the TT genotype seemed to be protective from the disease (OR = 0.41, 95% CI: 0.21–0.80); see Supporting Information.

Genotype GG at SNP rs800292 was significantly elevated in AMD ($p = 9.11 \times 10^{-4}$ GG versus GA+AA; OR = 1.38, 95% CI: 1.12–1.70) conferring risk even if the genotype GA and AA could protect from the disease (OR = 0.61, 95% CI: 0.4–0.93 and OR = 0.18, 95% CI: 0.04–0.85, respectively).

The frequency of genotype GG at SNP rs529825 was also associated with AMD ($p = 3.14 \times 10^{-4}$, GG versus GA+AA) conferring 1.42-fold increased risk to the disease (OR = 1.42, 95% CI: 1.15–1.77) being similar for the dry form of the disease ($p = 5.64 \times 10^{-3}$, OR = 1.45, 95% CI: 1.14–1.83; Supporting Information).

The genotypes frequencies of SNP rs3753394, rs3766404, rs3753396 and rs1065489 did not differ significantly between groups ($p = 0.9643$, $p = 1.35 \times 10^{-2}$, $p = 0.7034$ and $p = 0.5397$, respectively; Table 2), suggesting that these SNPs are not associated with AMD.

The logistic regression multivariate analysis (multivariate linear regression analysis and backwards stepwise regression analysis) indicated that the covariates sex and age are not predictors of disease in an individual and only rs529825, rs800292 and rs1065489 appear to be essential in this model. The eight SNPs studied are not completely independent among themselves, as they are part of the same gene; thus, the logistic regression analysis detects collinearity among them, providing redundant information in the model.

Haplotype analysis and linkage disequilibrium

Pairwise LD analysis identified two blocks (21 and 25 kb) (Fig. 1). The block 1 (21 kb) included three SNPs (rs3753394, rs529825 and rs800292) that were in strong LD, as observed by the D' value. The SNP rs3753394 was in complete LD with rs529825 and rs800292 (coefficient of LD [D'] = 1.00), while rs800292 was also in strong LD with rs529825 ($D' = 0.98$). The block 2 (25 kb) was defined by four SNPs (rs203674, rs1061170, rs3753396 and rs1065489). This analysis showed that rs1065489, rs3753396 and rs1061170 were in complete LD ($D' = 1.00$). Strong LD was found between SNPs pairs rs203674 and rs1061170, rs203674 and rs375396 and between rs203674 and rs1065489 ($D' = 0.98$, 0.96 and 0.97, respectively).

The haplotype analysis identified three of the nine theoretically possible haplotypes formed by three SNPs (rs3753394, rs529825 and rs800292) and four of the sixteen possible haplotypes formed by four SNPs (rs203674, rs1061170, rs3753396 and rs1065489). The frequency of these haplotypes and their association with AMD are shown in Table 3. The CGG haplotype, consisting of two risk alleles (rs529825 and rs800292), is significantly associated with AMD ($p = 4.00 \times 10^{-3}$) and confers 1.3-fold increased risk (OR = 1.32, 95% CI: 1.00–1.73). The

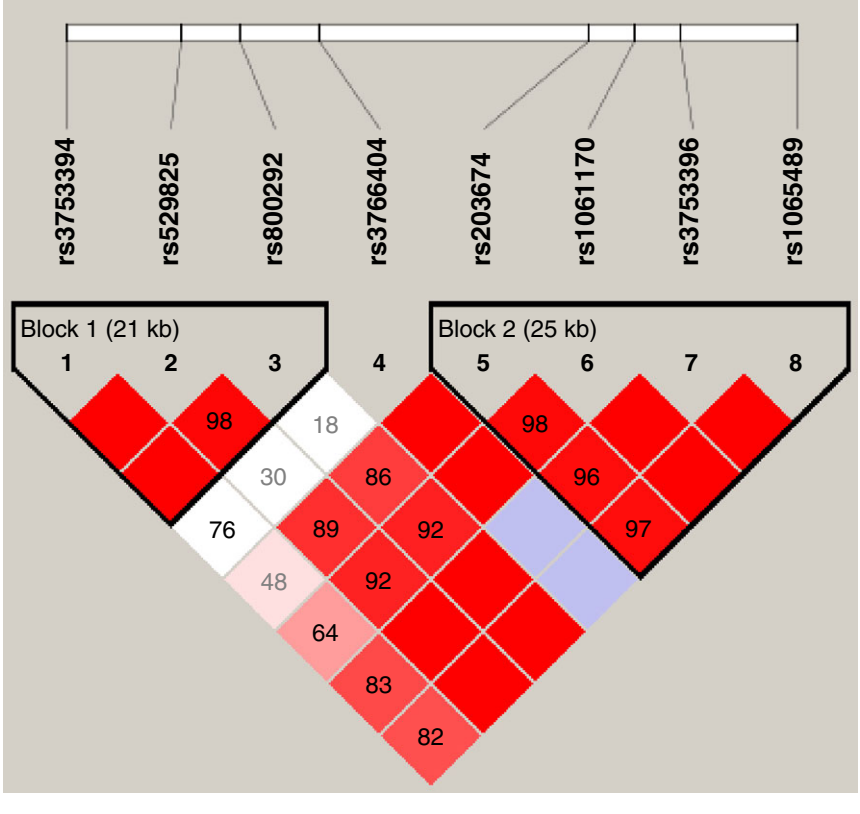

Fig. 1. Complement factor H (CFH) linkage disequilibrium (LD) plot. LD plot of the single-nucleotide polymorphisms rs3753394, rs529825, rs800292, rs3766404, rs203674, rs1061170, rs3753396 and rs1065489 of CFH. The number in the diamond refers to D' ($100 \times D'$). The LD block was defined according to the standard confidence intervals. The strength of LD is depicted by red intensity, which moves from white to light red as D' progresses from 0 to 100.

Table 3. Haplotype analysis for *CFH* SNPs.

SNPs alleles			Haplotype frequency		Association test between AMD and controls	
rs3753394	rs529825	rs800292	AMD $n = 130$	Control $n = 96$	p-Value	OR (95% CI)
C	G	G	0.569	0.432	4.0×10^{-3}	1.32 (1.00–1.73)
T	G	G	0.304	0.297	0.8730	1.02 (0.68–1.53)
C	A	A	0.123	0.260	2.00×10^{-4}	0.47 (0.27–0.84)
rs203674	rs1061170	rs3753396	rs1065489			
G	C	A	G	0.569 0.333	6.74×10^{-7}	1.71 (1.24–2.35)
T	T	A	G	0.189 0.402	6.24×10^{-7}	0.47 (0.31–0.72)
T	T	G	T	0.207 0.217	0.7833	0.95 (0.57–1.60)
G	T	A	G	0.026 0.030	0.7977	0.87 (0.18–4.08)

n = number of subjects; SNP = single-nucleotide polymorphisms; AMD, age-related macular degeneration.
Individual p values and odds ratios (OR) between age-related macular degeneration and control are provided for each of the haplotypes compared with all the other haplotypes.

CAA haplotype, containing two protective alleles (rs529825 and rs800292), is significantly associated with AMD ($p = 2.00 \times 10^{-4}$) conferring protection against the disease (OR = 0.47, 95% CI: 0.27–0.84). The GCAG haplotype, containing two risk alleles (rs203674 and rs1061170), is a risk haplotype ($p = 6.74 \times 10^{-7}$, OR = 1.71, 95% CI: 1.24–2.35) that confers more than 1.5-fold increased risk. The TTAG haplotype, which is composed of the protective alleles of rs203674 and rs1061170, was significantly associated with AMD ($p = 6.24 \times 10^{-7}$) and appeared as a protective haplotype of AMD (OR = 0.47, 95% CI: 0.31–0.72). The rest of haplotypes shown in Table 3 not presented an association with AMD.

Discussion

The present study describes the association of genetic variants in the *CFH* gene with AMD in a Northern Spanish population. In this analysis, we compared AMD cases in three clinical subgroups: subjects with the dry form only, subjects with the wet form only and subjects with both forms (mixed). When the allelic and genotypic frequencies of all the SNPs (rs3753394, rs529825, rs800292, rs3766404, rs203674, rs10671170, rs3753396 and rs1065489) were analysed, we confirmed that four of them (rs529825, rs800292, rs203674, rs1061170) exhibited the strongest association with AMD in this cohort (Northern Spanish population) when compared to healthy controls. However, no significant differences were found between the three clinical subgroups of AMD cases, that is dry, neovascular and mixed. These results were confirmed after using the Bonferroni correction.

In recent years, a large body of information suggested that there are significant differences between SNPs of the *CFH* gene and their association with AMD cases from different ethnic groups. For example, the C allele of the SNP rs1061170 or the G allele of rs203674 has been found strongly associated with AMD in Caucasian (Hageman et al. 2005; Francis et al. 2007; Spencer et al. 2007) and in Spanish cases (Brión et al. 2011; Martínez-Barricarte et al. 2012), but they lack significant association with Japanese and Chinese populations, respectively (Ng et al. 2008). Our studies, in the present Spanish population enriched for a Northern cohort including AMD patients from the regions of Galicia, Asturias and Basque Country, are in agreement with studies carried out with other Caucasian populations where the SNP rs1061170 carrying the *CFH* variant (Y402H) conferred similar risk of developing soft drusen in both forms of advanced AMD (i.e. GA or neovascular AMD) (Magnusson et al. 2006; Sepp et al. 2006).

In contrast, the T variant of the SNP rs3753394 that has been associated to AMD cases in Asian populations, especially in the wet form (Kim et al. 2008; Xu et al. 2008; Dong et al. 2011), does not show association with AMD cases in Caucasian populations (Hageman et al. 2005; Spencer et al. 2007), including the present study. Finally, there are other polymorphisms such as rs800292 that has been found associated with AMD cases in many ethnic groups including Caucasian (Hageman et al. 2005; Li et al. 2006; Francis et al. 2007; Spencer et al. 2007), Chinese (Ng et al. 2008; Yang et al. 2010; Tian et al. 2012; Wu et al. 2013; Huang et al. 2014; Liu et al. 2014; ; Zhuang et al. 2014), Japanese (Goto et al. 2009; Mori et al. 2010; Arakawa et al. 2011; Tanaka et al. 2011), Korean (Kim et al. 2008, 2013) and Spanish (Brión et al. 2011; Martínez-Barricarte et al. 2012; Caire et al. 2014; and present study).

To explore further whether the SNPs frequencies of the *CFH* gene associated with AMD exhibited significant differences between the dry and wet forms, we compared their frequencies among AMD cases from the Northern Spanish population. With the exception of the study reported by Brión et al. (2011) that is a multicentric study which comprises AMD patients from all the entire Spanish population, not other studies have compared their allelic forms between the distinct clinical forms of AMD. Our study shows some new SNPs differences in the northern cohort associated with AMD (rs203674), but no differences were found in the frequencies of any the SNPs studied between the dry and wet forms of AMD in subjects exhibiting either one or the other forms, or both (Supporting Information). This is in contrast with earlier reports that showed, for example, contradicting results on whether the C variant of SNP rs1061170 in the *CFH* gene exhibited significant association differences between the dry and wet forms. For instance, it has been reported that in the Caucasian population, the C variant of rs1061170 was associated with the exudative form of the disease (Souied et al. 2005; Weger et al. 2007; Wegscheider et al. 2007; Baatz et al. 2009; Ricci et al. 2009), whereas, in the Spanish population, the C variant of rs1061170 was associated with the dry form (Caire et al. 2014). Similarly, in Chinese cohorts, there are studies where this C variant has been shown associated with the exudative form of AMD (Lau et al. 2006; Dong et al. 2011), whereas in other studies, it has shown not association (Chen et al. 2006; Xu et al. 2008).

Interestingly, the T allele of the SNP rs3753394 has been associated with the

exudative form of AMD in all Chinese populations so far studied (Chen et al. 2006; Ng et al. 2008; Liu et al. 2010; Dong et al. 2011). This allele has not been reported to be significantly associated with AMD in Caucasian populations (Hageman et al.2005; Spencer et al. 2007). The G allele of the SNPs rs800292 has been shown associated with both clinical forms of AMD (dry or exudative) in Chinese (Chen et al. 2006; Ng et al. 2008; Yang et al. 2010; Huang et al. 2014; Liu et al. 2014; Zhuang et al. 2014), Japanese (Mori et al.2007; Goto et al. 2009; Arakawa et al. 2011; Tanaka et al. 2011) and Korean (Kim et al. 2008, 2013) cohorts.

In contrast, within the Spanish population, two studies reported contradictory results; Caire et al. (2014) suggested that the G allele of SNPs rs800292 is associated with the dry form, whereas Bri´on et al. (2011) suggested it is associated with the wet form. In our present study, the G allele of SNPs rs800292 was found not associated with either form of AMD.

Finally, the G allele of rs3753396 has been associated with the exudative form of AMD in a Chinese cohort (OR 1.60 95% CI: 1.16–2.20) (Ng et al. 2008), while in others studies (Tian et al. 2012) or in others cohorts (Caucasian), G allele of rs3753396 had not been found association with AMD (Spencer et al. 2007).

The haplotype analysis indicated that carriers of the haplotypes of CFH, Crs3753394 Grs529825 Grs800292 and Grs203674 Crs1061170 Ars375396 Grs1065489 were associated with increased risk of AMD while the two haplotypes Crs3753394 Ars529825 Ars800292 and Trs203674 Trs1061170 Ars3753396 Grs1065489 were identified as protective for AMD. Earlier work by Hageman et al. (2005) identified three haplotypes for AMD, one conferring risk, named H1 (Crs3753394 Crs529825 Grs800292 Trs3766404 Trs203674 Crs1061170 Ars3753396 Grs1065489) and two protective named H2 (Crs3753394 Trs529825 Ars800292 Trs3766404 Grs203674 Trs1061170 Ars3753396 Grs1065489) and H4 (Crs3753394 Crs529825 Grs800292 Trs3766404 Grs203674 Trs1061170 Ars3753396 Grs1065489. The haplotype Trs3753394 Grs529825 Grs800292 which not present significant differences associated with AMD is similar than the risked haplotype Grs800292 and Grs203674 Crs1061170 in the G alleles of rs203674 and rs1061170 but differs in the T allele of rs800292 which is not associated with AMD in the present study. This observation may suggest that these three variants are not disease-causing, and they could be in LD with other CFH variant(s) responsible for disease.

In conclusion, this study has demonstrated a positive correlation between the polymorphisms rs529825, rs800292, rs203674 and rs1061170 in the *CFH* gene and the susceptibility of AMD in the Northern Spanish population. We also controlled potential confounding variables in the analysis. Furthermore, we identified four haplotypes with significant association with AMD, two associated with disease susceptibility and two that seemed to be protective. We did not find any specific polymorphism for the different forms of AMD in this cohort. Collectively, our results confirmed the strong association between *CFH* polymorphisms and AMD, suggesting that *CFH* represents a strong genetic risk factor for this disease in the Northern Spanish population.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Allelic and genotypic association analysis.

Case Reports in Ophthalmology

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Blockade of Tumor Necrosis Factor-Alpha: A Role for Adalimumab in Neovascular Age-Related Macular Degeneration Refractory to Anti-Angiogenesis Therapy?

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Key Words
Anti-vascular endothelial growth factor · Tumor necrosis factor-alpha inhibitors · Wet age-related macular degeneration

Abstract
Aims: To report a case of wet age-related macular degeneration (wet-AMD) refractory to intravitreal anti-vascular endothelial growth factor (anti-VEGF) therapy in a patient who showed visual and anatomical improvement and stabilization after starting a subcutaneous treatment course with adalimumab, an anti-tumor necrosis factor-alpha (TNF-α) drug, for concomitant Crohn's disease. ***Methods:*** Observational case report of a female patient. Ophthalmological evaluation was performed by slit lamp and ophthalmoscopy (posterior pole and anterior segment). Best-corrected visual acuity (BCVA) was determined, and imaging was performed by fluorescein angiography, indocyanine green angiography, and optical coherence tomography (OCT). Intravitreal therapies used and treatment with anti-TNF-α were recorded. ***Results:*** A 64-year-old woman with wet-AMD was treated with fourteen intravitreal injections of ranibizumab (0.5 mg) for a period of 40 months with intervals of 1–6 months.

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She initially showed a good visual and anatomical response to periodic anti-VEGF treatment but during check visits, anatomical and functional responses deteriorated. At the 40-month follow-up, the patient had developed Crohn's disease, and her rheumatologist started treatment with adalimumab (40 mg subcutaneously every 2 weeks). During the 25 months of treatment with adalimumab, the patient did not require any additional intravitreal anti-VEGF treatments because her BCVA, clinical, and OCT findings improved and remained stable. ***Conclusions:*** We described a case of a patient with wet-AMD refractory to anti-VEGF therapy, which clinically benefited from subcutaneous adalimumab therapy. Treatment with subcutaneous anti-TNF-α in combination with anti-VEGF therapy avoids the high cost and risks related to multiple intravitreal anti-VEGF injections with good functional and anatomic outcomes.

Introduction

Age-related macular degeneration (AMD) is the principal cause of central visual loss in individuals over 55 years [1]. In neovascular AMD with choroidal neovascularization (CNV), damage to the outer retinal cells and retinal pigment epithelium (RPE) elicits a cascade of inflammatory and angiogenic responses that lead to neovascularization under the macula [2]. Vascular endothelial growth factors (VEGFs) are the most specific and crucial regulators of angiogenesis [3]. Ranibizumab, a high-affinity recombinant antigen-binding fragment (Fab) that neutralizes all isoforms of VEGF-A, blocks VEGF-A-induced angiogenesis, has been approved for intravitreal therapy for the treatment of neovascular AMD [4]. However, intravitreal therapies can bring rare but serious risks, such as endophthalmitis, vascular occlusions, crystalline lens injury, and increased intraocular pressure among others [5]. Tumor necrosis factor-alpha (TNF-α) is the prototypical member of a family of cytokines that also include FasL, CD40L, and TRAIL. These cytokines are involved in proapoptosis, proinflammatory responses, differentiation, and cell activation [6]. The concentration of TNF-α is elevated in several rheumatic diseases, such as rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, and psoriatic arthritis [7]. TNF-α seems to participate in the pathogenesis of multiple types of uveitis [8]. Inflammatory cytokines, including interleukin 1 beta, interferon gamma, and TNF-α, increase the secretion of VEGF in RPE cells and choroidal fibroblasts [9]. TNF-α receptors are expressed in many cell types in the retina and choroid plexus, including the RPE, Müller cells, and choroidal vascular cells [2, 10, 11]. Systemic and intravitreal injections of anti-TNF-α administered alone or in combination with anti-VEGF drugs have been used to treat multiple eye diseases such as uveitis [12–14], neovascular AMD [15–19], diabetic macular edema (DME) [20–23], cystoid macular edema due to cataract surgery [22, 23], branch retinal vein occlusion [23], and central retinal vein occlusion (CRVO) [23]. The clinical results of these applications have been variable. Markomichelakis et al. [15] reported three cases of regression of CNV secondary to AMD in patients receiving systemic treatment with infliximab, an anti-TNF-α drug for inflammatory arthritis. All 3 patients had an increase in visual acuity (VA) after treatment. This sets a precedent for the use of anti-TNF-α in these types of ophthalmic patients. Here, we describe a patient with neovascular AMD who required multiple intravitreal injections of ranibizumab to maintain a precarious clinical stability, but for whom there was clear deterioration during 40 months of follow-up. However, after administration of subcutaneous adalimumab (Humira®) injections for concurrent Crohn's disease, her vision and the clinical appearance of her neovascular AMD improved significantly during the next 25 months.

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Case Report

In August 2008, a 64-year-old woman with no known systemic diseases visited the Fernandez-Vega Ophthalmological Institute, Oviedo, Spain, with metamorphopsia and a decrease of central vision in her left eye that had occurred over the preceding year. The best-corrected visual acuity (BCVA) was 0.3 in the affected eye, and slit-lamp examination showed no alteration in the anterior segment. The intraocular pressure was 16 mm Hg in both eyes. Dilated fundus examination showed subretinal yellowish deposits and drusen in the macular area in both eyes. The deposits were associated with pigmentary changes in her left eye (fig. 1a, left). Fluorescein angiography showed no significant alterations in the right eye, but in the left eye, there was early macular hyperfluorescence that increased in the last stages of the angiography (fig. 1a, middle). Indocyanine green angiography showed central hyperfluorescence throughout the examination (fig. 1a, right). Optical coherence tomography (OCT) of the right eye demonstrated only subfoveal drusen and some RPE disruption, whereas OCT of the left eye showed a macular central thickness of 237 μm and intraretinal and subretinal fluid associated with shallow RPE detachment and subfoveal deposits and drusen (fig. 1b). The patient was diagnosed with dry AMD in her right eye and wet-AMD in her left eye. We started treatment on the left eye with intravitreal injections of ranibizumab (0.5 mg once a month for 3 months). Subsequent booster injections (0.5 mg) were given according to the ophthalmological findings in follow-up visits in a pro re nata treatment modality. The right eye was evaluated at each visit to assess the stability of the dry AMD. After the initial doses of ranibizumab (August–October 2008) in her left eye, the patient experienced clinical improvement with the disappearance of the subretinal fluid (fig. 2), and her BVCA improved to 0.9. However, during the following 40 months, she experienced decompensation of the neovascular AMD (fig. 2b, d, f), requiring rescue with eleven additional injections of ranibizumab (0.5 mg intravitreal) with intervals of 1–6 months. Improvement was maintained with BVCA ranging between 0.4 and 0.8. We decided not to switch to another antiangiogenic drug such as bevacizumab or aflibercept throughout the monitoring period because we believed that with ranibizumab the clinical response was quite good. In December 2011, the patient was diagnosed to have Crohn's disease, and began treatment with adalimumab (Humira®) (40 mg subcutaneously every 2 weeks) ordered by her rheumatologist (Hospital Universitario Central de Asturias, Oviedo, Spain), which is the standard treatment for this disease. For the next 25 months after the initiation of adalimumab treatment, the patient did not require any new injections of ranibizumab to maintain vision and clinical stability. Vision in her left eye maintained a VA of 0.7 from the first month after initiation of therapy with adalimumab, and no neovascular AMD activity was apparent (fig. 3a–c). The patient only required an annual booster injection of ranibizumab in 2014 and 2015 to maintain clinical stability associated with adalimumab.

Discussion

TNF-α has been implicated in the pathogenesis of inflammatory, edematous, neovascular, and neurodegenerative diseases of the eye [24]. Several anti-TNF-α drugs are currently available for the therapeutic management of these conditions, including infliximab (Remicade®), adalimumab (Humira®), etanercept (Enbrel®), certolizumab pegol (Cimzia®), and golimumab (Simponi®) [6]. Since introduction, anti-TNF-α drugs have been used by ophthalmologists to treat several ocular diseases 'off-label', i.e., out of the indications approved by a regulatory agency but which can be used in the context of compassionate therapy [6]

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(see Appendix 1). The anti-inflammatory effects of TNF-α inhibitors make these agents an obvious therapeutic alternative for treating noninfectious ocular inflammation, especially when conventional therapy has failed or carries the risk of adverse effects with chronic use [12–14]. One study reported positive outcomes without any ocular adverse events after intravitreal administration of infliximab in patients with chronic persistent noninfectious uveitis [13]. Another study reported that a single intravitreal injection of 1.0 mg/0.05 ml of infliximab controlled intraocular inflammation among patients with relapsing posterior uveitis associated with Behçet's disease [14]. Considering the inflammatory process underlying the development of AMD, anti-TNF-α therapy could potentially offer benefits for the treatment of this disease. In the present case, we believe that the observed BVCA and anatomical improvements can be attributed to subcutaneous systemic adalimumab administration as an anti-VEGF therapy adjuvant. This conclusion is supported by the fact that the patient did not attain optimal responses to the prior intravitreal ranibizumab injections, even after 14 doses. Several case studies have described treatment of patients with neovascular AMD using intravitreal anti-TNF-α therapy, albeit with variable outcomes [15–19]. However, to date, there have been no published studies of subcutaneous administration of adalimumab in patients with neovascular AMD. One study reported regression of three cases of CNV secondary to AMD in patients receiving intravenous administration of infliximab for inflammatory arthritis [15]. All three cases had an increase in VA. Arias et al. [16] conducted a prospective interventional trial of 4 patients with neovascular AMD that was refractory to anti-VEGF agents. Intravitreal infliximab was administered at a dosage of 2 mg but produced no visual or anatomical benefit. Furthermore, 2 of the patients in that study developed a severe intraocular inflammatory reaction. On the other hand, Theodossiadis et al. [18] described 3 patients with improved VA after treatment with intravitreal infliximab for neovascular AMD. Giganti et al. [19] used low-dose intravitreal infliximab (0.5 mg/0.05 ml) to treat 2 patients with neovascular AMD and 2 patients with DME. Pretreatment of all 4 patients included anti-VEGF therapy, and/or laser coagulation, and/or photodynamic therapy. The VA in only 1 patient with neovascular AMD improved. The other patients showed decreases in VA and developed intraocular inflammation. Wu et al. [20] conducted a multicenter and retrospective study in patients with DME. They treated 39 eyes with either intravitreal adalimumab (2 mg/0.1 ml) or intravitreal infliximab (1 mg/0.05 ml or 2 mg/0.1 ml). All patients had received at least three injections of VEGF inhibitors prior to receiving the TNF-α inhibitor. VA did not increase significantly in either the adalimumab or the 1-mg infliximab group after 3 months. In the 2-mg infliximab group, VA actually deteriorated. Macular thickness decreased in both infliximab groups but was unchanged in the adalimumab group. In the 2-mg infliximab group, 42% of patients developed severe uveitis that resolved with medical therapy and surgery. Sfikakis et al. [21] used intravenous infliximab to treat 11 patients with AMD that was refractory to laser treatment. They observed marked VA improvement in 8 eyes. A descriptive study found that in seven cases of refractory pseudophakic cystoid macular edema, a single intravitreal injection of infliximab (1.0 mg/0.10 ml) produced improvement of VA after 6 months [22]. In all cases, there was no intraocular inflammation. Several studies have investigated the potential role of TNF-α in the pathogenesis of eye diseases. Yoshimura et al. [25] collected vitreous specimens from the eyes of patients with diabetic retinopathy, CRVO, rhegmatogenous retinal detachment, and DME. They did not find elevated vitreous concentrations of TNF-α in any of these specimens. Suzuki et al. [26] analyzed the expression of cytokines in vitreous fluid and found a higher concentration of TNF-α in patients with CRVO than in patients with diabetic retinopathy. These studies do not support the hypothesis that TNF-α plays a major role in primary noninflammatory retinal diseases [25, 26]; however, the choroid plexus or retina themselves might be the locus of increased TNF-α concentration,

rather than the vitreous body [6]. In view of these studies [20–22], it is not surprising that the results obtained following intravitreal administration of TNF-α inhibitors are so heterogeneous [6]. Intravitreal injection of infliximab could be effective for severe intraocular inflammation, but it does not appear to benefit patients with refractory AMD or partially responsive neovascular AMD. Nevertheless, infliximab can be of some benefit in cases of persistent noninfectious posterior uveitis and refractory pseudophakic cystoid macular edema [27]. Intravitreal injections of adalimumab do not appear to benefit patients with dry AMD or neovascular AMD. Intravitreal administration of adalimumab and bevacizumab in combination might be effective in the management of patients with partially responsive neovascular AMD and macular edema of various etiologies [27]. Nevertheless, further preclinical and clinical studies are necessary to obtain firm conclusions regarding the use of intravitreal anti-TNF-α drugs with anti-VEGF drugs in the management of retinal diseases. To our knowledge, this is the first report of recurrent wet-AMD that improved following subcutaneous administration of an anti-TNF-α drug, adalimumab, for the treatment of an associated systemic rheumatic disease. These findings are very important because the use of this drug could be beneficial for the treatment of neovascular AMD in selected patients, while avoiding both the high cost and the risks related to repeated intravitreal injections of anti-VEGF agents. Future clinical studies could determine if patients with wet-AMD but without rheumatic diseases would benefit from subcutaneous adalimumab anti-VEGF in association with intravitreal anti-VEGF.

Statement of Ethics

This study was done according to the Declaration of Helsinki after written patient consent.

Disclosure Statement

There is no financial support from any organization. None of the authors has any financial/conflicting interests to disclose.

Appendix 1

Guideline on compassionate use of medicinal products, pursuant to Article 83 of Regulation (EC) No. 726/2004 (doc ref: EMEA/27170/2006). London: European Medicines Agency; 2007. Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Regulatory_and_ procedural_guideline/2009/10/WC500004075.pdf (accessed January 20, 2011).

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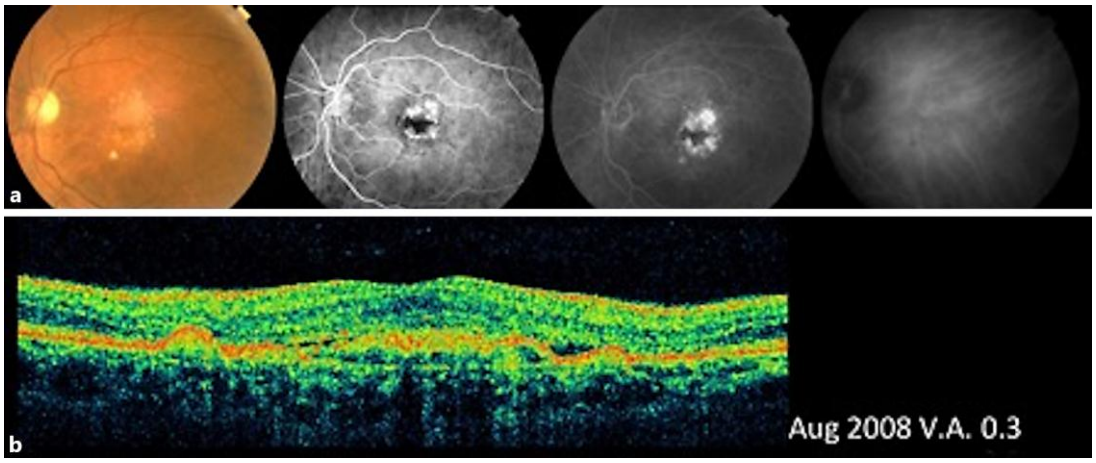

Fig. 1. Features of neovascular AMD in the patient's left eye at diagnosis. **a** Left: fundus photograph of the left eye showing subretinal confluent drusen in the macular area. Center-left: fluorescein angiography showing macular hyperfluorescence in the early stages (center-right) that increases in the late stages. Right: indocyanine green angiography showing late hyperfluorescence. **b** OCT of the left eye showing RPE detachment with intraretinal, subretinal, and sub-RPE fluid.

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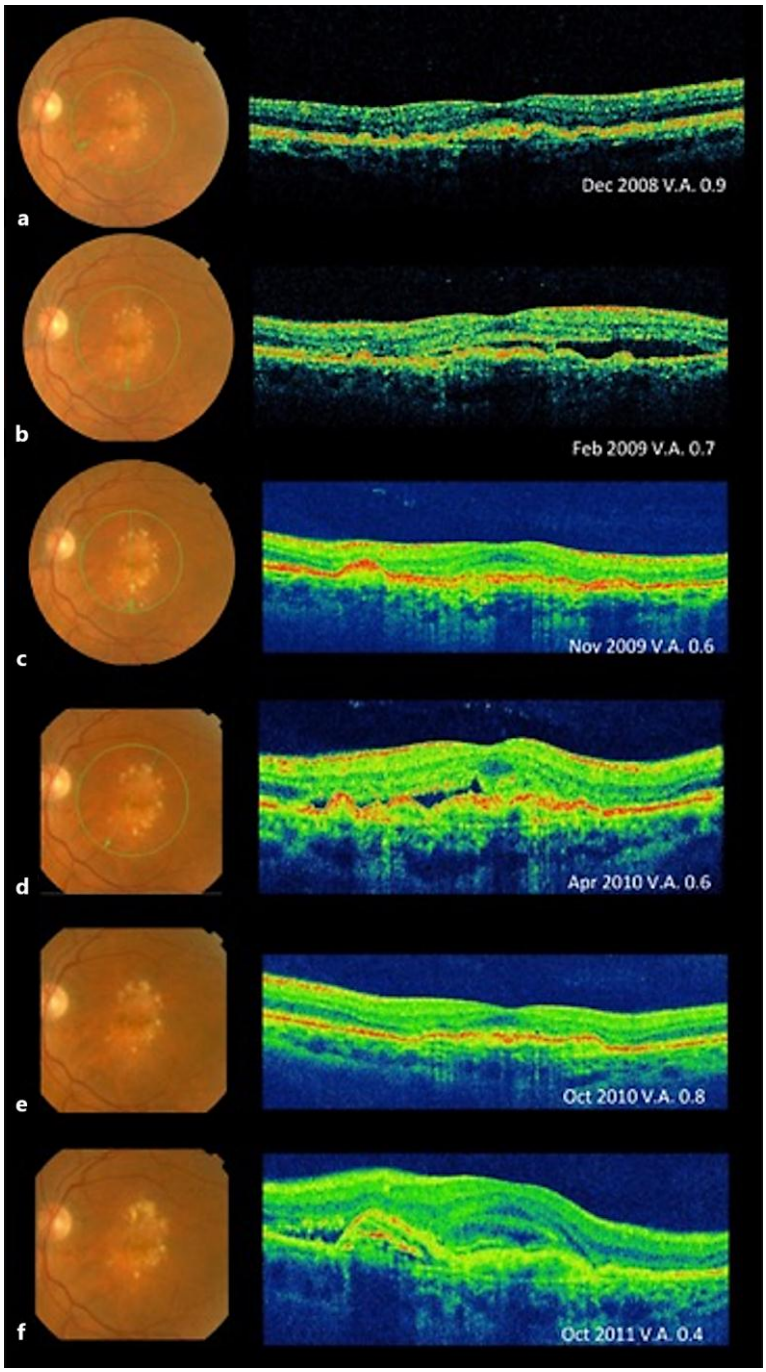
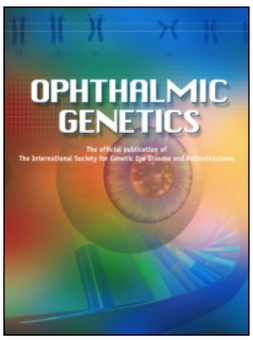


Fig. 2. Changes in the macula and VA while treated with intravitreal ranibizumab alone. **a** December 2008: fundus photography and OCT of the left eye after three doses of intravitreal ranibizumab. OCT showing drusen, but with marked improvement in the previously observed intraretinal, subretinal, and sub-RPE fluid. **b** February 2009: fundus photography and OCT of the left eye showing subretinal fluid and RPE detachment due to reactivation of CNV. **c–f** Fundus photography and OCT showing successive reactivations and improvements after the completion of the corresponding treatments in a pro re nata basis of treatment with ranibizumab during 40 months of follow-up. OCT in panels **b**, **d** and **f** showing the recurrences of intraretinal, subretinal, and sub-RPE fluid. OCT in panels **a**, **c** and **e** showing the subsequent improvement after anti-VEGF treatment.



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RESEARCH REPORT

Metallothionein polymorphisms in a Northern Spanish population with neovascular and dry forms of age-related macular degeneration

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ABSTRACT

Background: To elucidate the potential role of single nucleotide polymorphisms (SNPs) in the metallothionein (MT) genes in Northern Spanish patients with age-related macular degeneration (AMD).
Methods: A total of 130 unrelated Northern Spanish natives diagnosed with AMD (46 dry, 35 neovascular, and 49 mixed) and 96 healthy controls, matched by age and ethnicity, were enrolled in a case–control study. DNA was isolated from peripheral blood and genotyped for 14 SNPs located at 5 MT genes (*MT1A*: rs11076161, rs 11640851, rs8052394, and rs7196890; *MT1B*: rs8052334, rs964372, and rs7191779; *MT1M*: rs2270836 and rs9936741; *MT2A*: rs28366003, rs1610216, rs10636, and rs1580833; *MT3*: rs45570941) using TaqMan probes. The association study was performed using the HaploView 4.0 software.
Results: The allelic and genotypic frequencies analysis revealed that rs28366003 at *MT2A* gene is significantly associated with dry AMD. The frequency of genotype AG was significantly higher in dry AMD than in control cases ($p = 2.65 \times 10^{-4}$; AG vs. AA) conferring more than ninefold increased risk to dry AMD (OR = 9.39, 95% CI: 2.11–41.72), whereas the genotype AA confers disease protection (OR = 0.82, 95% CI: 0.71–0.95). No statistically significant differences were observed between AMD subjects and controls in the rest of the 14 SNPs analyzed.
Conclusions: The present study is the first to investigate the potential association of SNPs at MT genes with susceptibility to AMD. We found a significant association of SNP rs28366003 at *MT2A* gene with susceptibility to the dry form of AMD in a Northern Spanish population.

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Introduction

Age-related macular degeneration (AMD) is a neurodegenerative eye disease characterized by a progressive loss of central vision, and is the leading cause of irreversible blindness among people over the age of 60 years in developed countries.[1,2] It affects approximately 50 million individuals worldwide and this number is expected to increase to 196 million in 2020 and to 288 million in 2040.[3]

The pathogenesis of AMD remains poorly understood, it is generally accepted that this is a multifactorial disease, where genetic, environment, age, smoking,[4–6] obesity, and dietary fat consumption[7–9] are significant risk factors in the development and progression of the disease.[10–13] Rare single nucleotide polymorphisms (SNPs) in genes involved in the complement system,[13–16] lipid metabolism, extracellular matrix remodeling, and angiogenesis[13] are also associated with increased risk of AMD. There are two main clinical forms of AMD: dry and wet (neovascular or exudative). Most AMD cases start as the dry form and in 10–20% of individuals it progresses to the wet form.[17] One of the clinical hallmarks in early and dry forms of AMD is the abundance of extracellular deposits, called drusen, located between the retinal pigmented epithelium (RPE) and Bruch's membrane in the macula region. Drusen are composed of proteins, including vitronectin, apolipoproteins, crystallins, lipids, and elevated levels of zinc.[18–21] The mechanism leading to the formation of drusen and the accumulation of zinc is still unclear. Zinc is an essential chemical element in the physiology of the retina, photoreceptors and RPE being the ocular cells with the highest levels of this metal in the human eye.[19,22] Zinc dyshomeostasis has been related with retinal diseases,[23] and it has been suggested to contribute to the aggregation of proteins and lipids, triggering the formation and growth of drusen in AMD.[24,25] Recent studies from our laboratory and others have shown that metallothioneins (MTs), the main cytosolic zinc-ion binding proteins, so far known, are abundantly expressed in ocular tissues.[25,26] There are four main gene subfamilies of MTs expressed in humans: MT1, MT2, MT3, and MT4, and this cluster of genes is located on chromosome 16 (16q12-22).[27] MTs are low-

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molecular-mass (6–7 kDa)[28]cysteine-rich proteins involved in many physiological and pathophysiological processes, including the modulation of oxygen-free radicals and nitric oxide and protection against UV/ionic radiation,[29,30] and cytotoxic alkylating agents including chemotherapeutics.[31–34] MTs levels decrease with aging and AMD, which may result in an improper control of zinc homeostasis.[22,35,36] SNPs in MT genes have been shown to be associated with an increased susceptibility to pathological conditions including neurodegenerative disorders,[37] cardiovascular disease,[38,39] diabetic neuropathy,[40] cancer,[41–43] atherosclerosis,[44] chronic inflammation,[38] hyperlipidaemia,[40] hyperglycemia,[38] and renal dysfunction through chronic heavy metals exposure.[45,46]

Because of the potential relevance of MTs and zinc homeostasis in the pathogenesis of AMD, we have examined, in the present work, the possible association of the SNPs at MT genes and AMD in a Spanish population from North Spain. The criteria for choosing the SNPs of the MT gene family (*MT1A*: rs11076161, rs11640851, rs8052394, and rs7196890; *MT1B*: rs8052334, rs964372, and rs7191779; *MT1M*: rs2270836 and rs9936741; *MT2A*: rs28366003, rs1610216, rs10636, and rs1580833; *MT3*: rs45570941) and analyzed in the present study was based on earlier reports,[47] describing their association with pathological processes other than AMD.

Material and methods

Study subjects

The present case–control study involved 130 unrelated native Spanish patients diagnosed with AMD and 96 healthy controls recruited at the Fernández-Vega Ophthalmological Institute (Asturias, Spain). Complete ophthalmic examinations were performed for both patients and controls, including slit-lamp biomicroscopy and funduscopy in both eyes. AMD-diagnosed patients were further examined by fluorescence fundus angiography, indocyanine green angiography, or optical coherence tomography. Individuals were classified as follows: dry AMD, with evidence of geographic atrophy (GA) in any eye; wet AMD, with evidence of choroidal neovascularization (CNV) in any eye; and mixed AMD, subjects with CNV and GA in any eye (in the same eye or in the contralateral one). Control subjects were selected from patients undergoing cataract surgery who did not show indications of other relevant ocular pathologies such as retinopathies or maculopathies. To avoid possible misclassification, considering that AMD is a late-onset disorder, only people aged 60 years or above were recruited as controls. The number of subjects, gender, and the mean, and age range in each group (AMD with dry form, AMD with wet form, AMD with dry and wet forms, and controls) are shown in Table 1.

Table 1. Demographic characteristics of AMD patients and controls.

Study population (*n*)	Age (mean ± SD)	Age range (years)	Gender (female/male)
Controls (96)	73.3 ± 8.03	60–92	53 (55.2%)/43
AMD (130)	77.7 ± 7.84	52–99	79 (60.8%)/51
Dry AMD (46)	77.0 ± 9.20	52–99	28 (60.9%)/18
Wet AMD (35)	77.3 ± 6.55	65–91	24 (68.6%)/11
Mixed AMD (49)	78.7 ± 7.33	64–93	27 (55.1%)/22

n, number of subjects; SD, standard deviation.

The study adheres to the tenets of the Declaration of Helsinki on Biomedical Research Involving Human Subjects, and was approved by the Clinical Research Ethics Committee at the Hospital Universitario Central de Asturias (Oviedo, Spain). All participants signed an informed consent.

Genotyping

Peripheral blood was collected in 6 mL K2EDTA tubes (Vacuette, Madrid, Spain). Tubes were stored at −20°C until use. Genomic DNA was obtained from the blood samples of all studied subjects using a commercial DNA extraction kit (FlexiGene DNA Kit; Qiagen, Hilden, Germany) according to the manufacturer's protocol.

The present study included 14 SNPs located in 5 different MTs genes: (i) *MT1A*: rs11076161, rs11640851, rs8052394, and rs7196890; (ii) *MT1B*: rs8052334, rs964372, and rs7191779; (iii) *MT1M*: rs2270836 and rs9936741; (iv) *MT2A*: rs28366003, rs1610216, rs10636, and rs1580833; and (v) *MT3*: rs45570941. The localizations of these variants along the MTs genes are shown in Figure 1.

Allelic discrimination was performed with TaqMan probes (C__25996927_10 (rs11076161), C__1430037_10

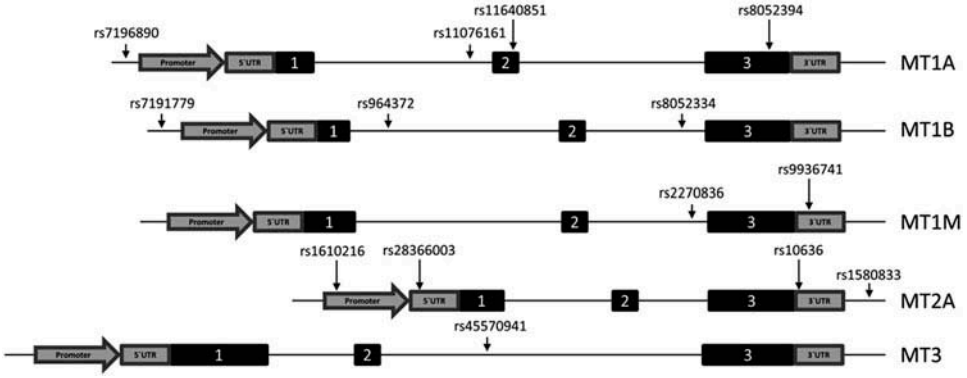

Figure 1. Metallothionein (*MT1A, MT1B, MT1M, MT2A*, and *MT3*) polymorphisms. Localization of the SNPs analyzed. SNP names according to NCBI dbSNP. UTR – untranslated region. Exons are represented by black boxes.

(rs8052334), C__60284591_10 (rs28366003), C__1429975_10 (rs1610216), C__1402094_10 (rs10636), C__1429997_10 (rs9936741), C__1429976_10 (rs1580833), and Custom TaqMan (R) SNP genotyping assays (AH1SDMB (rs11640851), AH21BSJ (rs8052394), AH399YR (rs964372), AH5I74Z (rs2270836), AH6R6A7 (rs45570941), AH4AAAS (rs7196890), and AHLJ1QD (rs7191779)) provided by the manufacturer (Applied Biosystems, Inc., Foster City, CA, USA), in the 7500 Real Time PCR System (Applied Biosystems, Inc.). All PCR amplifications were performed with the thermal cycling conditions of 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. The genotyping results were confirmed in a random subgroup of our samples (seven samples per group) using direct DNA sequencing.

Statistical analysis

All the SNPs were assessed for Hardy–Weinberg equilibrium (HWE) by a χ^2 test in both groups (cases and controls) with HaploView 4.0 software.[48] The comparison of the SNPs allelic frequencies between both groups was performed using a standard χ^2 test, with a p-value of less than 3.57×10^{-3} (0.05/14) being considered as statistically significant (Bonferroni method was used for the adjustment of multiple comparisons). Additionally, we used Sigmaplot version 11 software (Systat Software, Inc., San Jose, CA) to run a logistic regression analysis in order to control for potential confounders.

The comparison of genotypic frequencies between the AMD and control groups was performed using a χ^2 test (Pearson correction) with SPSS version 15.0 software (IBM Corporation, Armonk, NY). Relative risk association was estimated by calculating odds ratios (OR) along with 95% confidence intervals (CI) using the methods described in Armitage et al.[49] and PLINK (version 1.07) as described by Purcell et al.[50] Linkage disequilibrium (LD) plot was generated with HaploView 4.0 software,[48] and blocks were defined according to the algorithm developed by Gabriel et al.[51] Individual haplotypes and their estimated population frequencies were performed using HaploView 4.0 software[48] with all of the parameters set at the default values. Haplotype association analysis were performed using a standard χ^2 test with a p-value <0.05 considered statistically significant with HaploView 4.0 software.[48]

Results

The demographic characteristics of the three AMD subgroups (i.e., dry, wet, and mixed, totaling 130 cases) and the controls (96 subjects) recruited in the present study are shown in Table 1.

Association study of MT SNPs

The allelic and genotype frequencies for each of the 14 sequence variants in the MT genes (*MT1A*: rs11076161, rs11640851, rs8052394, and rs7196890; *MT1B*: rs8052334, rs964372, and rs7191779; *MT1M*: rs2270836 and rs9936741; *MT2A*: rs28366003, rs1610216, rs10636, and rs1580833; *MT3*: rs45570941) were analyzed in all the AMD cases (n = 130) and controls (n = 96; Table 2). Results from the association study of the SNPs with each of the different AMD forms separately (dry, wet, or mixed forms) are presented in the Supporting Information Table (online only).

The SNPs studied located along the MT genes are shown in Figure 1, including coding and non-coding regions. The observed genotype frequencies of 13 out of 14 MT SNPs were in HWE ($p > 0.01$) in both AMD and control groups, with the exception of rs1610216 ($p < 0.01$).

MT1A *polymorphisms*

Allele and genotype frequencies of the *MT1A* SNPs analyzed in this study (rs11076161, rs11640851, rs8052394, and rs7196890) did not significantly differ in AMD cases (AMD, dry, wet, or mixed forms) when compared with controls (see Supporting Information Table—online only).

MT1B *polymorphisms*

No association was found between the *MT1B* SNPs analyzed (rs8052334, rs964372, and rs7191779) and AMD. The genotypic frequencies of rs8052334 and rs7191779 showed small differences between AMD mixed cases and controls under recessive model ($p = 2.68 \times 10^{-2}$, CC vs. CT+TT; $p = 1.13 \times 10^{-2}$, CC vs. GC+GG). These differences were not significant after the stringent Bonferroni correction ($p < 3.57 \times 10^{-3}$; see Supporting Information Table—online only).

MT1M *polymorphisms*

The comparison between AMD cases (AMD, dry, wet, or mixed forms) and controls in the allele and genotype frequencies of the *MT1M* variants analyzed in this study (rs2270836 and rs9936741) did not show significant differences between AMD cases and controls (see Supporting Information Table—online only).

MT2A *polymorphisms*

Allele frequencies of rs28366003 in *MT2A* were significantly different in AMD cases when compared with controls. The allele G was detected in a statistically significant higher frequency in patients with AMD than in controls ($p = 0.009$; Table 2). After Bonferroni correction for multiple testing ($p < 3.57 \times 10^{-3}$), these differences did not remain significant.

The frequency of genotype AG at rs28366003 was significantly higher (not after Bonferroni correction) in AMD than in control under a dominant association model ($p = 7.72 \times 10^{-3}$; AG + GG vs. AA) conferring approximately 5.54-fold increased risk for AMD (OR = 5.54, 95% CI: 1.30–23.65), whereas the genotype AA could protect from the disease (OR = 0.90, 95% CI: 0.84–0.97). None of the studied individuals presented genotype GG at this SNP.

The comparison between the allelic and genotypic frequencies of this SNP in each of the clinical subgroups of AMD compared with controls showed significant differences (Supporting Information). Allele frequencies of rs28366003 showed significant association with dry form of AMD ($p = 4.40 \times 10^{-4}$; Table 3). The G allele confers more than ninefold increased disease susceptibility (OR = 9.80, 95% CI: 1.11–86.25). This association remained significant after the Bonferroni correction for multiple testing ($p < 3.57 \times 10^{-3}$). The frequency of genotype AG was significantly higher in dry AMD cases than in

Table 2. Allelic and genotypic association analysis.

	SNP ID		AMD (%)	Control (%)	*p value*	OR (95% CI)
MT1A	rs11076161		(*n* = 130)	(*n* = 96)		
	Allele	G	76.2	74	0.593	1.03 (0.88–1.20)
		A	23.8	26		0.91 (0.58–1.44)
	Genotype	GG	60	56.25	0.5716	1.07 (0.85–1.34)*
		GA	32.3	35.42		0.91 (0.63–1.32)
		AA	7.7	8.33		0.92 (0.38–2.25)
		Total	78/42/10 (GG/GA/AA)	54/34/8 (GG/GA/AA)	0.8521	
	rs11640851		(*n* = 130)	(*n* = 96)		
	Allele	C	39.6	39.1	0.9053	1.01 (0.73–1.41)
		A	60.4	60.9		0.99 (0.80–1.23)
	Genotype	AA	37.69	41.67	0.5801	0.90 (0.65–1.25)*
		AC	45.39	38.54		1.18 (0.86–1.61)
		CC	16.92	19.79		0.85 (0.49–1.49)
		Total	49/59/22 (AA/AC/CC)	40/37/19 (AA/AC/CC)	0.5824	
	rs8052394		(*n* = 130)	(*n* = 96)		
	Allele	A	86.5	83.9	0.4244	1.03 (0.92–1.15)
		G	13.5	16.1		0.84 (0.45–1.57)
	Genotype	AA	76.15	68.75	0.2152	1.11 (0.94–1.31)*
		AG	20.77	30.21		0.69 (0.44–1.08)
		GG	3.08	1.04		2.95 (0.34–26.01)
		Total	99/27/4 (AA/AG/GG)	66/29/1 (AA/AG/GG)	0.1796	
	rs7196890		(*n* = 130)	(*n* = 96)		
	Allele	A	63.1	64.6	0.742	0.98 (0.80–1.19)
		C	36.9	35.4		0.77 (0.53–1.12)
	Genotype	AA	40.77	44.79	0.5452	0.91 (0.67–1.23)*
		AC	44.62	39.59		1.13 (0.82–1.54)
		CC	14.61	15.62		0.93 (0.50–1.74)
		Total	53/58/19 (AA/AC/CC)	43/38/15 (AA/AC/CC)	0.7493	
MT1B	rs8052334		(*n* = 130)	(*n* = 96)		
	Allele	C	52.3	49.5	0.5521	1.06 (0.81–1.37)
		T	47.7	50.5		0.94 (0.72–1.23)
	Genotype	CC	30	19.79	0.0824	1.52 (0.94–2.45)*
		CT	44.62	59.38		0.75 (0.58–0.97)
		TT	25.38	20.83		1.22 (0.74–1.99)
		Total	39/58/33 (CC/CT/TT)	19/57/20 (CC/CT/TT)	0.0783	
	rs964372		(*n* = 130)	(*n* = 96)		
	Allele	G	82.7	84.4	0.6345	0.98 (0.87–1.10)
		C	17.3	15.6		1.11 (0.61–2.02)
	Genotype	GG	70	70.83	0.8933	0.99 (0.83–1.17)*
		GC	25.38	27.09		0.94 (0.60–1.46)
		CC	4.62	2.08		2.21 (0.46–10.74)
		Total	91/33/6 (GG/GC/CC)	68/26/2 (GG/GC/CC)	0.5865	
	rs7191779		(*n* = 130)	(*n* = 96)		
	Allele	G	51.2	47.4	0.4296	1.08 (0.82–1.41)
		C	48.8	52.6		0.93 (0.72–1.20)
	Genotype	GG	28.46	17.71	0.061	1.61 (0.96–2.68)*
		GC	45.39	59.37		0.76 (0.59–0.98)
		CC	26.15	22.92		1.14 (0.71–1.82)
		Total	37/59/34 (GG/GC/CC)	17/57/22 (GG/GC/CC)	0.0816	
MT1M	rs2270836		(*n* = 130)	(*n* = 96)		
	Allele	C	60.4	62.5		0.97 (0.78–1.19)
		T	39.6	37.5	0.6481	1.06 (0.76–1.47)
	Genotype	CC	36.15	39.58	0.8671	0.91 (0.65–1.28)*
		CT	48.47	45.84		1.06 (0.80–1.40)
		TT	15.38	14.58		1.05 (0.56–1.98)
		Total	47/63/20 (CC/CT/TT)	38/44/14 (CC/CT/TT)	0.8707	
	rs9936741		(*n* = 130)	(*n* = 96)		
	Allele	T	98.1	97.4	0.6265	1.01 (0.97–1.05)
		C	1.9	2.6		0.73 (0.13–4.16)
	Genotype	TT	96.15	95.83	0.9025	1.00 (0.95–1.06)*
		TC	3.85	3.13		1.23 (0.30–5.02)
		CC	0	1.04		NA
		Total	125/5/0 (TT/TC/CC)	92/3/1 (TT/TC/CC)	0.4877	
MT2A	rs28366003		(*n* = 130)	(*n* = 96)		
	Allele	A	94.2	99		0.95 (0.91–1.00)
		G	5.8	1	0.009	5.80 (0.70–47.72)
	Genotype	AA	88.46	97.92	7.72x10-3	0.90 (0.84–0.97) #
		AG	11.54	2.08		5.54 (1.30–23.65)
		GG	0	0		NA
		Total	115/15/0 (AA/AG/GG)	94/2/0 (AA/AG/GG)	7.72x10-3	
	rs1610216		(*n* = 130)	(*n* = 96)		
	Allele	C	66.9	64.1	0.5265	1.04 (0.86–1.26)
		T	33.1	35.9		0.92 (0.64–1.32)
	Genotype	CC	33.85	28.12	0.3597	1.20 (0.81–1.79)*
		CT	66.15	71.88		0.92 (0.77–1.10)

(*Continued*)

Table 2. (Continued).

	SNP ID		AMD (%)	Control (%)	*p value*	OR (95% CI)
		TT	0	0		NA
		Total	44/86/0 (CC/CT/TT)	27/69/0 (CC/CT/TT)	0.3597	
	rs10636		(*n* = 130)	(*n* = 96)		
	Allele	G	75.4	81.2		0.93 (0.81–1.06)
		C	24.6	18.8	0.1375	1.31 (0.78–2.19)
	Genotype	GG	56.92	67.71	0.7623	0.84 (0.69–1.03)*
		GC	36.93	27.08		1.36 (0.92–2.03)
		CC	6.15	5.21		1.18 (0.40–3.50)
		Total	74/48/8 (GG/GC/CC)	65/26/5 (GG/GC/CC)	0.28512	
	rs1580833		(*n* = 130)	(*n* = 96)		
	Allele	C	70.4	66.7	0.399	1.05 (0.88–1.26)
		A	29.6	33.3		0.89 (0.60–1.31)
	Genotype	CC	47.69	44.79	0.6654	1.06 (0.80–1.42)*
		AC	45.39	43.75		1.04 (0.77–1.40)
		AA	6.92	11.46		0.60 (0.26–1.40)
		Total	62/59/9 (CC/AC/AA)	43/42/11 (CC/AC/AA)	0.4926	
MT3	rs45570941		(*n* = 130)	(*n* = 96)		
	Allele	G	89.6	86.5	0.3024	1.04 (0.94–1.14)
		C	10.4	13.5		0.77 (0.38–1.57)
	Genotype	GG	80.77	76.04	0.3903	1.06 (0.92–1.22)*
		GC	17.69	20.84		0.85 (0.50–1.45)
		CC	1.54	3.12		0.49 (0.08–2.89)
		Total	105/23/2 (GG/GC/CC)	73/20/3 (GG/GC/CC)	0.5854	

n, number of subjects; OR, odds ratio; CI, confidence interval. The Bonferroni-corrected significance level for the allelic frequencies comparisons was 3.57x10-3 (0.05/14). Total indicates the general test of association in the 2 x 3 table of disease-by-genotype.
*OR values and *p* values derived from comparison of the genotypic frequencies under the recessive model (GG vs. GA+AA at rs11076161, CC vs. AC+AA at rs11640851, AA vs. AG+GG at rs8052394, CC vs. AC+AA at rs7196890, CC vs. CT+TT at rs8052334, CC vs. GC+GG at rs964372, GG vs. GC+CC at rs7191779, TT vs. CT+CC at rs2270836, TT vs. TC+CC at rs9936741, CC vs. CT+TT at rs1610216, CC vs. GC+GG at rs10636, CC vs. AC+AA at rs1580833, and GG vs. GC+CC at rs45570941).
#OR values and *p* values derived from comparison of the genotypic frequencies under the dominant model (AA vs. AG+GG at rs28366003).
NA, the odds ratio was not available where the number of individuals with two copies of the risk allele was zero.

Table 3. Allelic and genotypic association analysis between the SNP rs2836003 and dry AMD.

SNP ID		Dry AMD %	Control %	*p* value	OR (95% CI)
rs28366003		(*n* = 46)	(*n* = 96)		
Allele	A	90.2	99		0.91 (0.82–0.99)
	G	9.8	1	4.40 × 10–4	9.80 (1.11–86.25)
Genotype	AA	80.43	97.92	2.65 × 10–4	0.82 (0.71– 0.95)*
	AG	19.57	2.08		9.39 (2.11–41.72)
	GG	0	0		NA
	Total	37/9/0 (AA/AG/GG)	94/2/0 (AA/AG/GG)	2.65 × 10–4	

n, number of subjects; OR, odds ratio; CI, confidence interval. The Bonferroni-corrected significance level for the allelic frequencies comparisons was 3.57 × 10–3 (0.05/14). Total indicates the general test of association in the 2 × 3 table of disease-by-genotype.
*OR values and *p* values derived from comparison of the genotypic frequencies under the dominant model (AG + GG vs. AA at rs28366003).
NA, the odds ratio was not available.

control under dominant association model ($p = 2.65 \times 10^{-4}$; AG +GG vs. AA) being over nine times more likely to suffer dry AMD (OR = 9.39, 95% CI: 2.11–41.72), whereas the genotype AA could protect from the disease (OR = 0.82, 95% CI: 0.71–0.95).

No association was found between the other *MT2A* SNPs analyzed (rs1610216, rs10636, and rs1580833) and AMD. The variant rs10636 showed differences in the allelic frequencies between dry AMD cases and controls ($p = 0.0443$; C allele; Supporting Information Table—online only), but these differences were not significant after Bonferroni correction.

MT3 *polymorphisms*

Allele and genotype frequencies of the SNP rs45570941 (*MT3*) did not significantly differ in AMD cases (AMD, dry, wet, or mixed forms) when compared with controls (Supporting Information Table—online only).

The logistic regression multivariate analysis (multivariate linear regression analysis and backwards stepwise regression analysis) indicated that the covariates sex and age are not predictors of disease in an individual and only the SNP rs28366003 (at *MT2A* gene) appear to be essential in this model (data not shown). The 14 SNPs studied are not completely independent among themselves, since they are part of the same gene or they are located in close genes, thus the logistic regression analysis detects collinearity among them, providing redundant information in the model.

Haplotype analysis and linkage disequilibrium

Pairwise linkage disequilibrium (LD) analysis of the analyzed SNPs identified only one linkage block (Figure 2). This block included three SNPs (rs11076161, rs11640851, and rs8052394, located at *MT1A* gene) that were in strong LD, as observed by the coefficient of LD (D' value). The SNP rs11076161 was in strong LD with rs11640851 ($D' = 0.98$) and rs8052394 ($D' = 0.95$), while rs11640851 was also in strong LD with rs8052394 ($D' = 0.96$).

The haplotype analysis inferred three of the nine theoretically possible haplotypes formed by these three SNPs

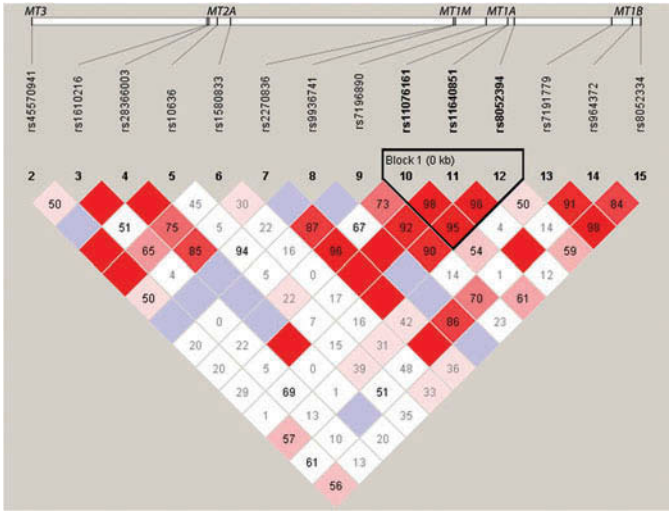

Figure 2. Linkage disequilibrium (LD) plot. LD plot of fourteen MT SNPs (rs11076161, rs 11640851, rs8052394, and rs7196890 at *MT1A* gene; rs8052334, rs964372, and rs7191779, at *MT1B* gene; rs2270836 and rs9936741 at *MT1M* gene; rs28366003, rs1610216, rs10636, and rs1580833 at *MT2A* gene; and rs45570941 at *MT3* gene). The number in the diamond refers to D′ (100xD′). The LD block was defined according to the standard confidence intervals. The strength of LD is depicted by red intensity, which moves from white to light red as D` progresses from 0 to 100.

(rs11076161, rs11640851, and rs8052394), but none of them presented association with AMD (Table 4).

Discussion

To our knowledge, the present study is the first attempt to investigate the association of the SNPs at MT genes with AMD. In this analysis, we stratified AMD cases in three clinical subgroups: subjects with the dry form, subjects with the wet form, and subjects with both forms (mixed). When the allelic and genotypic frequencies of all SNPs (rs11076161, rs11640851, rs8052394, and rs7196890 at *MT1A* gene; rs8052334, rs964372, and rs7191779 at *MT1B* gene; rs2270836 and rs9936741 at *MT1M* gene; rs28366003, rs1610216, rs10636, and rs1580833 at *MT2A* gene; and rs45570941 at *MT3* gene) were analyzed, we observed that none of them exhibited a significant association with AMD in this Spanish cohort, considering together the clinical groups, and using the stringent Bonferroni correction. Furthermore, the haplotype analysis did not show haplotypes associated with the disease.

The most important finding of this study is the strong association of the G allele of the SNP rs28366003, at *MT2A* gene, with the dry form of AMD, suggesting a high and specific disease risk for carriers of this allele in our cohort. Additionally, the frequency of the heterozygote genotype AG was significantly higher in dry AMD cases than in controls, indicating that one copy of the risk allele (the G allele) is sufficient to have an impact on the disease. As far as we know, the association between this genetic variant and AMD has not been explored. Kayaalti et al.[52,53] showed a link between the rs28366003 polymorphism and longevity, describing that the AA genotype is associated with longevity in the Turkish population. Moreover, the association between rs28366003 and prostate cancer was shown in a study of the Polish population.[42] *MT2A* variant rs28366003 may provoke the enhancement of tumor development and growth; contribute to deregulated cell proliferation, apoptosis, and oxidative stress; and increase genomic instability through the variation of the cellular activity and the signaling of MT-dependent pathways.[47,52–54] The presence of the G allele in the core promoter region of the *MT2A* gene (rs28366003) may be an indicator of higher sensitivity for metal toxicity and carcinogenicity, as well as a useful potential marker for increased risk of tumor.[42,52,54–56] The SNP rs28366003, located in the center of the consensus sequence TGCACTC, between the TATA box and the site of initiation of transcription in the *MT2A* gene, has been shown to play a critical role in regulating metal concentration (cadmium, zinc, and lead, but not copper) in blood samples taken from Turkish population, showing that the presence of the GG genotype promotes significantly the increment of cadmium and lead levels and the decline of zinc compared with A allele carriers.[54] Besides, Adams et al.[57] reported that the G allele of rs28366003 influences urinary excretion of metals that bind MTs (Cd, Cu, and Zn).[57]

The metal regulatory transcription factor-1 (MTF-1) induces the expression of metallothioneins by binding to the consensus sequence (TGCRCNC) of the metal-responsive elements in the *MT2A* promoter region. However, the presence of the variant G of rs28366003 in the center of this sequence inhibits the MTF-1 binding to the core promoter region causing a decrease in the induction of *MT2A* gene transcription.[53,58] The decline in protein MT2A, one of the most highly expressed MT in ocular tissues, including RPE and retina,[26] might affect the proper zinc homeostasis in dry AMD patients.

In summary, the present study is the first one that analyzed the association of 14 genetic variants (the most relevant SNPs at MT genes according to Raudenska et al.[47]) distributed along 5 MT genes, with AMD in the Spanish population. Our results showed no association with AMD for 13 of the SNPs studied. However, a strong association was found between the SNP rs28366003 (*MT2A* gene) and the

Table 4. Haplotype analysis.

SNPs alleles			Haplotype frequency		Association test between AMD and controls	
rs11076161	rs11640851	rs8052394	AMD (n = 130)	Control (n = 96)	p value	OR (95% CI)
G	A	A	0.599	0.604	0.9289	0.99 (0.80–1.23)
G	C	A	0.158	0.13	0.4098	1.21 (0.63–2.33)
A	C	G	0.13	0.156	0.4457	0.83 (0.44–1.59)
A	C	A	0.104	0.105	0.9834	0.99 (0.46–2.14)

n, number of subjects. Individual *p* values and odds ratios (OR) between AMD and control are provided for each of the haplotypes compared with all the other haplotypes.

dry form of AMD in our population. This variant has been linked to longevity,[52,53] cancer, and higher sensitivity for metal toxicity and carcinogenesis.[42,52,54] The findings suggest that rs28366003 may reduce the MT2A gene transcription, and probably alter the proper regulation of zinc homeostasis.

It should be also mentioned that there are several limitations of our study. Due to the relatively small sample size, the frequencies of some homozygous variants were low or null in subgroups therefore reduced the statistical power and limited us from evaluating the effects in stratified analysis (especially in the different forms of AMD). Besides, the risk allele of rs28366003 (allele G) is presented only in 9.8% of patients with dry AMD, being a limitation to be used as a biomarker of the disease. Replication of the present study in other AMD population cohorts is warranted.

In conclusion, this study explores for the first time the association of AMD and MT polymorphisms and shows a positive correlation between the SNP rs28366003 (*MT2A* gene) and the dry form of AMD in our cohort, suggesting that this SNP represents a genetic risk factor for dry AMD in the Northern Spanish population.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Anexo 3.

Comunicaciones a congresos relacionadas con la tesis realizadas durante el periodo del doctorado

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Complement factor H polymorphisms associated with exudative or dry AMD in the Spanish population.

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Abstract

Purpose: To elucidate the potential role of single nucleotide polymorphisms (SNPs) in complement factor H (*CFH*) gene in Spanish patients with aged-related macular degeneration (AMD).

Methods: A case-control study of 136 (46 dry, 37 wet, 53 dry + wet) unrelated native Spanish diagnosed with AMD and 106 controls matched by age and ethnicity were enrolled. DNA was isolated from peripheral blood and subjected to genotyping for AMD-associated SNPs (rs3753394, rs529825, rs800292, rs3766404, rs203674, rs3753396, rs1065489 and rs10671170) using TaqMan probes and restriction fragment-length polymorphism (RFLP). The association study was performed using the HaploView 4.0 software.

Results: The allelic frequency analysis revealed that rs529825, rs800292, rs3766404, rs203674 and rs10671170 were significantly associated with an increased risk for AMD. The haplotypes CGG (rs529825, rs800292 and rs233674) and GCAG (rs203674, rs1061170, rs3752296 and rs1065489), were significantly associated with AMD whereas the haplotypes CAA (rs529825, rs800292 and rs233674) and TTAG (rs203674, rs1061170, rs3752296 and rs1065489) were found to be protective. Small differences in allelic frequencies were found between dry or exudative cases, these differences were not significant and did not distinguish the two clinical forms of AMD

Conclusions: This study confirmed significant association of SNPs rs529825, rs800292, rs3766404, rs203674 and rs1061170 in the *CFH* gene with susceptibility to AMD in the Spanish population. We identified haplotypes that confer protection or increased risk of AMD. We did not find any specific genetic variant in *CFH* capable to distinguish the different clinical forms of AMD in this cohort. Collectively, our results confirmed that *CFH* represents a strong genetic risk factor for this disease in the Spanish population.

Complement factor H polymorphisms associated with exudative or dry AMD in the Spanish population.

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INTRODUCTION

Aged-related macular degeneration (AMD) is the leading cause of blindness in elderly population in developed countries [1], affecting 50 million individuals worldwide.

The association of *CFH* SNPs with AMD has been studied in populations of diverse geographic origins, including Spanish population [2-4] confirming the *CFH* gene as the most important genetic risk factor known so far for this pathology. The present study describes the association of genetic variants in the CFH gene with AMD in a Northern Spanish population.

The present study was designed: i) To investigate the association of the most relevant SNPs described to date in the CFH gene (rs3753394, rs529825, rs800292, rs3766404, rs203674, rs1061170, rs3753396 and rs1065489) with AMD cases with either, the dry form, the wet form or both, from the Northern region of Spain; ii) To identify risk and protective haplotypes among these AMD cases; and iii) to compare our results with earlier studies carried out with a Spanish multi-centre group of AMD cases [2].

MATERIALS & METHODS

A case-control study of 130 unrelated native Northern Spanish diagnosed with AMD (46 dry, 35 neovascular, and 49 mixed), and 96 healthy controls matched by age and ethnicity were enrolled. DNA was isolated from peripheral blood and genotyped for AMD-associated SNPs (rs3753394, rs529825, rs800292, rs3766404, rs203674, rs10671170, rs3753396 and rs1065489) using TaqMan probes and restriction fragment–length polymorphism (RFLP). The association study was performed using the HaploView 4.0 software.

RESULTS

The allelic frequency analysis revealed that rs529825, rs800292, rs203674 and rs10671170 were significantly associated with an increased risk for AMD. The haplotypes CGG (rs3753394, rs529825 and rs800292) and GCAG (rs203674, rs1061170, rs3753396 and rs1065489) were significantly associated with AMD while the haplotypes CAA (rs3753394, rs529825 and rs800292) and TTAG (rs203674, rs1061170, rs3753396 and rs1065489) were found to be protective. Small differences in allelic frequencies were found between dry and neovascular cases; however, these differences were not significant and did not distinguish one form the other

CONCLUSIONS

This study found significant association of SNPs rs529825, rs800292, rs203674 and rs1061170 in the CFH gene with susceptibility to AMD. We identified haplotypes that confer protection or increased risk of AMD, but not specific genetic variants in CFH capable to distinguish the different clinical forms of AMD in this cohort. Collectively, our results confirmed that CFH represents a strong genetic risk factor for this disease in the Northern Spanish population.

Conflict of interest statement: None of the authors have commercial interest in the results of this manuscript

RESULTS

SNP ID			AMD % (N=130)	Control % (N=96)	p-value	OR (95% CI)
rs3753394	**Allele**	C	69.6	69.8		1.00 (0.84-1.19)
		T	30.4	30.2	0.9678	1.01 (0.67-1.50)
	Genotype	CC	49.23	48.96	0.9643	1.00 (0.77- 1.31)*
		CT	40.77	41.67		0.98 (0.71-1.34)
		TT	10	9.37		1.07 (0.48-2.39)
		Total	64/53/13 (CC/CT/TT)	47/40/9 (CC/CT/TT)	0.9831	
rs529825	**Allele**	G	87.7	73.4	$1.00x10^{-4}$	1.19 (1.04-1.37)
		A	12.3	26.6		0.46 (0.26-0.81)
	Genotype	GG	76.92	54.17	$3.14x10^{-4}$	1.42 (1.15-1.77)*
		GA	21.54	38.54		0.56 (0.37-0.84)
		AA	1.54	7.29		0.21 (0.04-0.99)
		Total	100/28/2 (GG/GA/AA)	52/37/7 (GG/GA/AA)	$7.49x10^{-4}$	
rs800292	**Allele**	G	87.3	73.4	$2.00x10^{-4}$	1.19 (1.04-1.36)
		A	12.7	26.6		0.48 (0.27-0.84)
	Genotype	GG	76.15	55.21	$9.11x10^{-4}$	1.38 (1.12-1.70)*
		GA	22.31	36.46		0.61 (0.40-0.93)
		AA	1.54	8.33		0.18 (0.04-0.85)
		Total	99/29/2 (GG/GA/AA)	53/35/8 (GG/GA/AA)	$1.31x10^{-3}$	
rs3766404	**Allele**	T	93.8	87	$1.2x10^{-2}$	1.08 (0.99-1.18)
		C	6.2	13		0.48 (0.20-1.11)
	Genotype	TT	87.7	75	$1.35x10^{-2}$	1.17 (1.03-1.33)*
		TC	12.3	23.96		0.51 (0.29-0.92)
		CC	0	1.04		NA
		Total	114/16/0 (TT/TC/CC)	72/23/1 (TT/TC/CC)	$3.37x10^{-2}$	
rs203674	**Allele**	G	59.6	37	$1.96x10^{-6}$	1.61 (1.20-2.17)
		T	40.4	63		0.64 (0.49-0.83)
	Genotype	GG	33.08	14.58	$1.55x10^{-3}$	2.27 (1.32-3.90)*
		GT	53.08	44.79		1.18 (0.90-1.56)
		TT	13.84	40.63		0.34 (0.21-0.56)
		Total	43/69/18 (GG/GT/TT)	14/43/39 (GG/GT/TT)	$6.29x10^{-6}$	
rs1061170	**Allele**	C	56.9	34.4	$2.07x10^{-6}$	1.65 (1.21-2.26)
		T	43.1	65.6		0.66 (0.51-0.84)
	Genotype	CC	31.54	11.46	$3.92x10^{-4}$	2.75(1.49-5.07)*
		CT	50.77	45.83		1.11 (0.84-1.46)
		TT	17.69	42.71		0.41 (0.27-0.64)
		Total	41/66/23 (CC/CT/TT)	11/44/41 (CC/CT/TT)	$1.54 x10^{-5}$	
rs3753396	**Allele**	A	79.2	77.6	0.6771	1.02 (0.89-1.17)
		G	20.8	22.4		0.93 (0.56-1.53)
	Genotype	AA	60	62.5	0.7034	0.96 (0.78-1.18)*
		AG	38.46	30.21		1.27 (0.88-1.85)
		GG	1.54	7.29		0.21 (0.04-0.99)
		Total	78/50/2 (AA/AG/GG)	60/29/7 (AA/AG/GG)	$5.72x10^{-2}$	
rs1065489	**Allele**	G	78.5	77.6	0.8275	1.01 (0.88-1.16)
		T	21.5	22.4		0.96 (0.58-1.58)
	Genotype	GG	58.46	62.5	0.5397	0.93 (0.76-1.16)*
		GT	40	30.21		1.32 (0.91-1.92)
		TT	1.54	7.29		0.21 (0.04-0.99)
		Total	76/52/2 (GG/GT/TT)	60/29/7 (GG/GT/TT)	$4.47x10^{-2}$	

Table 1. Allele and genotype frecuencies in individuals with AMD and controls in a Spanish cohort. Comparison of allelic and genotipic frequencies was performed using χ^2 test. The Bonferroni-corrected significance level for the allelic frequencies comparisons was 0.01 (0.05/5). Total indicate the general test of association in the 2- by-3 table of disease-by-genotype. The asterisk indicate the OR values and p values derived from comparison of the genotypic frequencies under the recessive model.

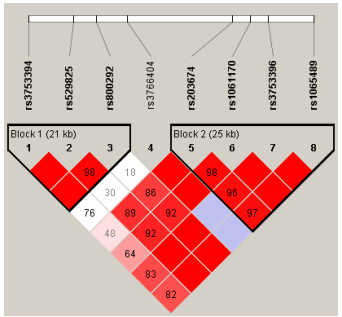

Figure 1. CFH linkage disequilibrium (LD) plot. HaploView 4.0 software was used. The number in the diamond refers to D´(100xD´). The LD block was defined according to the standard confidence intervals. The strength of LD progresses from 0 to 100 (white to red)

SNPs alleles				Haplotype frequency		Association test between AMD and Controls	
rs3753394	**rs529825**	**rs800292**		**AMD n=130**	**Control n= 96**	**P-Value**	**OR (95% CI)**
C	G	G		0.569	0.432	$4.0x10^{-3}$	1.32 (1.00-1.73)
T	G	G		0.304	0.297	0.873	1.02 (0.68-1.53)
C	A	A		0.123	0.26	$2.00x10^{-4}$	0.47 (0.27-0.84)
rs203674	**rs1061170**	**rs3753396**	**rs1065489**				
G	C	A	G	0.569	0.333	$6.74x10^{-7}$	1.71 (1.24-2.35)
T	T	A	G	0.189	0.402	$6.24x10^{-7}$	0.47 (0.31-0.72)
T	T	G	T	0.207	0.217	0.7833	0.95 (0.57-1.60)
G	T	A	G	0.026	0.03	0.7977	0.87 (0.18-4.08)

Table 2. Haplotype frequencies of SNPs rs3753394, rs529825, rs800292, rs203674, rs1061170, rs3753396 and rs1065489 and their association with AMD. HaploView 4.0 software was used. Individual p-values and odds ratios (OR) between AMD and control are provided for each of the haplotypes compared with all the other haplotypes..

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ABSTRACT BOOK

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• T073

Metallothionein polymorphisms in a Northern Spanish population with Age-Related Macular Degeneration (AMD)

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Purpose To elucidate the potential role of single nucleotide polymorphisms (SNPs) in the metallothionein (MT) genes in Northern Spanish patients with aged-related macular degeneration (AMD).
Methods A case-control study of 130 unrelated Northern Spanish natives diagnosed with AMD (46 dry, 35 neovascular, and 49 mixed) and 96 healthy controls matched by age and ethnicity were enrolled. DNA was isolated from peripheral blood and genotyped for fourteen MT SNPs (MT1A: rs11076161, rs 11640851, rs8052394 and rs7196890; MT1B: rs8052334, rs964372 and rs7191779; MT1M: rs2270836 and rs9936741; MT2A: rs28366003, rs1610216, rs10636 and rs1580833; MT3: rs45570941) using TaqMan probes. The association study was performed using the HaploView 4.0 software.
Results The allelic frequency analysis revealed that rs28366003 in MT2A gene, showed the unique significant association with AMD (dry form; p=4.40x10-4), increased disease susceptibility ranged from approximately 9.80 for the allele G (OR= 9.80, 95% CI: 1.11-86.25). The frequency of genotype AA at SNP rs28366003 was significantly lower in dry AMD cases than in control under a recessive association model (p=2.65x10-4; AA vs AG+GG) conferring protection from the disease (OR=0.82, 95% CI: 0.71-0.95). No statistically significant differences were observed between AMD subjects and controls in the rest of the thirteen SNPs analyzed.
Conclusions The present study is the first to investigate the potential association of SNPs at MT genes with susceptibility to AMD. We found a significant association of SNP rs28366003 in the MT2A gene with susceptibility to the dry form of AMD in the Northern Spanish population

• T074

Classification and heritability of macular pigment spatial profile phenotypes using two-wavelength fundus autofluorescence

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Purpose We investigated the frequency and heritability of macular pigment (MP) spatial profile phenotypes determined by objective and subjective profile classification based on fundus autofluorescence (FAF).
Methods Between scans Coefficient of Repeatability (CoR) of MP optical density (MPOD) was calculated from two FAF scans (Spectralis, Heidelberg, Germany) of 40 participants (39±8.6 years) acquired in a single session. We then analyzed two FAF scans acquired in a single session from 314 twins (157 pairs; 39±8.8 years) and classified each MP profile as exponential, ring-like or central dip by subjective visual assessment. Profiles were also classified objectively based on deviations larger than the CoR away from the exponential fit. We calculated kappa agreement of the profiling methods, case-wise concordance of non-exponential profiles for the 88 mono- (MZ) and 69 dizygotic (DZ) twin pairs, and profile heritability.
Results Following visual subjective profiling, 64% showed an exponential profile, 27% presented ring-like and 9% central dip profiles; case-wise concordance was 0.80 for MZ and 0.41 for DZ twins. Following objective classification, 71% showed an exponential profile, 29% ring-like profile and no central dip profiles were identified; case-wise concordance was 0.74 for MZ and 0.36 for DZ twins. Heritability was calculated as 81.5% (95% CI 61.1 to 93.1). Between scan repeatability of profile classification showed good agreement objectively (κ=0.85, 95% CI 0.69 to 1.00; P<0.0005) and moderate agreement visually (κ=0.48, 95% CI 0.23 to 0.73; P<0.0005). Agreement of subjective versus objective profiling was low (κ=0.23, 95% CI 0.04 to 0.42; P=0.02).
Conclusions MP profiles showed high heritability. Compared to visual assessment, objective profile classification is a more reliable method for future experimental studies using two-wavelength FAF.

• T075

The zinc-metallothionein redox system in human retina and RPE

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Purpose The retina contains the highest concentration of zinc in the human eye and it is primarily associated with the photoreceptors and the RPE. Metallothioneins (MTs) are the main cytosolic zinc-ion-binding proteins, and their main roles include neuroprotection and maintenance of cellular zinc homeostasis. Zinc is the main regulator of MTs, and there is a tight control in the number of atoms of zinc bound to the MT proteins (stoichiometry), which could be related with their antioxidant and neuroprotective functions. The main purpose of this work is to study the Zn-MT system in the RPE and retina of the human eye.
Methods We first determined the total content of Zn by elemental mass spectrometry (i.e., ICP-MS), in RPE and retina from ***post mortem*** human donors, and compared its quantitative distribution by bio-imaging (i.e, laser ablation-ICP-MS), on cryogenic eye sections. Secondly, we carried out the quantitative speciation of zinc in the water-soluble protein fractions of RPE and neural retina, to study its protein binding profile. Finally we studied the effects of metals (i.e., ZnSO4), inflammatory cytokines (i.e., IL1a) and glucocorticoids (i.e., dexamethasone), by ICP-MS, in an ***in vitro*** cellular model of human RPE cells (HRPEsv).
Results We found preferential quantitative distribution of zinc in the RPE, followed by the retina in lesser levels. Zinc is mainly associated to high and low molecular mass proteins in both RPE and retina. Exogenous zinc, interleukin and dexamethasone increased MT proteins synthesis and induced a stoichiometric change in MT proteins in HRPEsv cells.
Conclusions The stoichiometric transition on Zn-MT proteins in HRPEsv and its potential implication against oxidative stress processes will be discussed.

• T076

Retinal function and morphology in Mitf mutant mice

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Purpose The Mitf (microphthalmia-associated transcription factor) gene that is essential for the normal development of the retinal pigment epithelium (RPE). Mutations in this gene can cause hypopigmentation, microphthalmia and blindness. The purpose of this work was to analyze the retinal function and morphology in mice with specific Mitf mutations.
Methods The following Mitf mutations were used: Mitfmi-enu122 (398), Mitfmi-wh/+, Mitfmi-wh/Mitfmi and wild type (C5BL/6J) mice as a control. Mice were anesthetized by an intraperitoneal injection of 40 mg/kg-1 Ketamine and 4 mg/kg-1 Xylazine. Flash electroretinography (ERG), from mice with pupils dilated, with a corneal electrode and a reference electrode placed in the mouth, was used to determine the role of the MITF protein in retinal function. Histological retinal sections were stained with hematoxylin and eosin.
Results ERG recordings revealed that only one of the four mutants had any retinal function. The wild type mice had significantly higher mean amplitudes of the photopic a-waves and scotopic oscillatory potentials than the Mitfmi-enu122 (398) animals (α= 0,05). Furthermore, Mitfmi-enu122 (398) had significantly shorter implicit times for the photopic b-waves and c-waves. Histology revealed that the RPE layer in the Mitfmi-enu122 (398) and shows localized thinning of the RPE and their retinas look normal. However, the Mitfmi-wh/+ showed a profound RPE degeneration and this layer is missing from the Mitfmi-wh/Mitfmi animals. Furthermore, Mitfmi-wh/+ and Mitfmi-wh/Mitfmi have an immense retinal degeneration, lacking the photoreceptor and outer plexiform layers.
Conclusions This study demonstrates that the Mitf gene has an impact on retinal function in mice, and the morphology of the neuroretina and the RPE.

The zinc-metallothionein redox system in human retina and RPE

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T075

INTRODUCTION

The retina contains the highest concentration of zinc in the human eye and it is primarily associated with the photoreceptors and the RPE. Metallothioneins (MTs) are, so far, the main cytosolic zinc-ion-binding proteins known, involved in functions including neuroprotection and maintenance of cellular zinc homeostasis. Zinc is the main regulator of MTs, and there is a tight control in the number of atoms of zinc bound to the MT proteins (stoichiometry), which could be related with their antioxidant and neuroprotective functions. The main purpose of this work was to study the Zn-MT system in the RPE and retina of the human eye.

EXPERIMENTAL PROCEDURES

i) ***Total quantification of Zn***: Retina, RPE-choroids and sclera were dissected from normal human eye donors, mineralized, and Zn was quantified by elemental mass spectrometry (ICP-MS) and isotope dilution (ID) analysis. **ii)** ***Zinc quantitative bioimaging***: Quantitative distribution of zinc on cryogenic eye sections (35-µm) was carried by laser ablation (LA)-ICP-MS. **iii)** ***Zn quantitative speciation***: Quantification of Zn in the water-soluble cellular proteins, extracted from Retina and RPE, was carried out by ID-ICP-MS, following separation of zinc binding proteins by size exclusion chromatography. **iv)** ***Localization of MTs in HRPEsv cells* (*in vitro* human RPE cell line)**: Cellular distribution of MT proteins in HRPEsv cells was visualized by indirect immunofluorescence using a MTI/II isoform-specific antibody. **v)** ***Zinc distribution in HRPEsv cells***: Elemental distribution of Zn in HRPEsv cells was carried out by LA-ICP-MS on glass slides at -20ºC. **vi)** ***Study of Zn-MT system in HRPEsv cells***: HRPEsv cells were treated, in a dose-dependent manner with $^{68}ZnSO_4$ (25, 50 and 100 µM), glucocorticoids (500 nM Dexomethasone), and EPO (350 mU/mL), during 24h. The concentration of zinc, the absolute quantification and the stoichiometry of intracellular MTs, were determined by HPLC-ICP-MS.

RESULTS AND CONCLUSIONS

Zinc distribution in eye tissues: Zinc is highly concentrated in the RPE-choroids, followed by the retina and the sclera (Table 1). The quantitative bioimages obtained by LA-ICP-MS showed preferential distribution of Zn in the RPE, with up to 25 µg Zn/g tissue in certain regions (Fig 1). ***Quantitative Zn speciation***: Zinc in retina is mostly bound to low and medium molecular weight (LMW and MMW) proteins, whereas in RPE is mainly associated to LMW, probably MTs (Fig 2, Table 2). ***MTs and Zn localization in HRPEsv cells***: Intense labeling with MT-1/2 antibody was detected in both the cytoplasm and nuclei, and zinc is broadly distributed in the HRPEsv cells (Fig 3 and 4). ***Study of Zn-MT system in HRPEsv cells***: Exogenous zinc and dexamethasone increased MT proteins synthesis and induced a stoichiometric change in MT proteins in HRPEsv cells (Table 3).

Under control conditions, we found $Zn_{1.5}Cu_{0.7}$MT redox system in HRPEsv cells, while in previous studies we identified $Zn_3Cu_{0.2}$MT and Zn_6Cu_1MT in lens (in preparation) and cornea (Álvarez et al., 2012) epithelial cell lines, respectively. Our data suggest that MT proteins are in different redox states in the natural barriers combating free radicals, which may be related with their different antioxidant capacity. Functional studies are required to confirm this hypothesis.

Absolute quantification of Zn

Sample	Zn (µg Zn /g tissue)
Retina	7.3± 0.8
RPE-Choroids	14 ± 2
Sclera	4.8 ± 0.2

Table 1. Total concentration of Zn (in micrograms of Zn per gram of wet tissue) determined in human retina, RPE-choroids and sclera from 10 eyes (5 postmortem donors), by ID-ICP-MS.

Quantitative bioimaging of Zn in human eye tissue sections by LA-ICP-MS

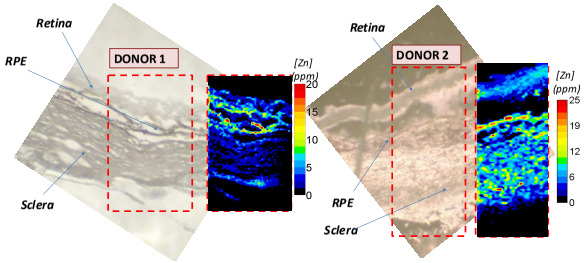

Fig 1. Quantitative distribution of zinc in two representative frozen sections of retina, RPE, choroids and sclera from human eyes obtained by single line scanning LA-ICP-MS (80% energy, 20 Hz, 25 µm, 10 µm·s-1, -20ºC). Matrix-matched laboratory standards, containing 0 to 100 ppm of Zn diluted in gelatin, were used for external calibration.

MTs localization in HRPEsv cells

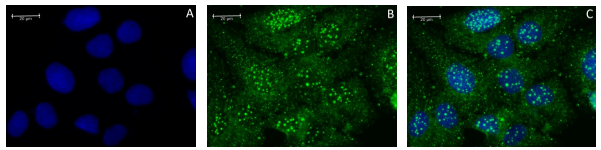

Fig 3. Cellular distribution of MTs in HRPEsv cells by Indirect immunofluorescence. (A) DAPI staining of cell nuclei. (B) MT1/2 labeled. (C) Merged image.

Zn distribution in HRPEsv cells

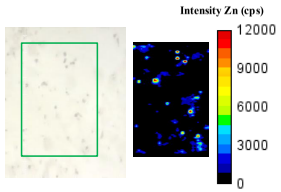

Fig 4. Elemental distribution of Zn in HRPEsv cells, obtained by LA-ICP-MS (Energy: 5.6 mJ/pulse; Repetition Rate: 20 Hz; Spot size: 10 µm; Scan speed: 4.31 µm s^{-1}; Carrier gas: He (1.0 L min^{-1}); Cell temperature: -20°C).

Quantitative speciation of Zn in human retina and RPE by HPLC-ID-ICP-MS.

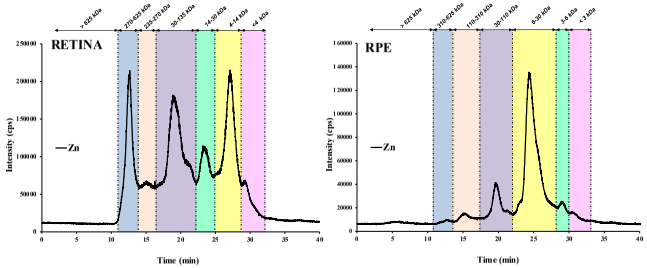

Fig 2. Example of Zn chromatographic profiles in the water-soluble protein fraction of retina and RPE, obtained by size exclusion (SEC)-ICP-MS. Y-axis represent counts per second of Zn, while X-axis indicates the retention time (in min). Dashed lines and colored rectangles denote the different regions of molecular mass of proteins.

RETINA	
MW	µg Zn/ g protein
Total Zn	120 ± 38
270- 625 kDa	17 ± 6
135- 270 kDa	10 ± 3
30- 135 kDa	36 ± 9
14- 30 kDa	19 ± 6
4- 14 kDa	37 ± 21

RPE	
MW	µg Zn/ g protein
Total Zn	76 ± 38
310- 625 kDa	4.0 ± 0.6
110- 310 kDa	7 ± 3
30- 110 kDa	15 ± 5
6- 30 kDa	38 ± 26
3- 6 kDa	6 ± 3
< 3 kDa	6 ± 3

Table 2. Quantitative speciation of Zn-bound to proteins (in micrograms of Zn per gram of extracted protein), in the water-soluble protein fraction of retina and RPE, obtained by HPLC-ID-ICP-MS. Data shown the average of 10 retinas and 10 RPEs from 5 postmortem donors analyzed (three analytical replicates per sample).

Differential induction of MTs in HRPEsv cells by EPO, Dexamethasone and Zn

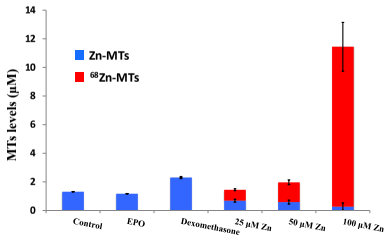

Fig 5. Absolute concentration of MTs (µM) in HRPEsv cells. HRPEsv cells were either not treated (control) or exposed to $^{68}ZnSO_4$ (at 25, 50 or 100 µM), Dexomethasone (500 nM), and EPO (350 mU/mL), for 24h. The enriched stable isotope $^{68}ZnSO_4$ preferentially binds to newly synthesized MT isoforms (**^{68}Zn-MTs**), and is distinguished from the natural form of Zinc bound to endogenous forms of MTs ( ) by HPLC-ICP-MS.

The elemental stoichiometry of MTs in HRPEsv cells

Treatment	Zn:Cu:MT stoichiometry
Control	1.5±0.3 : 0.7±0.3 : 1
EPO (350 mM)	2.0±0.4 : 0.14±0.01 : 1
Dexomethasone (500 nM)	3.0±0.1 : 0.2±0.1 : 1
25 µM $^{68}ZnSO_4$	2.4±0.1 : 0.60±0.01 : 1
50 µM $^{68}ZnSO_4$	4.6±0.3 : 0.05±0.02 : 1
100 µM $^{68}ZnSO_4$	6.8±0.4 : 0.05±0.02 : 1

Table 3. Stoichiometry of Zn-Cu-MT proteins in HRPEsv cells. The elemental stoichiometric composition of the MTs complexes in HRPEsv cells was calculated by the determination of the sulfur to metal (Zn, Cu) ratio, i.e., Zn:Cu:MT, considering that the number of sulfur atoms per MT molecule is known to be 21.

SUPPORTED BY:

Conflict of interest statement: None of the authors have commercial interest in the results of this work