The presence of CFH, HTRA1, ARMS2, VEGF-A and VEGF-R and the appearance of age-related macular degeneration sub-types

F. Cruz-González a,*, L. Cabrillo Estévez b, C. Cañete Campos a, A. Sánchez-Jara Sánchez a, L. Juan Marcos a, R. González-Sarmiento c

a Servicio de Oftalmología, Hospital Universitario de Salamanca, Salamanca, Spain
b Servicio de Oftalmología, Instituto Salmantino de Oftalmología, Salamanca, Spain
c Departamento de Medicina Molecular, Facultad de Medicina, Universidad de Salamanca, Salamanca, Spain

Objective: To demonstrate the genetic influence in the onset of the different age-related macular disease (AMD) subtypes by analysing the genotype distribution of CFH, ARMS2, HTRA1, VEGF-A and VEGF-R polymorphisms in patients with neovascular and atrophic AMD.

Materials and methods: The study was conducted on 101 consecutive patients with AMD diagnosis (74 exudative, 27 atrophic) following Wisconsin international classification criteria. The CFH rs1410996, ARMS2 rs10940923, VEGF-A rs833061, rs699947, and VEGF-R rs2071559 polymorphisms were analyzed using real time PCR with Taqman probes, and HTRA1 rs112000638 using restriction endonucleases digestion.

A study was made of the genotype distribution of the different polymorphisms in our group of patients with neovascular AMD and those with the atrophic type, and a comparison was made of the results for each one of the genes studied.

Results: No statistically significant differences (p > .05) were found in the genotype distribution of the different polymorphisms between patients with neovascular AMD and patients with atrophic AMD in our population, although the “risk” genotypes tended to appear more frequently in patients with neovascular AMD, despite the lack of statistical significance.

Conclusions: Allelic variants of CFH, ARMS2, HTRA1, VEGF-A or VEGF-R genes are not associated with the different AMD subtypes. This suggests that, although the polymorphisms seem to be associated with the disease susceptibility, they are not involved in the onset of the different clinical variants of AMD. Further studies in different populations, and with a larger cohort of patients, are needed to confirm these results.

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Relación de la presencia de polimorfismos de CFH, HTRA1, ARMS2, VEGF-A y VEGF-R con la aparición de los subtipos de degeneración macular asociada a la edad

RESUMEN

Objetivo: Demostrar la influencia genética en el desarrollo de los distintos tipos de degeneración macular asociada a la edad (DMAE) analizando las distribuciones genotípicas de polimorfismos de CFH, ARMS2, HTRA1, VEGF-A y VEGF-R en pacientes con DMAE exudativa y DMAE atrófica.

Método: Se tomaron 101 pacientes diagnosticados de DMAE (74 exudativa y 27 atrófica) según las normas del sistema internacional de clasificación Wisconsin. Analizamos los polimorfismos rs1410996 del gen CFH, rs10940923 de ARMA2, rs833061 y rs699947 de VEGF-A y rs2071559 de VEGF-R mediante PCR a tiempo real con sondas Taqman y el HTRA1 rs112000638 mediante digestión con endonucleasas de restricción.

Analizamos la distribución genotípica de los distintos polimorfismos en nuestro grupo de pacientes con DMAE exudativa y los que presentan DMAE atrófica y comparamos los resultados para cada uno de los genes a estudio.

Resultados: No encontramos diferencias estadísticamente significativas (p > 0,05) en la distribución genotípica de los distintos polimorfismos entre pacientes con DMAE atrófica y pacientes con DMAE exudativa en nuestra población, si bien los genotipos considerados «de riesgo» por otros estudios tienden a aparecer de forma más frecuente en la DMAE exudativa, a pesar de no obtener diferencias significativas.

Conclusiones: Las variantes alélicas de los genes CFH, ARMS2, HTRA1, VEGF-A o VEGF-R no se asocian con los diferentes subtipos de DMAE, lo que indica que, aunque parecen que están implicados en la susceptibilidad a padecer la enfermedad, no están implicados en el desarrollo de las variantes clínicas en nuestra población. Son necesarios nuevos estudios en diferentes poblaciones y con un mayor tamaño muestral para confirmar estos resultados.

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Introduction

Age-related macular degeneration (AMD) is a disorder of the photoreceptor-pigment epithelium complex of the retina-Bruch membrane-choriocapillaries. The importance of AMD lies in its prevalence and incidence as well as in the significant limitation it produces, being the first cause of legal blindness in the European population.

Genetic influence in the pathogeny of AMD is known due to studies carried out in families and twins. First degree relatives of individuals with AMD are at greater risk of having the disease, in addition to being affected at an earlier age and with higher probabilities of exhibiting the most severe forms of AMD.

Developments in the last decade in the study of macular and retinal dystrophies with monogenic inheritance provided important clues for initiating a study of the relationship of different genes with the onset of AMD. Some genes like CFH, ARMS2 and HTRA1 have evidenced a direct association with the disease. If said results are confirmed, the variations of these genes would be related with only a small fraction of the susceptibility to AMD.

A range of complete genome linking analyses have pointed toward the presence of a locus in 1q25–q31 associated with the disease. Case studies and controls have identified complement factor H (CFH) as the culprit. It has been consistently demonstrated that the CFHY402H variants, located in a fixation site for reactive C-protein, exhibit a strong association with the appearance of AMD.

The connection of said gene with AMD is not clear, but it has been verified that CFH is an important complement cascade regulator. Hageman et al. discovered that CFH and C3b/C3b are located between the drusen and pointed out that these regions are activation surfaces for complement cascade between the drusen and Bruch membrane. Researchers of the Rotterdam Eye Study have demonstrated that CFH is associated with all stages of AMD, from the first signs of early AMD such as drusen up to the late lesions that cause significant vision impairment.

Various studies affirm that there could be multiple susceptible alleles in the chromosome region of the CFH gene, with uncoded variants playing an important role in the susceptibility to the disease.

Two studies have pointed out the LOC387715/HTRA1 locus in 10q26 as the second most important locus in the pathogenesis of AMD. The Rivera study found the strongest association of ARMS2 with the increased risk of having AMD, demonstrating a probability increase of 7.6 for having the disease in homozygote individuals for the risk allele. These findings have been confirmed independently by various authors.

No differences were found in the distribution of risk alleles in ARMA2 between groups of patients with high risk early...
AMD and advanced AMD. This was also demonstrated for geographic atrophy and the exudative form of the disease. At present, it is not known whether the risk alleles of ARMA2 and CFH have a direct correlation with the severity of the disease. No studies have been made in this area with Spanish populations.

Parallel studies to those carried out in the adjacent regions of ARMS2 pointed at HTRA1 as one of the genes related to AMD susceptibility in chromosome 10q26. Based on the location of the ARMS2 rs10490924 polymorphism between PLEKHA1 and HTRA1, it was suspected that this simple nucleotide polymorphism (SNP) could be related to the variant of a different gene.

The implication of HTRA1 with the risk of AMD was subsequently confirmed in a Chinese cohort, where the presence of this protein was discovered in the drusen of patients with AMD. In addition, a higher expression of messenger RNA (mARN) of HTRA1 was found in the rs1200638 risk allele in lymphocytes and pigment epithelium of the retina of patients with AMD. The risk of disease associated with this gene demonstrated to be higher in the Asian population compared with Caucasians. A meta-analysis of 14 case and control studies on the polymorphism of this gene confirmed a strong association with the appearance of AMD, with high risk in homozygote subjects for the risk allele.

Since the initial discovery of the VEGF/VPF gene (known at present as VEGF-A), multiple additional closely related genes have been identified, such as VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PIGC). However, the role of said genes in angiogenesis seems more limited. The human VEGF-A gene is located in chromosome 6p21.3 and comprises 8 exons and 7 introns. VEGF-165 is the main isoform and the primary mediator for neovascularization in the eye.

The main promoter for the expression of VEGF-A in the eye is hypoxia. This gene contains a sequence in 5 which joins in the hypoxia-induced factor 1 (HIF). In a study comprising 512 patients with exudative AMD and 253 controls, the genetic influence of the expression of VEGF-A in the appearance of late disease lesions was studied. Overall, 29 SNP were studied without finding a statistically significant association with the appearance of wet AMD.

The VEGF protein joins 3 tyrosine kinase receptors that are closely related with each other: VEGF-R-1, VEGF-R-2 and VEGF-R-3. The VEGF-R-1 receptor was the first to be identified although its function is not yet clear. The VEGF-R-2 receptor is the main mediator of the pathological effects of VEGF in the eye. Joining the VEGF ligand with the VEGF-R-2 receptor produces dimerization and autophosphorylation, followed by the phosphorylation of numerous proteins related to the transduction of cellular signs such as phospholipase C, PI3-kinase, GTPase activation protein and the SRC protein family. A study was carried out in 2010 that verified the relationship of 22 SNP of the VEGF-R-2 gene with the susceptibility of exhibiting exudative AMD, finding a statistically significant association only with the infrequent haplotype (TTT).

New studies have found different loci related to the complement cascade that increase the risk of expression of AMD, which demonstrate its relationship with the disease and opening new pathways for genetic research.

Genetic polymorphisms would only explain a small part of AMD etiology. Being a very complex disease, various disease-modifying environmental factors have been studied, including tobacco and body mass index. Associating said environmental facts with the presence of different polymorphisms, it has been observed that the risk of having the disease increases exponentially, thus confirming the importance of epigenetics in its development.

Due to the results obtained by other authors in similar studies and that there is no similar study in the Spanish population, the authors have decided to study the polymorphisms of the above-described genes in relation to the appearance of atrophic and exudative AMD in the Spanish population. The selected polymorphisms were studied previously by this group in relation to the appearance of the disease, comparing them with a control group, obtaining statistically significant differences in the distribution of genotypes of the CFH rs1410996, ARMS2 rs10940923, HTRA1 rs11200638 and VEGFR-2 rs2071559 polymorphisms between the control group and the patients group. Due to the results it was decided to continue the research comparing said genetic variants between patients with different types of AMD. Even though other studies on genetics and AMD have been carried out in the Spanish population, this is the first to compare the differences in the genotypic distribution between patients with different types of AMD.

**Patients and methods**

The present study selected patients with any type of AMD according to the criteria of the Wisconsin classification system. After signing the informed consent in accordance with the legal rules for clinical studies in Spain and those of the Ethics Committee of the University Clinic Hospital of Salamanca, peripheral blood samples were taken of all patients diagnosed consecutively with any type of AMD in the Ophthalmology Dept. of the University Clinic Hospital of Salamanca from September 1, 2007 to September 4, 2009.

The study comprised 101 Caucasian patients, 56 males and 45 females, with a mean age of 77.5 years (±7.189). Of these, 74 were diagnosed with wet AMD whereas 27 exhibited atrophic AMD. Of the former, 50 were treated with intravitreal ranibizumab injection. No differences were appreciated concerning age or sex between patients with exudative and atrophic AMD (Table 1).

All patients underwent a complete examination in the ophthalmological practice comprising: data collection including risk factors, visual acuity, anterior and posterior pole exploration, retinography of both eyes and optic coherence tomography for all patients. Fluorescein angiography was performed when activated neovascularization was suspected.

The researchers analyzed polymorphisms rs1410996 of genes CFH, rs10940923 of ARMA2, rs833061 and rs699947 of VEGF-A and rs2071559 of VEGF-B by means of real time PCR utilizing probes marked with fluorochrome, utilizing the following commercial probes: C_2530294_10, C_31895102_10, C_1647381_10, C_8311602_10, C_15869271_10, respectively, manufactured by Applied Biosystems® (California, United States).
PCR with Taqman probes provide simultaneous amplification and detection processes in the same closed vial, without requiring subsequent actions. In addition, by means of fluorescence detection, it is possible to measure during amplification the amount of DNA being synthesized at all times, as the fluorescence emission produced in the reaction is proportional to the amount of formed DNA. This allows the assessment and recording of the amplification reaction kinetics at all times. The thermal cyclers for PCR with Taqman probes include a fluorescence reader and are designed for measuring at any point in time the fluorescence issued in each vial in which amplification is carried out. For allele discrimination, specific probes marked with fluorochrome were utilized.

For studying HTRA1 rs112000638 a different process was applied, utilizing digestion by means of restriction endonucleases due to the absence in the market of probes for this polymorphism. Endonuclease EaG1 was utilized as it recognizes the specific sequence of this polymorphism. Restriction endonucleases recognize specific DNA sequences and escind them at this point. The study of restriction fragment length polymorphisms is a technique that allows to discriminate different alleles of a gene, analyzing the size of fragments generated after the digestion of DNA with restriction enzymes. Digestion is carried out incubating 17 μL of the PCR product with 1μL of the selected restriction endonuclease (in the present case, EAG-1), utilizing as digestion tamponade that which is specific for each endonuclease or a universal tamponade, placed at the specific temperature during a period ranging between 5 and 7 h. The fragments obtained after digestion was separated with electrophoresis in 1% or 3% agarose gel stained with Syber-safe® (Thermo Fisher scientific, Waltham Massachusets, United States). All the gels included the size marker. For monitoring the migration of DNA in the gel, 2 stains were included in the loading tamponade: xylene-cyanol and bromophenol blue. Subsequently, a digital photograph was taken under ultraviolet lighting utilizing the Kodak Science ID application (Kodak SA, Rochester, NY, United States).

The Hardy–Weinberg equilibrium was assessed for determining the genotypes that exceeded the standard distribution. The analyses between 2 groups were carried out utilizing the T for student test, applying ANOVA for over 2 variables. The qualitative variables were analyzed with Pearson’s χ² test. The magnitude of association was expressed with odds ratio and precision with a confidence interval of 95%. Statistical data were processed with the SPSS application (version 19.0. SPSS Inc., Chicago, IL, United States).

### Results

The differences in the distribution of polymorphisms studied between patients with atrophic AMD and exudative AMD were analyzed on the basis of a co-dominant genetic model.

No significant differences were found when comparing within the group of patients the distribution of CFH rs141996 polymorphism between patients with the exudative and atrophic form of AMD (Table 2). Likewise, when grouping A and G allele patients, no statistically significant differences were found (Table 3).

No differences were found in the distribution of ARMA2 rs10490923 polymorphism between patients exhibiting the

| Table 1 - Age and sex distribution of patients with exudative and atrophic AMD. |
|-----------------|-----------------|-----------------|
|                 | Exudative AMD   | Atrophic AMD    | p    |
| Age             |                 |                 |      |
| 78.93 ± 7.004   | 74.52 ± 6.835   | 0.356           |
| Sex %           |                 |                 |      |
| 47.3 males      | 37 males        | 0.123           |
| 52.7 females    | 63 females      |                 |
| Total           | 74              | 27              |      |

| Table 2 - Genotypic distribution of CFH, ARMA2, HTRA1, VEGF-A and VEGF-R polymorphisms in the group of patients with exudative and atrophic AMD. |
|-----------------|-----------------|-----------------|-----------------|
| Gene            | Genotype        | Exudative n (%) | Atrophic n (%) | p    |
| CFH             | AA              | 5 (6.8)         | 2 (7.4)        | 0.504|
| rs1410996       | AG              | 37 (50)         | 10 (37)        |      |
|                 | GG              | 32 (43.2)       | 15 (55.6)      |      |
| ARMS2           | GG              | 23 (31.1)       | 14 (51.9)      | 0.149|
| rs10490923      | GT              | 36 (48.6)       | 10 (37)        |      |
|                 | TT              | 15 (20.3)       | 3 (11.1)       |      |
| HTRA1 -625      | AA              | 15 (20.3)       | 9 (33.3)       | 0.307|
|                 | AG              | 37 (50)         | 13 (48.1)      |      |
|                 | GG              | 22 (29.7)       | 5 (18.5)       |      |
| VEGF-A          | AA              | 11 (14.9)       | 5 (18.5)       | 0.277|
| rs833061        | AC              | 37 (50)         | 17 (63)        |      |
|                 | CC              | 26 (35.1)       | 5 (18.5)       |      |
| VEGF-A          | CC              | 13 (17.6)       | 5 (18.5)       | 0.311|
| rs699947        | CT              | 36 (48.6)       | 17 (63)        |      |
|                 | TT              | 25 (33.8)       | 5 (18.5)       |      |
| VEGFR           | AA              | 16 (21.6)       | 8 (29.6)       | 0.549|
| rs2071559       | AG              | 33 (44.6)       | 9 (33.3)       |      |
|                 | GG              | 25 (33.8)       | 10 (37)        |      |
Exudative and atrophic form of AMD (Table 2). Similarly, no differences were found when grouping allele A patients or when grouping allele G patients (Table 3).

When analyzing the genotypic distribution of VEGF-A rs833061 and rs699947 polymorphisms and VEGF-R rs2071559 polymorphisms, no statistically significant differences were found (Table 2) patients carrying one of the polymorphism alleles (Table 3).

Finally, no differences were found between patients with atrophic AMD and those with exudative AMD in relation to HTRA1 rs112000638 polymorphism (Tables 2 and 3).

### Discussion

No significant differences were found in the genotypic distribution of rs1410996 polymorphism between the groups with exudative and atrophic AMD (Table 2). However, a tendency of the homozygote group was identified for allele G in a higher proportion of exudative AMD patients, matching other studies with various CFH SNPs. In the present case, the sample of patients with atrophic AMD is not very large and therefore, when dividing the group between different genotypes, a small number remains and this could explain why the differences were not statistically significant. As indicated above, in previous studies various CFH SNP had demonstrated an influence in the appearance of exudative AMD.

No differences were found in the genotypic distribution of rs10490924 between the group of patients with exudative and atrophic AMD. However, when grouping patients carrying the T allele, a tendency was found (p = 0.055) toward a greater presence of exudative AMD in this group (Table 3). Some studies did find a relationship between the expression of the exudative form of the disease with the expression of the T risk allele. However, this relationship was not consistently confirmed in other studies. In the present case, even though a statistically significant conclusion was not found, a tendency to an increased risk of neovascular AMD was appreciated in the group expressing the T allele.

No differences were observed when comparing the genotypic distribution of HTRA rs112000638 polymorphism in patients with exudative AMD with those having atrophic AMD (Table 2). No studies were found confirming this relationship. It seems that the increased expression of HTRA1 could enhance the appearance of the disease in any of its variants.

When analyzing the genotypic distribution comparing between patients with neovascular and atrophic AMD, no significant differences were found either (Table 2) for any of the studied VEGF-A polymorphism. Likewise, another study did not find differences in the genotypic expression that could confirm increased risk of neovascular AMD. Despite the important role played by VEGF in the appearance of choroidal neovascularization in patients with AMD, these data are confirmed in other studies.

Differences were found in the distribution of the polymorphism (rs2071559) of gene VEGF-R-2 when comparing patients with exudative and atrophic AMD (Table 2). Even though the appearance of choroidal neovascularization secondary to AMD is clearly influenced by the activity of the VEGF-R receptor, no differences were obtained in the genotypic distribution per AMD type. To date, no other studies on this matter have been found.

### Table 3 – Genotypic distribution of CFH, ARMA2, HTRA1, VEGF-A and VEGF-R polymorphisms in the group of patients with exudative and atrophic AMD, grouped per allele.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Exudative</th>
<th>Atrophic</th>
<th>p</th>
</tr>
</thead>
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<tr>
<td>CFH</td>
<td>AA</td>
<td>5 (6.8)</td>
<td>2 (7.4)</td>
<td>0.909</td>
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<tr>
<td></td>
<td>AG + GG</td>
<td>69 (93.2)</td>
<td>25 (92.6)</td>
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<tr>
<td></td>
<td>AA + AG</td>
<td>42 (56.8)</td>
<td>12 (44.4)</td>
<td>0.272</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>32 (43.2)</td>
<td>15 (55.6)</td>
<td></td>
</tr>
<tr>
<td>ARMS2</td>
<td>GG</td>
<td>23 (31.1)</td>
<td>14 (51.9)</td>
<td>0.055</td>
</tr>
<tr>
<td>rs10490923</td>
<td>GT + TT</td>
<td>51 (68.9)</td>
<td>13 (48.1)</td>
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<tr>
<td></td>
<td>GG + GT</td>
<td>59 (79.7)</td>
<td>24 (88.9)</td>
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</tr>
<tr>
<td></td>
<td>TT</td>
<td>15 (20.3)</td>
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<tr>
<td>HTRA1</td>
<td>AA</td>
<td>15 (20.3)</td>
<td>9 (33.3)</td>
<td>0.172</td>
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<tr>
<td>–625</td>
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<td>18 (66.7)</td>
<td></td>
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<tr>
<td></td>
<td>AA + AG</td>
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<td>GG</td>
<td>22 (29.7)</td>
<td>5 (18.5)</td>
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<tr>
<td>VEGF-A</td>
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<td>5 (18.5)</td>
<td>0.912</td>
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<tr>
<td>rs833061</td>
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<tr>
<td></td>
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<td>49 (66.2)</td>
<td>22 (81.5)</td>
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<tr>
<td></td>
<td>TT</td>
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<tr>
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<td></td>
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<tr>
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<td>8 (29.6)</td>
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The contribution of genetics in the appearance of any AMD subtype is probably related to different loci. The combined effects of the different genetic variants and the interactions between genes and environmental factors make it very complicated to obtain more significant results. The polymorphisms of different genes would only explain a small part of the risk of expressing the disease because AMD has a very complex etiology. Various risk factors such as body mass index and tobacco smoking should be considered in subsequent studies.

Accordingly, even though the sample size advises precaution in the interpretation of the results, it can be concluded that the allele variations of CFH, ARMS, HTRA, VEGF-A or VEGF-R genes are not associated with the different subtypes of AMD in the studied sample. This indicates that, even though said genes are involved in the vulnerability for expressing AMD, they are not involved in the development of the clinical variants in the studied population. Even so, the results of this study should be confirmed in subsequent studies with different populations and larger sample sizes. The importance of this study lies in being the first to analyze the genetic differences between AMD subtypes in the Spanish population.

Conflict of interest

The authors state that there is no conflict of financial or personal interests related to the present study.

References


