CFH haplotypes and *ARMS2*, *C2*, *C3*, and *CFB* alleles show association with susceptibility to age-related macular degeneration in Mexicans

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Purpose: To evaluate the contribution of genetic variants of complement factor H (*CFH*), complement component 2 and 3 (*C2* and *C3*), complement factor B (*CFB*), and age-related maculopathy susceptibility 2 (*ARMS2*) to age-related macular degeneration (AMD) risk in the Mexican Mestizo population.
Methods: Analysis included 282 unrelated Mexican patients with advanced AMD, 205 healthy controls, and 280 population controls. Stereoscopic fundus images were graded on the Clinical Age-Related Maculopathy System (CARMS). We designed a resequencing strategy using primers with M13 adaptor for the 23 exons of the *CFH* gene in a subgroup of 96 individuals clinically evaluated: 48 AMD cases and 48 age- and sex-matched healthy controls. Single nucleotide polymorphisms (SNPs) in *C3* (Arg80Gly and Pro292Leu), *C2* (rs547154), *CFB* (Leu9His), and *ARMS2* (Ala69Ser) were genotyped in all patients, healthy and population controls using TaqMan assay.
Results: All evaluated individuals were Mexican Mestizos, and their genetic ancestry was validated using 224 ancestry informative markers and calculating F_{st} values. The *CFH* resequencing revealed 19 SNPs and a common variant in the intron 2 splice acceptor site; three *CFH* haplotypes inferred from individual genotypes, showed significant differences between cases and controls. The risk alleles in *C3* (rs1047286, odds ratio [OR]=2.48, 95% confidence interval [CI]=1.64–3.75, p=1.59E-05; rs2230199, OR=2.15, 95% CI=1.48–3.13, p=6.28E-05) and in *ARMS2* (rs10490924, OR=3.09, 95% CI=2.48–3.86, p=5.42E-23) were strongly associated with risk of AMD. The protective effect of alleles in *C2* (rs547154) and *CFB* (rs4151667) showed a trend but was not significantly associated after correction for multiple testing.
Conclusions: Our results show that *ARMS2* and *C3* are major contributors to advanced AMD in Mexican patients, while the contributions of *CFH*, *C2*, and *CFB* are minor to those of other populations, reveling significant ethnic differences in minor allele frequencies. We provide evidence that two specific common haplotypes in the *CFH* gene predispose individuals to AMD, while another may confer reduced risk of disease in this admixed population.

Age-related macular degeneration (AMD) is a late-onset disease recognized as a serious condition that leads to vision loss in a growing number of elderly persons [1,2]. Early AMD is characterized by the presence of medium-size drusen or retinal pigment abnormalities; while advanced or late AMD can be either non-neovascular (dry, atrophic, or nonexudative) or neovascular (wet or exudative). The advanced non-neovascular form is characterized by drusen and geographic atrophy extending to the center of the macula, whereas advanced neovascular AMD is characterized by choroidal neovascularization and its sequelae [3]. In the U.S. population, more than 8 million have AMD, and the prevalence of the advanced form is projected to increase by more than 50% by 2020 [4]. In the United States, the highest prevalence of most AMD lesions was found in persons aged 60 years and older [5]. Prevalence of advanced AMD in self-identified Latinos from Los Angeles, California, increased from 0% in those 40–49 years of age to 8.5% in those 80 or older, and that of early AMD from 6.2% to 29.7% [6].

One of the earliest genome-wide association studies (GWASs) examined AMD and demonstrated that a common variant in the complement factor H (*CFH*) gene was strongly associated with the disease [7]. *CFH* resequencing revealed a polymorphism in linkage disequilibrium (LD) with the

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risk allele representing an amino acid change, Tyr402His (rs1061170) [7]. Later, several groups replicated the finding [8], and new *CFH* variants were reported [9,10]. In addition, other polymorphisms in *CFH* besides Tyr402His appear to increase the risk for developing AMD in Asians [11,12]. Multivariate models have shown that variables such as ethnic group are significantly associated with the incidence of AMD [13], indicating that the molecular basis of the disease is polygenic, involving multiple genes, and with different levels of severity by ethnicity [14,15]. In fact, racial differences between Latinos, Asian Americans, and Caucasians have been proposed to explain the differences in the risk of developing nonexudative and exudative AMD among these ethnic groups [16]. Thus, the AMD genetic risk and the frequency of associated genetic polymorphisms differ across ethnicities [15]. For instance, while white and black groups show the highest frequency for the Tyr402His polymorphism, Hispanics and Chinese have the lowest frequencies of this variant [17]. In addition, it has been suggested that although the Tyr402His variant appears to play a causal role in the etiology of AMD, this polymorphism could be simply in LD with additional nearby alleles that might show even stronger association [18].

However, additionally AMD susceptibility loci have been identified in several gene regions, including *ARMS2/HTRA1* (Ala69Ser, rs10490924) [19], as well as others related to complement pathway genes such as factor B (*CFB*) (Leu9His, rs4151667), *C2* (rs547154), and *C3* (Pro292Leu, rs1047286 and Arg80Gly, rs2230199) [20-22]. Similar to the differences in frequency reported for Tyr402His across populations, the risk allele frequencies of Ala69Ser diverge greatly between European and Asian populations from the HapMap International Project, with 45% in Asian populations compared to 21% in Caucasians (HapMap). The Mexican population is mainly composed of Mestizos, who are individuals with a genetic background consisting of Amerindian, European, and, to a lesser extent, African ancestries. As a result of admixture, the resulting linkage disequilibrium (LD) patterns in this locus may affect the frequency and effect of the variants previously associated with AMD, and may enable the identification of additional AMD-related variants [23,24].

Therefore, we aimed to analyze the genetic variation in *CFH,* and evaluate the role of known polymorphisms in *ARMS*, *CFB*, *C2,* and *C3* in AMD risk in a Mexican population. For this purpose, all 23 exons of *CFH* were resequenced in 96 Mexican subjects (48 AMD cases and 48 controls), and genotypes of seven previously AMD-associated single nucleotide polymorphisms (SNPs; rs10490924, rs4151667, rs547154, rs1047286, rs2230199, rs1410996, and rs1061170)

were investigated in 754 unrelated Mexican Mestizos: 273 patients with advanced AMD, 201 healthy controls, and 280 population controls. The results of our study strengthen the understanding of the genetic contribution of the *CFH, ARMS*, *CFB*, *C2*, and *C3* genes to susceptibility of AMD in Mexicans or other Latin American populations.

METHODS

Subjects: The protocol adhered to the tenets of the Declaration of Helsinki. This study was approved by the Scientific, Ethics, and Biosafety Review boards from the Instituto Nacional de Medicina Genómica (INMEGEN, Mexico). Informed consent was obtained from all individuals.

Patients with AMD and healthy controls were unrelated men and women aged >60 years enrolled at the Institute of Ophthalmology Conde de Valenciana and Hospital Dr. Luis Sánchez Bulnes de la Asociación Para Evitar la Ceguera in Mexico City. A total of 282 unrelated Mestizo Mexican patients with either of the two forms of advanced AMD (geographic atrophy or neovascular) and 205 healthy, age-matched controls were recruited and examined by an ophthalmologist. Stereoscopic fundus images of the macular region were obtained from each patient with AMD and healthy control for evaluation and diagnosis; thus, the groups of cases and healthy controls were equally rigorously clinically evaluated. The images were then graded based on the Clinical Age-Related Maculopathy System (CARMS), which categorizes patients into five mutually exclusive groups based on assessment of drusen, retinal pigment epithelial irregularities, geographic atrophy, retinal pigment epithelial detachment, and choroidal neovascularization [25]. In addition, we analyzed DNA samples from 280 population controls, individuals from six different regions in Mexico from the Mexican genome diversity project (MGDP) [24], and 156 Mexican Amerindians from two ethnic groups, Mayas and Zapotecos. The population controls were recruited with the aim of analyzing genetic population structure within Mexico and were not assessed for AMD status.

Genotyping: Genomic DNA was extracted from peripheral-blood leukocytes using the QIAamp DNA Blood Maxi Kit (Qiagen, Hilden, Germany). We selected seven SNPs that have been strongly associated with AMD in several ethnic groups, including two in the *CFH* gene (rs1061170 [Tyr402His] and rs1410996), two in *C3* (rs2230199 [Arg80Gly] and rs1047286 [Pro292Leu]), one in *C2* (rs547154 [IVS10]), one in *CFB* (rs4151667 [Leu9His]), and one in *ARMS2* (rs10490924 [Ala69Ser]). We performed genotyping with the use of an allelic discrimination assay with TaqMan SNP genotyping

probes (Applied Biosystems, Foster City, CA), following the manufacturer's protocol.

Complement factor H resequencing: The entire *CFH* gene was resequenced in a subgroup of 96 individuals clinically evaluated: 48 AMD cases and 48 age- and sex-matched healthy controls. The subset of 48 cases was AMD Grade 5 according to the CARMS system, and were selected based on age (75±8.0) and sex (same number of men and women), to minimize the age and gender differences between them and the healthy control group used in the same analysis. All 23 exons of *CFH* were amplified in a GeneAmp PCR System 9700 (Applied Biosystems). The amplifications (600 bp on average) were performed with a proofreading DNA polymerase, Pfu Ultra II Fusion HS DNA Polymerase (Stratagene, La Jolla, CA), in a total volume of 25 μl PCR. Final concentrations were the following: 0.3 mM deoxynucleotide triphosphate mixture (Takara Bio, Shiga, Japan), 1 mM Mg_2SO_4, 0.3 mM primer mix (10 uM each), 1 U Pfu Ultra II Fusion HS DNA Polymerase, and 50 ng DNA template. The PCR products were cleaned up and then sequenced using the BigDye Terminator V3.1 and M13 universal primers on a 3730×l DNA Analyzer (Applied Biosystems). Sequences were analyzed using Module SeqMan V7.0 (Lasergene software V7.0, DNASTAR, Madison, WI). The reference gene sequences were obtained from GenBank, accession number NG_007259, version: NG_007259.1 RefSeqGene, 102,494 bp. Primers pair sequences used for the 23 exons are shown in Appendix 1; 16 had been previously reported [26].

Complement factor H linkage disequilibrium and haplotypes inference: Linkage disequilibrium analysis was done with Haploview 4.0 [27]. Haplotypes were inferred from individual genotypes using the software PHASEv2.1 [28,29]. Conditions used for the haplotype inference procedure using 23 biallelic SNPs consisted of 100 iterations and one thinning interval step through the Markov chain.

Genetic ancestry analysis: To verify the distribution of the ancestral components between the case and control samples, we used 224 ancestry informative markers (AIMs) [24], which were SNPs with allele frequency differences (δ)≥0.4 for pairwise comparisons between the HapMap and Zapotecos groups. These AIMs were identified in the MGDP [24] and are publicly available upon request. Thus, we genotyped this set in our sample of cases and healthy controls using a customized microarray by Illumina (GoldenGate, San Diego, CA). The principal component analysis (PCA) and F_{st} were performed with EIGENSOFT 4.2 (Harvard, MA) [30,31].

Statistical analysis: PLINK association analysis toolset was used in data analysis [32]. Hardy–Weinberg equilibrium was checked for all SNPs evaluated in the population and healthy controls. Logistic regression analyses and further adjustment for appropriate covariates, such as age and sex, were applied to evaluate whether the SNPs were associated with AMD. The significance values were adjusted for multiple testing, and Bonferroni adjusted p values were reported.

RESULTS

Data on disease status, sex, and age are provided in Table 1. In our sample, 32.3% of cases had geographic atrophy (CARMS 4) while 63.7% had choroidal neovascularization (CARMS 5). All evaluated individuals were Mexican Mestizos, and their genetic ancestry was validated using a PCA and calculating the F_{st} values. The variation observed between our sample groups represents the distribution of ancestral components previously reported in the Mexican population [24], thus indicating that our study was not biased by a population stratification effect (Appendix 2 and Appendix 3).

Analysis of genetic structure of CFH coding region in a Mexican population: The *CFH* coding region (23 exons and the flanking sequences) was resequenced in 96 individuals (48 AMD cases and 48 healthy controls). This assay revealed 19 SNPs that have been observed previously, and a common variant in the intron 2 splice acceptor site, IVS2–18insTT. Two novel synonymous coding variants were found, one in the controls' exon 5 (Asp194Asp, minor allele frequency=1.06%), and the other one in cases' exon 17 (Gln816Gln, minor allele frequency=2.2%). The frequency distributions of the minor allele for all variants and their respective disease odds ratios (ORs) are provided in Appendix 4. Eleven SNPs showed significant allele frequency differences between the cases and controls; eight of them were more frequent in the cases than in the controls and had ORs ≥2.84, including Tyr402His. Interestingly, SNPs rs551397 in intron 1, Val62Ile (rs800292) in exon 2, and Ala473Ala (rs2274700) in exon 11 were observed more frequently in the controls than in the cases (OR=0.45, 95% confidence interval [CI]=0.22–0.93, p=3.17E-02; OR=0.45, 95% CI=0.22–0.93, p=3.17E-02; and OR=0.47, 95% CI=0.23–0.94, p=3.49E-02, respectively), suggesting that they confer a protective effect against AMD. However, after adjusting for multiple testing, these three suggestive protective variants were not significant.

LD analysis performed on 96 Mexican individuals showed extensive LD across an extended region of *CFH* (Figure 1). Five SNPs located in exons 7–9 were in virtually complete LD, and the same was observed for variants in exon 14 and exon 19 (r^2=0.82) and SNPs in exon 7 and intron 16 (r^2=0.82). Haplotype construction was done considering all polymorphisms found with direct sequencing. The haplotypes were inferred from the genotypes of 96
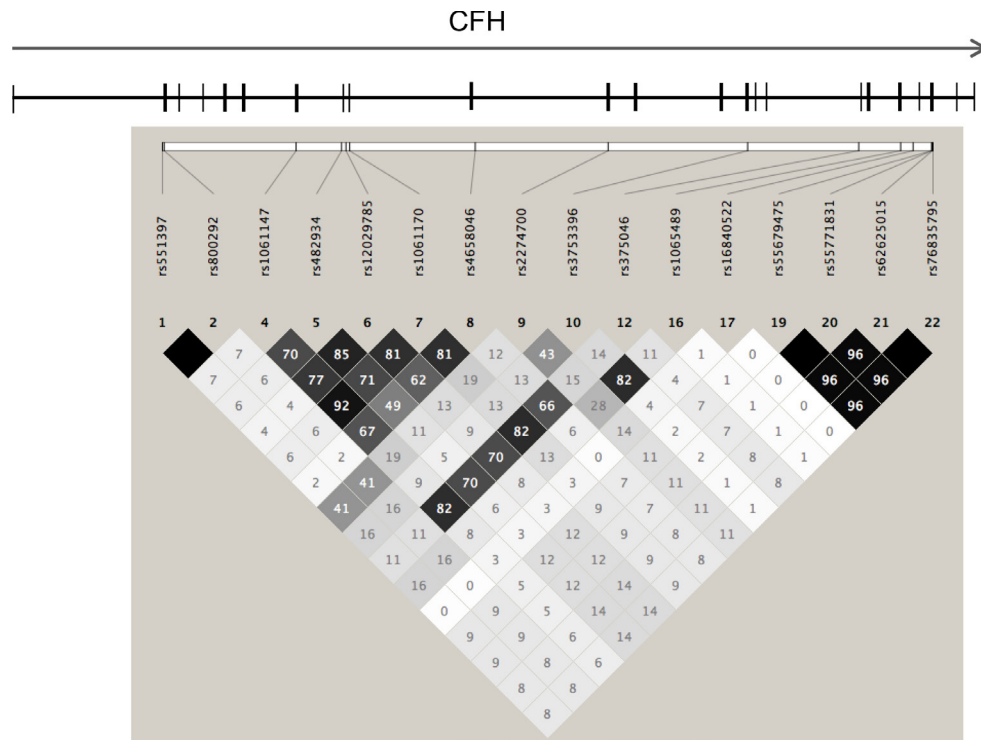
Figure 1. Linkage disequilibrium between the single nucleotide polymorphisms identified by resequencing in the *CFH* gene in Mexicans. The LD for SNPs is displayed in white for $r^2=0$; shades of gray for $0<r^2<1$; and black for $r^2=1$. The r^2 values between any two SNPs is listed in the cross cells. At the top, a schematic representation of *CFH* shows exons and introns where are located each SNP.

individuals, and the eight haplotypes that had a frequency greater than 4% are shown in Table 2. *CFH* haplotypes 6, 7, and 8 showed differences between the cases and the controls, 0.0% versus 4.2%, 9.4% versus 2.1% and 11.5% versus 0%, respectively. Haplotype 6, which was not present in the cases, comprised the three protective SNPs (rs551397, rs800292

and rs2274700). *CFH* haplotype 7 included the six AMD risk SNPs that conserved significant p values after adjustment for multiple testing (rs1061147, rs482934, rs12029785, rs1061170, rs4658046, and rs375046) and the four SNPs identified in exon 21. Haplotype 8 was not present in the controls and comprised the six risk alleles.

Variable	Cases	Controls	
		Healthy	Population
Disease status - no.	282	205	280
CARMS			
1	5		
2	39		
3	6		
4	41		
5	179		
Sex - no. (%)			
Male	91 (32.0)	62 (30.2)	140 (50.0)
Female	191 (68.0)	143 (69.8)	140 (50.0)
Mean age* - yr	76±8.0	65.5±9.8	NA

TABLE 1. DISEASE STATUS, SEX AND AGE OF AMD PATIENTS AND CONTROLS.

Stereoscopic fundus images of the macular region were obtained from each AMD patient and healthy control for evaluation and diagnosis. The population controls were recruited with the aim of analyzing genetic population structure within Mexico and were not assessed for AMD status. *Plus-minus values are means ± Standard Deviation (SD). NA: Not available.

TABLE 2. FREQUENCIES OF HAPLOTYPES IDENTIFIED IN *CFH* GENE IN MEXICANS AMD PATIENTS AND CONTROLS.

Haplo- type number	SNP*																Frequency (%)		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Cases (n=48)	Healthy controls (n=48)	*p*-value
1	*T*	*A*	C	T	A	T	T	G	A	A	G	T	T	G	C	G	20.8	25	NS
2	C	G	C	T	A	T	*C*	G	*G*	A	*T*	T	T	G	C	G	26.0	17.7	NS
3	C	G	C	T	A	T	T	*A*	*G*	A	*T*	T	*C*	*C*	*G*	*A*	5.2	6.3	NS
4	C	G	C	T	A	T	T	G	A	A	G	*C*	T	G	C	G	9.4	6.3	NS
5	C	G	C	T	A	T	T	G	A	A	G	T	T	G	C	G	2.1	6.3	NS
6	*T*	*A*	C	T	A	T	T	*A*	*G*	A	*T*	T	T	G	C	*A*	0.0	4.2	0.043
7	C	G	*A*	*G*	*G*	*C*	*C*	*A*	A	*C*	G	T	*C*	*C*	*G*	*A*	9.4	2.1	0.03
8	C	G	*A*	*G*	*G*	*C*	*C*	*A*	A	*C*	G	T	T	G	C	G	11.5	0	0.001

Capital letter represent the common allele and capital letters in bold and italic represent the allelic change of the SNP. *SNP1= rs551397; SNP2= rs800292; SNP3= rs1061147; SNP4= rs482934; SNP5= rs12029785; SNP6= rs1061170; SNP7= rs4658046; SNP8= rs2274700; SNP9= rs3753396; SNP10= rs375046; SNP11= rs1065489; SNP12= rs16840522; SNP13= rs55679475; SNP14= rs55771831; SNP15= rs62625015; SNP16= rs76835795 NS= no significant

AMD susceptibility alleles in CFH, C3, C2, CFB, and *ARMS2* genes: Seven SNPs previously associated with AMD in the *CFH, C3, C2, CFB*, and *ARMS2* genes were analyzed in 273 unrelated patients with advanced AMD (geographic atrophy or choroidal neovascularization) and 201 healthy controls. Nine cases and four healthy controls were discarded for genotyping because of quality control of call rate <70% SNPs per individual. Moreover, we adopted an intraethnic experimental design by analyzing 280 population controls from six different regions in Mexico, northern, southern, central and both coastal, western-Pacific and east, with ancestral component differences previously described [24].

All 754 samples were genotyped to determine the allele frequencies in three groups for the seven SNPs. The fact that we observed few differences between these two control groups indicates that there would be little bias due to use of both samples as a control group and justifies our combining two control groups to form a single group of 481 reference subjects. Screening of the seven SNPs revealed that the risk alleles in *CFH* (rs1410996, p=6.81E-09; rs1061170, p=1.02E-06), in *C3* (rs1047286, p=1.59E-05; rs2230199, p=6.28E-05) and in *ARMS2* (rs10490924, p=5.42E-23) were strongly associated with AMD (Table 3). Previously identified protective alleles in *C2* (rs547154, p=3.12E-02, OR=0.54, 95% CI=0.30–0.94) and *CFB* (rs4151667, p=5.06E-02, OR=0.34, 95% CI=0.12–1.00) were also found associated with reduced risk of AMD in this group. However, after adjusting for multiple testing, these two suggestive protective SNPs were not significant (Table 3).

DISCUSSION

To analyze the genetic structure at the *CFH* locus and identify additional susceptibility alleles for AMD, we resequenced all 23 exons of *CFH* in 96 Mexicans, 48 cases and 48 controls. We estimate that more than 99% of polymorphic sites with a minimal allele frequency of 1% were detected, in the 192 haploid genomes (96 individuals) evaluated in our study, and about 95% of all polymorphic sites with a similar frequency in the cases and the healthy controls [33]. We did not find novel alleles with potential functional effect in the resequenced region; however, we could not exclude the possibility of genetic contributions of unidentified polymorphisms in intron or regulatory regions to AMD.

We identified eight *CFH* haplotypes with frequencies higher than 4% in at least one of the groups (Table 2). Within these, haplotype 6 including three alleles that showed a suggestive protective effect for AMD development, T in rs551397 (intron 1), A in rs800292 (Val62Ile, exon 2), and A in rs2274700 (Ala473Ala, exon 11), was more frequent in the

controls (4.2% versus 0%) than in the cases (p value=0.043). Interestingly, this is consistent with the study by Ng et al., who showed these three alleles are part of haplotypes that conferred a reduced risk of AMD in a Chinese population [34]. In addition, one common extended haplotype in the *CFH* gene has been described as associated with lower risk if atypical hemolytic uremic syndrome (aHUS), membranoproliferative glomerulonephritis type II or dense deposit disease (MPGN2/DDD), and AMD [8,35]. This *CFH* haplotype carries the Val62Ile variant within the short consensus repeat 1 (SCR1) domain in the N-terminal region that is essential for factor H (fH) regulatory activities [8]. Tortajada et al. showed that the fH-Ile62 variant exhibits increased binding to C3b compared with fH-Val62 and is a more efficient cofactor for factor I in the proteolytic inactivation of C3b [36]. These findings provide evidence of *CFH* haplotypes that may have a protective effect for AMD; however, these results need further validation in an independent or larger sample size.

Haplotypes 7 and 8 were more frequent in the cases than in the controls (p value=0.030 and 0.001, respectively). Both haplotypes include two risk SNPs previously reported, Ala307Ala (rs1061147) in exon 7 and Tyr402His (rs1061170) in exon 9 [8,18], and the rs375046 in intron 16. Tyr402His has shown a functional effect, because it lies within SCR7 domain in fH, which binds heparin and C-reactive protein [8]. We found that rs375046 is in high LD with rs1061170 and rs1061147 (r²=0.82), and shows association with AMD risk, while in a Chinese population the frequency distribution of these variants is different and they are not associated [34].

In our population, the LD between Ala473Ala (rs2274700) and Gln672Gln (rs3753396) was less than that reported in a Chinese population, where both SNPs were significantly associated with exudative ADM [34]. The SNP Gln672Gln was not associated with AMD risk in our study and neither in Caucasians [8]. The frequencies pattern of this SNP and Ala473Ala suggests that the LD in our population is similar to that of Caucasians. Therefore, our results highlight the importance of considering LD differences observed in the *CFH* region and the frequencies patterns of genetic variants between populations to interpret the results of association studies and determine genetic susceptibility to AMD. In this context, haplotype and conditional analysis of individual variants should be performed to better understand the interaction between the different variants.

The AMD-associated variants in *CFH, C2, C3, CFB*, and *ARMS2* have been established in populations worldwide [10,12,19,20,22,37,38]. However, genetic risk and the frequency of associated genetic polymorphisms differ across ethnicities [15]. Thus, when we compared the frequency of

TABLE 3. ALLELIC ASSOCIATION OF SNPS IN *CFH*, *ARMS2*, *C2*, *CFB* AND *C3* GENES TO AMD IN MEXICANS.

Gene	SNP (Allele)	Allele frequency (%)			Association results			
		Cases n=273	Healthy controls n=201	Healthy and population controls n=481	Healthy controls*		Healthy and population controls	
					OR (CI 0.95)	*p*-value (*p*-value†)	OR (CI 0.95)	*p*-value (*p*-value†)
CFH	rs1410996 (T)	32.0	47.1	48.5	0.55 (0.39-0.77)	4.57E-04 (3.20E-03)	0.50 (0.40-0.63)	6.81E-09 (4.77E-08)
	rs1061170 (C)	26.9	15.7	15.0	2.26 (1.51-3.37)	6.66E-05 (4.66E-04)	1.96 (1.49-2.58)	1.02E-06 (7.14E-06)
ARMS2	rs10490924 (T)	53.9	27.6	24.4	2.40 (1.77-3.26)	1.59E-08 (1.11E-07)	3.09 (2.48-3.86)	5.42E-23 (3.79E-22)
C2	rs547154 (A)	3.0	5.0	5.4	0.48 (0.22-1.04)	6.20E-02 (NS)	0.54 (0.30-0.94)	3.12E-02 (NS)
CFB	rs4151667 (A)	0.7	1.8	2.3	0.26 (0.06-1.03)	5.52E-02 (NS)	0.34 (0.12-1.00)	5.06E-02 (NS)
C3	rs1047286 (T)	11.1	5.6	4.8	2.15 (1.13-4.07)	1.89E-02 (NS)	2.48 (1.64-3.75)	1.59E-05 (1.11E-04)
	rs2230199 (G)	12.1	5.0	5.9	2.60 (1.37-4.92)	3.23E-03 (2.26E-02)	2.15 (1.48-3.13)	6.28E-05 (4.40E-04)

* Association analyses were done using logistic regression assuming an additive model and adjusting by age and sex as covariates. † p-value adjusted for multiple testing by Bonferroni correction. OR= odds ratio; CI= confidence interval; NS= no significant

the seven genotyped SNPs in our control population group with two Mexican Amerindian groups (Zapotecos and Mayas) and individuals from three continental groups included in the HapMap International Project, Caucasians, Asians (Chinese and Japanese population), and individuals from a Nigerian region, we observed significant differences for rs1061170 (Tyr402His) in *CFH* between Mexican Mestizos and those populations (Appendix 5). In our population, the Caucasian ancestral contribution for this SNP seems to be important, although its frequency remains not as high as in Caucasians. The association for *CFH* rs1061170 has been reported mainly in Caucasians [39,40]. Our results showed that in Zapotecos, an Mexican Amerindian group, the Tyr402His frequency was as low as that reported for Japanese and Chinese, populations in which this variant is not associated with AMD [11,12,41] and where this allele may confer risk on just a few individuals in the population, and thus have a much lower contribution to overall genetic risk of AMD than in Caucasians [38].

Replication of genetic association studies in different populations helps in determining the effect size of the alleles associated with the disease and provides evidence of the underlying biologic mechanisms in the disease etiology [42,43]. Therefore, our analysis of these five genes provides additional support of their involvement in AMD in an admixed population. An important consequence of using population controls for which detailed phenotyping for the trait of interest is not available relates to the potential of misclassification bias: A proportion of the controls is likely to have AMD, and others will develop it in the future [44]. However, the effect that this has on power should be modest unless the extent of misclassification bias is substantial; for example, if 5% of controls met the definition of cases at the same age, the loss of power would be approximately the same as that due to a decrease in the sample size by 10% [45]. In Mexico, a report showed that the prevalence of AMD in a reference hospital at Mexico City was 3.3% [46]. Including controls from the same geographic area (population controls) is considered an effective approach and has been previously used in the analyses of multiple disease phenotypes, including AMD [44,47].

The SNPs showing strong associations with AMD (rs1410996, rs1061170, rs1047286, rs2230199, and rs10490924) using the combined groups of healthy plus population controls as reference were associated only when healthy controls were used. The SNPs in *C2* and *CFB* (rs547154 and rs4151667) were not as strongly associated as those in *CFH*, *C3*, and *ARMS*, but the power to detect the association is limited because their frequencies are low in the Mexican population (6% and 3%, respectively); however, the patients with AMD

had an allele frequency lower than that of the general population (3% versus 6% and 0.7% versus 3%, respectively). The previous observation indicates that these alleles may confer a protective effect in this population, but due to their low frequency, a larger sample size is needed to have the power to detect their association and confirm the protective effect previously described in other populations [20,37].

A previous study in Mexicans also reported significant association of *CFH* Tyr402His (OR=3.8, p=1.0E-05) and *ARMS2* Ala69Ser (OR=3.04, p=4.0E-07) [48]. The SNP evaluated in the *ARMS2* gene (rs10490924) showed a strong association with AMD in our sample (OR=3.09, 95% CI=2.48–3.86, p value=5.42E-23). Thus, as a result of logistic regression analysis to assess the conditioning effect of SNPs, we observed that for *ARMS* Ala69Ser (rs10490924) the BETA coefficient was still significant (p=5.23E-08) after entering *CFH* rs1410996, *CFH* Tyr402His (rs1061170), sex and age as covariates, suggesting that it does indeed have an effect independent of all these covariates (Appendix 6). This polymorphism had been reported to show high association with AMD [19,49,50]; Fritsche et al. proposed a functional role of *ARMS2* in mitochondrial homeostasis, although the precise mode of action remains to be elucidated [50].

In summary, our data show no evidence of the presence of novel alleles associated with AMD in the coding region of the *CFH* gene, although unidentified polymorphisms in the intron or regulatory regions may exist. In addition, we confirmed the association of *CFH*, *C3*, and *ARMS2* with the risk of developing AMD in Mexicans, and revealed significant ethnic differences in the allele frequency of SNPs, such as rs1061170 in *CFH*, rs2230199 in *C3*, and rs10490924 in *ARMS2*. The SNPs in *C2* and *CFB* (rs547154 and rs4151667) may confer a protective effect in our Mexican Mestizo population, but due to their low frequency, a larger sample size is needed to confirm this observation. Moreover, our results provide evidence that at least two specific common haplotypes in *CFH* predispose individuals to AMD, while another one may confer reduced risk of disease in this admixed population. In addition, other genetic factors not yet discovered may be responsible for AMD risk and protection in our Mexican Mestizo population. Gene–gene interactions between *CFH*, *C2*, and *CFB* and biologic and functional studies could be analyzed to identify the role of these three genes in conferring protection against AMD in admixed and parental populations.

APPENDIX 1. LIST OF PRIMER SEQUENCES USED FOR COMPLEMENT FACTOR H GENE RESEQUENCING.

All primers were linked to M13 universal primers. To access the data, click or select the words "Appendix 1."

APPENDIX 2. PRINCIPAL COMPONENT ANALYSIS FOR THE THREE SAMPLES GROUPS USED.

The distribution of the ancestral components between case and control samples was assessed using 224 ancestry informative markers (AIMs) [24], which were SNPs with allele frequency differences (δ) ≥0.4 for pairwise comparisons between HapMap and Zapotecos groups. PDGM-Control: Population controls from the Mexican Genomic Diversity Project; Control-DM: Healthy controls; Case-DM: AMD cases. Eigenvector 1 and 2 are vectors that represent the genetic diversity of the three groups analyzed. To access the data, click or select the words "Appendix 2."

APPENDIX 3. F_{st} VALUES BETWEEN AGE-MACULAR DEGENERATION CASES, HEALTHY CONTROLS AND POPULATION CONTROLS.

To verify the distribution of the ancestral components between case and control samples, pairwise F_{st} statistics between cases, healthy and population controls are performed. Calculations were done with Eigensoft software using 224 ancestry informative markers. To access the data, click or select the words "Appendix 3."

APPENDIX 4. SNPS IDENTIFIED IN THE COMPLEMENT FACTOR H GENE RE-SEQUENCING IN MEXICANS, 48 AGE-MACULAR DEGENERATION PATIENTS AND 48 HEALTHY CONTROLS.

*Association analyses were done using logistic regression assuming an additive model and adjusting by age and sex as covariates. †p-value adjusted for multiple testing by Bonferroni correction. OR=odds ratio; C.I.=confidence interval; NS=no significant. To access the data, click or select the words "Appendix 4."

APPENDIX 5. COMPARISON OF ALLELE FREQUENCIES OF SNPS IN *CFH*, *C2*, *CFB*, *C3* AND *ARMS2* GENES BETWEEN MEXICAN MESTIZOS AND MEXICAN AMERINDIANS AND HAPMAP POPULATIONS.

*Data from International HapMap Project; †p-value is for the comparison of the allele frequencies with Mexican Mestizo. NS=no significant; Freq.=Frequency; NA=Not available. To access the data, click or select the words "Appendix 5."

APPENDIX 6. GENETIC EFFECT OF RS10490924 TO AGE-MACULAR DEGENERATION RISK IN MEXICANS.

*Analyses were done using logistic regression assuming an additive model and adjusting by rs1410996, rs1061170, sex and age as covariates. To access the data, click or select the words "Appendix 6."

REFERENCES

1. Klein R, Peto T, Bird A, Vannewkirk MR. The epidemiology of age-related macular degeneration. Am J Ophthalmol 2004; 137:486-95. [PMID: 15013873].

2. Hampton T. Genetic research provides insights into age-related macular degeneration. JAMA 2010; 304:1541-3. [PMID: 20940375].

3. Jager RD, Mieler WF, Miller JW. Age-related macular degeneration. N Engl J Med 2008; 358:2606-17. [PMID: 18550876].

4. Friedman DS, O'Colmain BJ, Munoz B, Tomany SC, McCarty C, de Jong PT, Nemesure B, Mitchell P, Kempen J. Prevalence of age-related macular degeneration in the United States. Arch Ophthalmol 2004; 122:564-72. [PMID: 15078675].

5. Klein R, Chou CF, Klein BE, Zhang X, Meuer SM, Saaddine JB. Prevalence of age-related macular degeneration in the US population. Arch Ophthalmol 2011; 129:75-80. [PMID: 21220632].

6. Varma R, Fraser-Bell S, Tan S, Klein R, Azen SP. Prevalence of age-related macular degeneration in Latinos: the Los Angeles Latino eye study. Ophthalmology 2004; 111:1288-97. [PMID: 15234128].

7. Klein RJ, Zeiss C, Chew EY, Tsai JY, Sackler RS, Haynes C, Henning AK, SanGiovanni JP, Mane SM, Mayne ST, Bracken MB, Ferris FL, Ott J, Barnstable C, Hoh J.

Complement factor H polymorphism in age-related macular degeneration. Science 2005; 308:385-9. [PMID: 15761122].

8. Hageman GS, Anderson DH, Johnson LV, Hancox LS, Taiber AJ, Hardisty LI, Hageman JL, Stockman HA, Borchardt JD, Gehrs KM, Smith RJ, Silvestri G, Russell SR, Klaver CC, Barbazetto I, Chang S, Yannuzzi LA, Barile GR, Merriam JC, Smith RT, Olsh AK, Bergeron J, Zernant J, Merriam JE, Gold B, Dean M, Allikmets R. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. Proc Natl Acad Sci USA 2005; 102:7227-32. [PMID: 15870199].

9. Souied EH, Leveziel N, Richard F, Dragon-Durey MA, Coscas G, Soubrane G, Benlian P, Fremeaux-Bacchi V. Y402H complement factor H polymorphism associated with exudative age-related macular degeneration in the French population. Mol Vis 2005; 11:1135-40. [PMID: 16379025].

10. Maller J, George S, Purcell S, Fagerness J, Altshuler D, Daly MJ, Seddon JM. Common variation in three genes, including a noncoding variant in CFH, strongly influences risk of age-related macular degeneration. Nat Genet 2006; 38:1055-9. [PMID: 16936732].

11. Chen LJ, Liu DT, Tam PO, Chan WM, Liu K, Chong KK, Lam DS, Pang CP. Association of complement factor H polymorphisms with exudative age-related macular degeneration. Mol Vis 2006; 12:1536-42. [PMID: 17167412].

12. Okamoto H, Umeda S, Obazawa M, Minami M, Noda T, Mizota A, Honda M, Tanaka M, Koyama R, Takagi I, Sakamoto Y, Saito Y, Miyake Y, Iwata T. Complement factor H polymorphisms in Japanese population with age-related macular degeneration. Mol Vis 2006; 12:156-8. [PMID: 16541016].

13. Clemons TE, Milton RC, Klein R, Seddon JM, Ferris FL 3rd. Risk factors for the incidence of Advanced Age-Related Macular Degeneration in the Age-Related Eye Disease Study (AREDS) AREDS report no. 19. Ophthalmology 2005; 112:533-9. [PMID: 15808240].

14. Peter I, Seddon JM. Genetic epidemiology: successes and challenges of genome-wide association studies using the example of age-related macular degeneration. Am J Ophthalmol 2010; 150:450-2. [PMID: 20863920].

15. Spencer KL, Glenn K, Brown-Gentry K, Haines JL, Crawford DC. Population differences in genetic risk for age-related macular degeneration and implications for genetic testing. Arch Ophthalmol 2012; 130:116-7. [PMID: 22232482].

16. Vanderbeek BL, Zacks DN, Talwar N, Nan B, Musch DC, Stein JD. Racial differences in age-related macular degeneration rates in the United States: a longitudinal analysis of a managed care network. Am J Ophthalmol 2011; 152:273-82. [PMID: 21696700].

17. Klein R, Knudtson MD, Klein BE, Wong TY, Cotch MF, Liu K, Cheng CY, Burke GL, Saad MF, Jacobs DR Jr, Sharrett AR. Inflammation, complement factor h, and age-related macular degeneration: the Multi-ethnic Study of Atherosclerosis. Ophthalmology 2008; 115:1742-9. [PMID: 18538409].

18. Li M, Atmaca-Sonmez P, Othman M, Branham KE, Khanna R, Wade MS, Li Y, Liang L, Zareparsi S, Swaroop A, Abecasis GR. CFH haplotypes without the Y402H coding variant show strong association with susceptibility to age-related macular degeneration. Nat Genet 2006; 38:1049-54. [PMID: 16936733].

19. Rivera A, Fisher SA, Fritsche LG, Keilhauer CN, Lichtner P, Meitinger T, Weber BH. Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. Hum Mol Genet 2005; 14:3227-36. [PMID: 16174643].

20. Gold B, Merriam JE, Zernant J, Hancox LS, Taiber AJ, Gehrs K, Cramer K, Neel J, Bergeron J, Barile GR, Smith RT, Hageman GS, Dean M, Allikmets R. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. Nat Genet 2006; 38:458-62. [PMID: 16518403].

21. Maller JB, Fagerness JA, Reynolds RC, Neale BM, Daly MJ, Seddon JM. Variation in complement factor 3 is associated with risk of age-related macular degeneration. Nat Genet 2007; 39:1200-1. [PMID: 17767156].

22. Yates JR, Sepp T, Matharu BK, Khan JC, Thurlby DA, Shahid H, Clayton DG, Hayward C, Morgan J, Wright AF, Armbrecht AM, Dhillon B, Deary IJ, Redmond E, Bird AC, Moore AT. Complement C3 variant and the risk of age-related macular degeneration. N Engl J Med 2007; 357:553-61. [PMID: 17634448].

23. Sans M. Admixture studies in Latin America: from the 20th to the 21st century. Hum Biol 2000; 72:155-77. [PMID: 10721616].

24. Silva-Zolezzi I, Hidalgo-Miranda A, Estrada-Gil J, Fernandez-Lopez JC, Uribe-Figueroa L, Contreras A, Balam-Ortiz E, del Bosque-Plata L, Velazquez-Fernandez D, Lara C, Goya R, Hernandez-Lemus E, Davila C, Barrientos E, March S, Jimenez-Sanchez G. Analysis of genomic diversity in Mexican Mestizo populations to develop genomic medicine in Mexico. Proc Natl Acad Sci USA 2009; 106:8611-6. [PMID: 19433783].

25. Seddon JM, Sharma S, Adelman RA. Evaluation of the clinical age-related maculopathy staging system. Ophthalmology 2006; 113:260-6. [PMID: 16458093].

26. Abrera-Abeleda MA, Nishimura C, Smith JL, Sethi S, McRae JL, Murphy BF, Silvestri G, Skerka C, Jozsi M, Zipfel PF, Hageman GS, Smith RJ. Variations in the complement regulatory genes factor H (CFH) and factor H related 5 (CFHR5) are associated with membranoproliferative glomerulonephritis type II (dense deposit disease). J Med Genet 2006; 43:582-9. [PMID: 16299065].

27. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005; 21:263-5. [PMID: 15297300].

28. Stephens M, Donnelly P. A comparison of bayesian methods for haplotype reconstruction from population genotype data. Am J Hum Genet 2003; 73:1162-9. [PMID: 14574645].

29. Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 2001; 68:978-89. [PMID: 11254454].

30. Patterson N, Price AL, Reich D. Population structure and eigenanalysis. PLoS Genet 2006; 2:e190-[PMID: 17194218].

31. Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet 2006; 38:904-9. [PMID: 16862161].

32. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, Sham PC. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 2007; 81:559-75. [PMID: 17701901].

33. Kruglyak L, Nickerson DA. Variation is the spice of life. Nat Genet 2001; 27:234-6. [PMID: 11242096].

34. Ng TK, Chen LJ, Liu DT, Tam PO, Chan WM, Liu K, Hu YJ, Chong KK, Lau CS, Chiang SW, Lam DS, Pang CP. Multiple gene polymorphisms in the complement factor h gene are associated with exudative age-related macular degeneration in chinese. Invest Ophthalmol Vis Sci 2008; 49:3312-7. [PMID: 18421087].

35. Pickering MC, de Jorge EG, Martinez-Barricarte R, Recalde S, Garcia-Layana A, Rose KL, Moss J, Walport MJ, Cook HT, de Cordoba SR, Botto M. Spontaneous hemolytic uremic syndrome triggered by complement factor H lacking surface recognition domains. J Exp Med 2007; 204:1249-56. [PMID: 17517971].

36. Tortajada A, Montes T, Martinez-Barricarte R, Morgan BP, Harris CL, de Cordoba SR. The disease-protective complement factor H allotypic variant Ile62 shows increased binding affinity for C3b and enhanced cofactor activity. Hum Mol Genet 2009; 18:3452-61. [PMID: 19549636].

37. Kaur I, Katta S, Reddy RK, Narayanan R, Mathai A, Majji AB, Chakrabarti S. The involvement of complement factor B and complement component C2 in an Indian cohort with age-related macular degeneration. Invest Ophthalmol Vis Sci 2010; 51:59-63. [PMID: 19696172].

38. Chu J, Zhou CC, Lu N, Zhang X, Dong FT. Genetic variants in three genes and smoking show strong associations with susceptibility to exudative age-related macular degeneration in a Chinese population. Chin Med J (Engl) 2008; 121:2525-33. [PMID: 19187590].

39. Francis PJ, Schultz DW, Hamon S, Ott J, Weleber RG, Klein ML. Haplotypes in the complement factor H (CFH) gene: associations with drusen and advanced age-related macular degeneration. PLoS ONE 2007; 2:e1197-[PMID: 18043728].

40. Zareparsi S, Branham KE, Li M, Shah S, Klein RJ, Ott J, Hoh J, Abecasis GR, Swaroop A. Strong association of the Y402H variant in complement factor H at 1q32 with susceptibility to age-related macular degeneration. Am J Hum Genet 2005; 77:149-53. [PMID: 15895326].

41. Gotoh N, Yamada R, Hiratani H, Renault V, Kuroiwa S, Monet M, Toyoda S, Chida S, Mandai M, Otani A, Yoshimura N, Matsuda F. No association between complement factor H gene polymorphism and exudative age-related macular degeneration in Japanese. Hum Genet 2006; 120:139-43. [PMID: 16710702].

42. Edwards AO, Fridley BL, James KM, Sharma AK, Cunningham JM, Tosakulwong N. Evaluation of clustering and genotype distribution for replication in genome wide association studies: the age-related eye disease study. PLoS ONE 2008; 3:e3813-[PMID: 19043567].

43. Zhang H, Morrison MA, Dewan A, Adams S, Andreoli M, Huynh N, Regan M, Brown A, Miller JW, Kim IK, Hoh J, Deangelis MM. The NEI/NCBI dbGAP database: genotypes and haplotypes that may specifically predispose to risk of neovascular age-related macular degeneration. BMC Med Genet 2008; 9:51-[PMID: 18541031].

44. Consortium TWTCC. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 2007; 447:661-78. [PMID: 17554300].

45. Colhoun HM, McKeigue PM, Davey Smith G. Problems of reporting genetic associations with complex outcomes. Lancet 2003; 361:865-72. [PMID: 12642066].

46. Arturo Santos JAP. Ceguera legal: prevalencia y causas principales en México. Investigación en Salud 2001.

47. Cipriani V, Leung HT, Plagnol V, Bunce C, Khan JC, Shahid H, Moore AT, Harding SP, Bishop PN, Hayward C, Campbell S, Armbrecht AM, Dhillon B, Deary IJ, Campbell H, Dunlop M, Dominiczak AF, Mann SS, Jenkins SA, Webster AR, Bird AC, Lathrop M, Zelenika D, Souied EH, Sahel JA, Leveillard T, Cree AJ, Gibson J, Ennis S, Lotery AJ, Wright AF, Clayton DG, Yates JR. Genome-wide association study of age-related macular degeneration identifies associated variants in the TNXB-FKBPL-NOTCH4 region of chromosome 6p21.3. Hum Mol Genet 2012; 21:4138-50. [PMID: 22694956].

48. Buentello-Volante B, Rodriguez-Ruiz G, Miranda-Duarte A, Pompa-Mera EN, Graue-Wiechers F, Bekker-Mendez C, Ayala-Ramirez R, Quezada C, Rodriguez-Loaiza JL, Zenteno JC. Susceptibility to advanced age-related macular degeneration and alleles of complement factor H, complement factor B, complement component 2, complement component 3, and age-related maculopathy susceptibility 2 genes in a Mexican population. Mol Vis 2012; 18:2518-25. [PMID: 23112567].

49. Jakobsdottir J, Conley YP, Weeks DE, Mah TS, Ferrell RE, Gorin MB. Susceptibility genes for age-related maculopathy on chromosome 10q26. Am J Hum Genet 2005; 77:389-407. [PMID: 16080115].

50. Fritsche LG, Loenhardt T, Janssen A, Fisher SA, Rivera A, Keilhauer CN, Weber BH. Age-related macular degeneration is associated with an unstable ARMS2 (LOC387715) mRNA. Nat Genet 2008; 40:892-6. [PMID: 18511946].