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ORIGINAL RESEARCH

Investigation of associations of *ARMS2*, *CD14*, and *TLR4* gene polymorphisms with wet age-related macular degeneration in a Greek population

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Background: Age-related macular degeneration (AMD) is a multifactorial degenerative ocular disease that leads to loss of central vision. Functional gene polymorphisms have already been associated with the disease (for example, *ARMS2* A69S, rs10490924).

Aim: The goal of our study was to verify the correlation of the aforementioned *ARMS2* variation with the disease, to examine, for the first time, the role of the *CD14* C260T variation (rs2569190), and to investigate the association of two *TLR4* polymorphisms (Asp299Gly or rs4986790 and Thr399Ile or rs4986791) in a Greek population with the wet form of AMD.

Patients and methods: Genomic DNAs were isolated from blood samples of 103 healthy controls and 120 Greek patients with wet AMD who were age- and sex-matched, and all of whom were clinically evaluated. For the genotyping of all selected polymorphisms, polymerase chain reaction–restriction fragment length polymorphism analysis was performed.

Results and conclusions: This study confirmed the association between the *ARMS2* variation and AMD, detecting the T risk allele in a significantly higher frequency in the patient group, compared with the control subjects (45% vs 29.13%, $P<0.001$, odds ratio [OR] 1.99, confidence interval 1.34–2.95). For the *CD14* polymorphism, no statistically significant correlation was observed. As for the *TLR4* polymorphisms, the percentage of heterozygotes increased from 2.9% to 11.7% in the patient population for Asp299Gly and from 1.9% to 10% for the Thr399Ile polymorphism (ORs 4.40 [$P=0.01$] and 5.61 [$P=0.0088$], respectively). Although our *ARMS2* and *CD14* results provided definite conclusions, the role of innate immunity *TLR4* gene awaits further investigation in larger AMD populations with more clinical data collected on past microbial infections.

Keywords: AMD, *ARMS2*, *CD14*, *TLR4*, SNPs, PCR-RFLP

Introduction

Age-related macular degeneration (AMD) is a multifactorial, heterogeneous, degenerative disorder of the human eye that affects patients over 50 years of age and can lead to severe loss of central vision.[1] It is considered to be the leading cause of irreversible blindness in Western societies and, given the fact that life expectancy is constantly rising, the prevalence of the disease is expected to increase significantly.[2] Clinically, two forms of AMD are recognized: the dry form, which affects 80% of the patients and the more severe and wet form, which affects the remaining 20%. Choroidal neovascularization (CNV) is a typical sign of wet AMD.[3]

AMD is a highly complex disease resulting from interactions between genetic susceptibility, aging, oxidative damage due to a surplus of oxygen and light in the eye, environmental influences, and underlying diseases.[4,5] Initially through family-based

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and then, with genome-wide association studies the strong genetic component of the disease has been verified (eg, in the most recent 2016 study with the largest number of patients and healthy controls so far), 52 single-nucleotide polymorphisms (SNPs) in 34 genes have been associated with the disease with a *P*-value $<10^{-8}$.[6] However, since 2005, two loci stand out in most studies, and their polymorphisms have been verified in most populations and are associated with the highest odds ratios (OR) depending on the population:[5,7] the *CFH* gene is involved in the alternative pathway regulation with the Y402H polymorphism (rs1061170)[8,9] and a genomic locus at chromosome 10q26 (LOC387715) where the *ARMS2* gene is located. The *ARMS2* gene encodes for a small 107-amino acid 12 kDa protein and A69S SNP (rs10490924) in exon 1 of the *ARMS2* gene, resulting from a G to T change (NM_001099667.1:c205G>T), is a mutation associated with subsequent mitochondrial dysfunction, generation of reactive oxygen species (ROS) and accumulation of somatic mutations in mitochondrial DNA.[10] According to a meta-analysis study, TT mutant homozygotes carry a 7.5-fold increased AMD risk, and the TG heterozygotes carry a 2.4-fold increased AMD risk when compared to the wild-type GG homozygotes.[11] However, there is an ongoing debate whether it is this gene or the adjacent *HTRA1* gene that shows true association.[12] Evidence for the *ARMS2* gene include reverse-transcriptase polymerase chain reaction (PCR) experiments – which have demonstrated that *ARMS2* variant transcripts are expressed in the ellipsoid region of the photoreceptors in the human retina, where the majority of mitochondria are located – and immunohistochemistry experiments, showing co-localization with a mitochondrial marker, anti-MTCO2.[13,14]

Other genes that are involved in inflammation, immune response, cholesterol transport, ubiquitin proteolytic system, and oxidative stress seem to play a key role in the development of AMD.[5,12] Therefore, SNPs in genes that are involved in the aforementioned conditions are worthy of investigation.

Toll-like receptors (TLRs) are members of a family that recognizes conserved microbial components in pathogens and contributes to the activation of immune response.[15] *TLR4* is located on chromosome 9q33.1, contains 4 exons that encode an 839 amino acid protein of 96 kDa molecular mass. It is highly expressed on lymphocytes, monocytes, and neutrophils and mediates for lipopolysaccharide (LPS) recognition. This interaction requires the presence of CD14-MD2 co-receptors and produces proinflammatory cytokines through adaptor molecules (eg, MAL, MyD88).[16] Allelic variations such as Asp299Gly, resulting from an A to G change (rs4986790, NM_138554.4:c.896A>G), and Thr399Ile, resulting from a C to T change (rs4986791, NM_138554.4:c.1196C>T), have been associated with atherosclerosis, cardiovascular disease, and other inflammatory diseases;[17–20] the role of these *TLR4* gene polymorphisms in AMD susceptibility was first mentioned in Zareparsi et al.[21] The frequencies of Gly and Ile amino acids in the aforementioned SNPs were found to be increased in patients, compared to controls, indicating that carriers of the changed amino acids had a 2.0- and 2.4-fold increased risk of developing AMD, respectively.

The co-receptor of TLR4, CD14, is a glycosylphosphatidylinositol (GPI) receptor that mediates the inflammatory response to bacterial products by binding low doses of LPS[22] present and transfer it to the TLR4–MD2 complex in order to initiate the transduction of the NF-κB pathway.[23,24] The *CD14* gene is located on chromosome 5q31.1, contains three exons, and encodes for a 356 amino-acid glycoprotein that is expressed mainly in the liver and is anchored to the cellular membrane through the GPI linkage. CD14 exists in two forms – the membrane-expressed (mCD14) present on the surface of mature myeloid cells and macrophages, as well as the soluble molecule (sCD14) that mediates LPS activation of epithelial cells.[25] The allelic variant C260T (rs2569190, NM_000591.3:c-260 C to T change at position −260 to the start codon) in the region of the gene promoter is associated with enhanced transcriptional activity and results in a higher density of the receptor.[18,22,26]

Thus far, no other study has examined the role of *CD14* gene in AMD, to the best of our knowledge. Considering its role in innate immunity and its role in cooperation with *TLR4*, we examined in this study, for the first time, whether C260T variation in *CD14* gene is correlated with AMD, and we investigated *TLR4* Asp299Gly and Thr399Ile variations in a Greek population with the wet AMD form. In the same population, we additionally tried to verify the association of A69S variation in the *ARMS2* gene with AMD.

Patients and methods

Patients

The study was conducted in a cohort of 103 healthy controls and 120 Greek patients, with the wet form of AMD. Blood samples were collected in EDTA tubes in Athens "G. Gennimatas" General Hospital and Ioannina University General Hospital (40-patient enrollment) during a 4-year period (2010–2013) and stored in −20°C until DNA extraction. All participants were selected after ophthalmologic evaluation and all clinical data were collected on cataract

surgery and smoking habits, and conditions such as glaucoma, diabetes, arterial hypertension, and heart disease that, wherever observed, were being treated with an appropriate medication regimen (Table 1). Ophthalmologic evaluation included visual acuity, ocular pressure measurement, slit-lamp anterior and posterior segment examination, optical coherence tomography (OCT), and fundus angiography (fluorescein or indocyanine). Inclusion criteria were: CNV in the fovea/perifoveal area due to AMD, active leakage of the new choroidal blood vessels, and decreased visual acuity. Exclusion criteria were: age <50 years, Snellen visual acuity better than 7/10 or worse than 1/10, and any other pathology leading to CNV (eg, angioid streaks, high myopia, presumed ocular histoplasmosis syndrome, choroidal rupture). The samples were obtained after approvals from the "G. Gennimatas" General Hospital and Ioannina University General Hospital ethics committees and after obtaining a signed informed consent from each participant.

Genomic DNA isolation

Genomic DNA was isolated from 200 µL blood by the NucleoSpin Blood kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. DNA purity and quantity were determined by fluorescence readings with the use of Quant-iT dsDNA-BR kit in the Qubit 1.0 fluorometer (ThermoFisher Invitrogen, Waltham, MA, USA).

Table 1 Clinical characteristics of all 223 participants in the study

Clinical characteristics	All, n (%)	Controls, n (%)	Patients, n (%)	χ^2
Sex				0.067
Male	110 (49.3)	44 (42.7)	66 (55.0)	
Female	113 (50.7)	59 (57.3)	54 (45.0)	
Smoking				0.287
Yes	73 (32.7)	30 (29.1)	43 (35.8)	
No	150 (67.3)	73 (70.9)	77 (64.2)	
Cataract surgery				0.155
Yes	51 (22.9)	28 (27.2)	23 (19.2)	
No	172 (77.1)	75 (72.8)	97 (80.8)	
Glaucoma				0.016*
Yes	23 (10.4)	16 (15.7)	7 (5.8)	
No	199 (89.6)	86 (84.3)	113 (94.2)	
Arterial hypertension				0.745
Yes	41 (18.4)	18 (17.5)	23 (19.2)	
No	182 (81.6)	85 (82.5)	97 (80.8)	
Diabetes				0.003*
Yes	55 (24.7)	35 (34.0)	20 (16.7)	
No	168 (75.3)	68 (66.0)	100 (83.3)	
Heart disease				0.742
Yes	37 (16.6)	18 (17.5)	19 (15.8)	
No	186 (83.4)	85 (82.5)	101 (84.2)	

Note: *Statistically significant.

Analysis of *ARMS2*-A69S, *CD14*-C260T, *TLR4*-Asp299Gly, and Thr399Ile gene polymorphisms

For the genotyping of all selected polymorphisms, PCR – restriction fragment length polymorphism analysis was performed on a fragment amplified using primers from the literature[27,28] (Table S1). Primers were synthesized by IDT (Integrated DNA Technologies, Inc. Coralville, IA, USA).

ARMS2 (A69S) genotyping

The PCR was performed in 0.2 mL tubes in a Primus 25 Advanced PCR engine (PEQLAB Biotechnologie GmbH, Erlangen, Germany). The amplification mixture of a total 20 µL volume included 1 µL of 20 pmol/µL LOC1-forward primer and 1 µL of 20 pmol/µL LOC2-reverse primer, 1× Go Taq Green Master Mix (Promega, USA) and ~2 µL of genomic DNA (≥50 ng). Sterile water was used to supplement up to 20 µL. The cycling protocol for *ARMS2* comprised a pre-incubation step at 94°C for 10 min hot-start polymerase activation, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 45 s, and a final extension step at 72°C for 7 min.

CD14 (C260T) genotyping

The PCR protocol for the C260T polymorphism was the same as described earlier (with CD14_F and CD14_R primers instead); the cycling conditions were: initial denaturation at 95°C for 5 min, followed by 35 cycles at 92°C for 40 s, 62°C for 35 s, and 72°C for 50 s. The final extension step was prolonged to 5 min.

TLR4 (Asp299Gly and Thr399Ile) genotyping

The PCR protocol was analogous as mentioned previously (with *TLR4* primers 1–2 and 3–4, respectively) and cycling conditions: 95°C for 5 min followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final incubation at 72°C for 5 min.

Restriction enzyme incubations

After confirming proper amplifications by running 5 µL PCR products in a 1.5% agarose gel, the rest of the PCR products (15 µL) were digested with the appropriate restriction enzymes for 4 h at 37°C.

After *Pvu*II digestion (ThermoFisher Fermentas, Lithuania), the 449-bp PCR amplicon for A69S in the *ARMS2* gene was cut into fragments of 259 and 190 bp in the case of the wild-type G allele whereas it was left uncut in the case

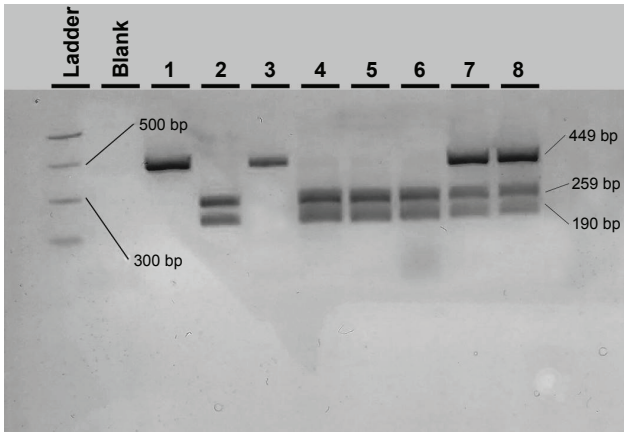

Figure 1 Agarose electrophoresis result of the *ARMS2* A69S PCR-RFLP genotyping method.
Notes: In the first two wells, the MW PCR marker and the PCR blank are shown. Wells 2 and 4–6 show wild-type GG samples; wells 7 and 8 show heterozygous G/T samples, and wells 1 and 3 show homozygote TT samples.
Abbreviations: PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; MW, molecular weight.

of the altered (mutant) T allele (449 bp). Therefore, a G/T heterozygote sample was cut into three fragments of 449, 259, and 190 bp (Figure 1).

The 561-bp amplified region for *CD14* was digested with *Hae*III restriction enzyme (NEB, USA). The wild-type C allele separated into fragments of 204, 201 (run as one band), and 156 bp. The T minor allele showed a loss of one *Hae*III cleavage site, resulting in the presence of fragments of 360 and 201 bp. Therefore the C/T heterozygote was cut into three fragments of 360, 204/201, and 156 bp (Figure 2).

With regard to the *TLR4* amplicons after digestion with the *Nco*I (Takara, Japan) restriction enzyme for the 299 residue and with *Hinf*I (NEB, USA) for the 399 residue, fragment

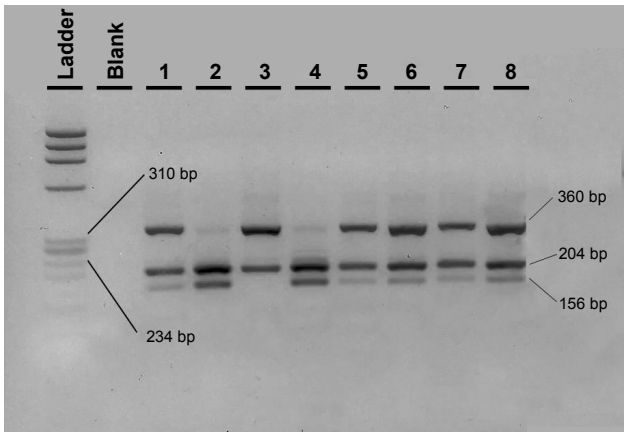

Figure 2 Agarose electrophoresis result of the *CD14* C260T PCR-RFLP genotyping method.
Notes: In the first two wells, the ϕχ174 *Hae*III DNA ladder and the PCR blank are shown. Wells 1 and 5–8 show the C/T heterozygous samples, wells 2 and 4 the wild-type CC samples, and well 3 a homozygote TT sample.
Abbreviations: PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

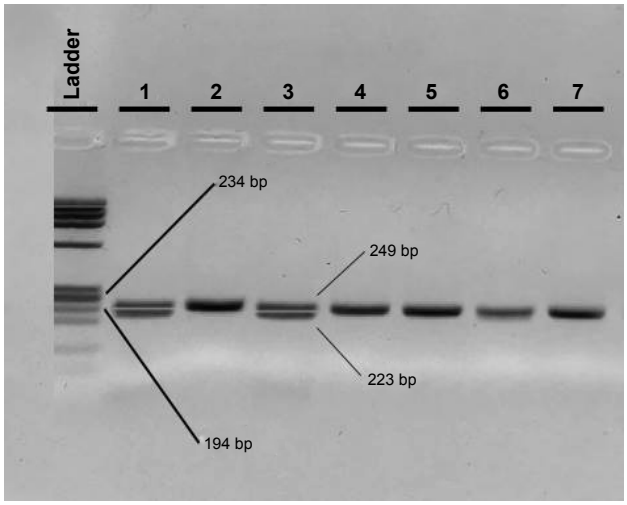

Figure 3 Agarose electrophoresis result of the *TLR4* Asp299Gly PCR-RFLP genotyping method.
Notes: In the first well, the ϕχ174 *Hae*III DNA ladder is shown. Wells 2 and 4–7 show the wild-type AA samples and wells 1 and 3 the A/G heterozygous samples.
Abbreviations: PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

sizes for carriers of the polymorphic allele decreased from 249 (wild-type) to 223 bp for the 299 residue and from 406 (wild-type) to 377 bp for the 399 residue (Figures 3 and 4). All restricted amplicons were analyzed by 3% agarose gel electrophoresis (2:1 Nusieve/Seakem, Lonza, Basel, Switzerland), visualized by ethidium bromide and sized by a MW marker (PCR Marker or ϕχ174 *Hae*III, both New England Biolabs, Ipswich, MA, USA).

DNA sequencing

For the verification of the PCR-restriction fragment length polymorphism results, the gold standard method of DNA sequencing was used for method comparison (Figures S1–S3).

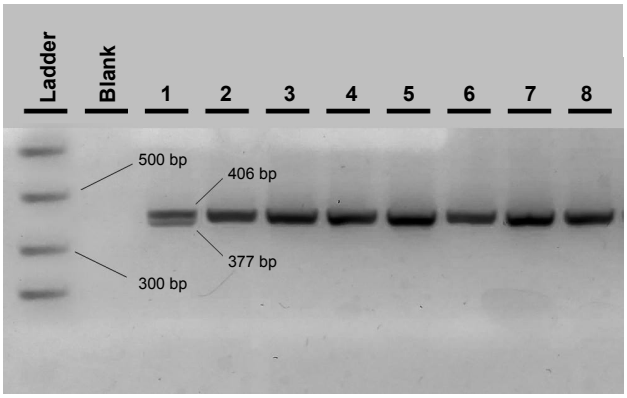

Figure 4 Agarose electrophoresis result of the *TLR4* Thr399Ile PCR-RFLP genotyping method.
Notes: In the first two wells, the MW PCR marker and the PCR blank are shown. Wells 2–8 show the wild-type CC samples and well 1 the heterozygous C/T sample.
Abbreviations: PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; MW, molecular weight.

After purification of the amplicons of conventional PCR (High Pure PCR Cleanup Micro kit, Roche Applied Science, Penzberg, Germany), cycle sequencing reaction was performed with the Big Dye 1.1 reagent in both directions with the use of either the forward or the reverse primer (ThermoFisher Scientific, Waltham, MA, USA); 10 µL of the purified cycle sequencing reactions (by NucleoSeq columns, Macherey-Nagel, Germany) were heated at 95°C for 2 min and cooled immediately at 4°C for 2 min with 10 µL formamide and then run in capillaries of the ABI Prism 310 Genetic Analyzer. For the analysis of DNA sequencing electropherograms, the Chromas 2.01 software was used (Technelysium Pty Ltd, Brisbane, QLD, Australia) and results were compared with the expected gene sequences with the NCBI BLAST (https://blast.ncbi.nlm.nih.gov).

Statistical analysis

Adequacy of the number of total samples and statistical power for all χ^2 tests were estimated with the G*Power 3.1.9.2 software.[29]

Genotyping statistical analysis was performed through the SNPStats Internet platform (http://bioinfo.iconcologia.net/snpstats/start.htm).[30] The variants were tested for Hardy–Weinberg equilibrium (HWE) in either patients with AMD or controls for each studied SNP, and OR and 95% confidence intervals (CIs) for all genotypes were calculated for all inheritance models (dominant, co-dominant, recessive, or log-additive). The one with the lower Akaike information criterion was preferred.[30] Furthermore, haplotype analysis and linkage disequilibrium estimation was performed for the two variants of *TLR4* gene.

Analysis of the association between AMD and risk factors (and genotypes in the multivariate model) was performed with the SPSS statistical software (version 21.0, IBM, Armonk, NY, USA). Age distribution was tested initially for normality with the Kolmogorov–Smirnov test and, subsequently, the Mann–Whitney *U*-test for median comparison was used. For gender and for clinical and environmental categorical variables, comparisons between percentages of groups were performed with the χ^2 test. To evaluate the risk of developing AMD, binary logistic regression analysis was adjusted for the presence of specific polymorphisms, age, sex, visual acuity, smoking habits, diabetes, glaucoma, surgical cataract, hypertension, and heart disease status. All tests of significance were two-sided and *P*-values <0.05 were considered statistically significant.

Results

The total number of samples provided sufficient statistical power (92%) to even detect medium-sized effects (0.3) in all χ^2-tests performed as estimated by the G*Power software. In the group of patients with wet AMD, 20 subjects (16.7%) also had geographic atrophy. Age distribution was not normal in patients and controls; therefore, medians were compared between the two groups and no significance difference was detected; the median age of the patients was 77 years and, for the controls, it was 78 (interquartile range 73–81 and 75–82), respectively. Sex distribution was the same: males comprised 55% of the patients and 57% of the controls ($P>0.05$). When all covariates were studied, in two parameters, glaucoma and diabetes, a reduction was observed in patients with AMD (Table 1).

All genotypes in all genes were in HWE equilibrium for both controls and patients ($P>0.05$). The *ARMS2* genetic variation and its distribution in the studied cohort are shown in Table 2. The T risk allele was detected in 108 of 240 patient total alleles; in a much higher frequency (45%) than in control subjects (29.13%, 60 out of 206 control alleles, $P<0.001$, odds ratio [OR] 1.99, 95% CI 1.34–2.95) and TT homozygotes doubled from controls to patients with AMD (9.70%–20.8%). ORs and levels of significance for each one of all possible inheritance models were calculated

Table 2 Genotype frequencies, inheritance models, and calculated ORs for the A69S SNP in the *ARMS2* gene (G wild-type allele, T risk mutant allele)

Model	Genotype	AMD =0	AMD =1	OR (95% CI)	*P*-value	AIC
Codominant	G/G	53 (51.5%)	37 (30.8%)	1.00	0.0031	302.3
	G/T	40 (38.8%)	58 (48.3%)	**2.08 (1.16–3.72)**		
	T/T	10 (9.7%)	25 (20.8%)	**3.58 (1.54–8.34)**		
Dominant	G/G	53 (51.5%)	37 (30.8%)	1.00	0.0017	302
	G/T-T/T	50 (48.5%)	83 (69.2%)	**2.38 (1.38–4.11)**		
Recessive	G/G-G/T	93 (90.3%)	95 (79.2%)	1.00	0.02	306.5
	T/T	10 (9.7%)	25 (20.8%)	**2.45 (1.11–5.38)**		
Log-additive	N/A	N/A	N/A	**1.94 (1.31–2.88)**	7e-04	300.4

Notes: AMD =0 indicates the healthy control population and AMD =1 indicates the AMD patients. Bold values indicate statistically significant correlations.
Abbreviations: AIC, Akaike information criterion; AMD, age-related macular degeneration; CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism; N/A, not applicable.

Table 3 Genotype frequencies, inheritance models, and calculated ORs for the C260T SNP in the *CD14* gene (C wild-type allele, T mutant allele)

Model	Genotype	AMD =0	AMD =1	OR (95% CI)	*P*-value	AIC
Codominant	C/C	34 (33%)	29 (24.2%)	1.00	0.34	311.7
	C/T	53 (51.5%)	71 (59.2%)	1.57 (0.85–2.89)		
	T/T	16 (15.5%)	20 (16.7%)	1.47 (0.64–3.34)		
Dominant	C/C	34 (33%)	29 (24.2%)	1.00	0.14	309.7
	C/T-T/T	69 (67%)	91 (75.8%)	1.55 (0.86–2.78)		
Recessive	C/C-C/T	87 (84.5%)	100 (83.3%)	1.00	0.82	311.8
	T/T	16 (15.5%)	20 (16.7%)	1.09 (0.53–2.23)		
Log-additive	N/A	N/A	N/A	1.26 (0.84–1.89)	0.26	310.6

Note: AMD =0 indicates the healthy control population and AMD =1 indicates the AMD patients.
Abbreviations: AIC, Akaike information criterion; AMD, age-related macular degeneration; CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism; N/A, not applicable.

and a robust statistically significant correlation was found a between AMD and the A69S SNP. In the model with the lower Akaike information criterion, the log-additive model, the calculated OR is 1.94 (95% CI 1.31–2.88).

The association for C260T variation in the *CD14* gene did not reach any statistical significance (P=0.14) and is shown in Table 3.

The Asp299Gly and Thr399Ile polymorphisms in the *TLR4* gene are rare in the total population, but certainly, the heterozygotes are quite enriched in the patient population (Table 4): from 2.9% in controls to 11.7% in patients in the first SNP and, in the latter, from 1.9% in controls to 10% in patients. No homozygote was detected in both polymorphisms (GG in 299 and TT in the 399 *TLR4* polymorphisms respectively). The calculated ORs were 4.40 (P=0.01) and 5.61 (P=0.0088), respectively (but with broad CIs). Haplotype analysis was performed only for the *TLR4* gene (Table 5) because both genetic variations are located on the same chromosome and showed that the risk GT haplotype is again correlated with the disease (OR =8.66, P=0.045), but the two polymorphisms are not linked (r^2=0.398, not shown). No difference in the percentage of genotype distribution in all studied genes exists within the subgroups of the clinical variables (all χ^2 tests with $P>0.05$).

When examining the effect of all significant SNPs determined in this study (*ARMS2* and both SNPs of the *TLR4* genes) and all clinical variables with binary logistic regression for the prediction of AMD, it was found that, among the three genes, only *ARMS2* shows a strong effect on the development of AMD due to the rarity of *TLR4* risk alleles (Table 6). If we also include the results for *CFH* and *FCGR2A* SNPs in the same population that were obtained from our previous study,[31] these two SNPs and *ARMS2* remain strong in the model of AMD prediction, albeit with reduced ORs (Table S2).

Concerning the clinical variables, the majority have no effect on the development and progression of the disease, except for glaucoma and diabetes that seem protective not only as shown in the logistic regression of Table 6 but also as expected from the χ^2 tests of Table 1.

Discussion

AMD is a complex, degenerative disease of the human eye that apparently fits the multi-hit "threshold model".[5] Numerous defects in genes affecting chronic inflammation, lipid transfer and recycling, response to oxidative stress, proteasome system functionality and extracellular matrix remodeling will eventually lead to drusen creation and Bruch's membrane breakdown and take their toll on photoreceptors and retinal pigment epithelial cells. The progress toward geographic atrophy and/or CNV will depend on the content of these hits.[4,5,12] However, even in the latest and largest of the genetic association studies, there is still an estimate of about 42% "missing heritability" (in the highest

Table 4 Genotype frequencies and calculated ORs for the Asp299Gly (A wild-type allele, G risk mutant allele) and the Thr399Ile (C wild-type allele, T risk mutant allele) SNPs in the *TLR4* gene

SNP	Genotype	AMD =0	AMD =1	OR (95% CI)	*P*-value	AIC
299	A/A	100 (97.1%)	106 (88.3%)	1.00	0.01	305.2
	A/G	3 (2.9%)	14 (11.7%)	**4.40 (1.23–15.78)**		
399	C/C	101 (98.1%)	108 (90%)	1.00	0.0088	305
	C/T	2 (1.9%)	12 (10%)	**5.61 (1.23–25.68)**		

Notes: Bold values indicate statistically significant correlations. AMD =0 indicates the healthy control population and AMD =1 indicates the AMD patients.
Abbreviations: AIC, Akaike information criterion; AMD, age-related macular degeneration; CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism.

Table 5 *TLR4* gene haplotype association with the disease

Haplotype association with response (n=223)				
TLR_299	**TLR_399**	**Freq**	**OR (95% CI)**	***P*-value**
A	C	0.9528	1.00	–
G	T	0.0223	**8.66 (1.06–70.82)**	0.045
G	C	0.0158	2.40 (0.46–12.67)	0.3
A	T	0.0091	2.88 (0.29–28.19)	0.36

Note: Bold values indicate statistically significant correlations.
Abbreviations: OR, odds ratio; CI, confidence interval; Freq, frequency.

disease prevalence group of people above 85 years of age).[6] Therefore, it is worthwhile investigating every plausible lead and testing it in various populations because minor allele frequencies and corresponding OR can differ significantly as seen, for example, in the *CFH* Y402H polymorphism between Caucasian and Asian populations.[5,7]

So far, in the Greek population genetic studies exist on *ARMS2*, *CFH*, *FCGR2A*, *C3*, *C2*, and *BF* genes,[31–33] but there were no data on *TLR4* (Asp299Gly and Thr399Ile) and *CD14* (C260T) allele frequencies in Greek patients with AMD. Both genes operate in the same pathway of inflammatory response, and the *TLR4* variations Asp299Gly and Thr399Ile affect the extracellular domain of the corresponding protein and result in a differential response to LPS.[34] In this study, findings for the aforementioned SNPs and investigation of their association with AMD susceptibility are reported. No other study has examined the role of the *CD14* SNP in AMD so far. The study was implemented on 103 control subjects and 120 Greek patients with wet AMD who were all evaluated with a full ocular examination. Our results showed no association with the *CD14* SNP but demonstrated that both *TLR4* polymorphisms are linked with an increased risk of wet AMD with ORs (95% CI), 4.40 (1.23–15.78) and 5.61 (1.23–25.68), respectively. In this way, our study corroborates the results from Zareparsi et al's investigation.[21] However, there exist other and larger studies that have presented contradictory conclusions concerning the association between *TLR4* variants and AMD.[6,35,36] Our *TLR4* findings in our control subjects are within the variation seen in various populations for these rare SNPs (data from dbSNP and ExAC browsers); however, our AMD well-ascertained population showed differences that cannot be explained unless there is a particular patient subpopulation. As TLR4 has a role in triggering innate immune defense against microbial agents, a limitation of our study might be the lack of clinical data concerning past infections, especially those that affected the eyes of some of our patients. This argument awaits further investigation in larger sample sizes in the future.

Concerning the *ARMS2* A69S variation, our results verify its association with AMD in any of the inheritance models as seen in many other studies worldwide.[10,11] In our study, the association reaches a *P*-value of 7×10^{-4} in the log-additive model with an OR of 1.94 (95% CI 1.31–2.88).

The binary logistic regression analysis including all the studied polymorphisms and all clinical parameters implicate that the *ARMS2* A69S polymorphism remains a strong, independent, and provisional factor for the development of the disease, whereas the two *TLR4* polymorphisms show weak predictability due to the rareness of these variations.

Furthermore, taking into consideration the results from our previous study on AMD that includes the *CFH* and *FCGR2A* genes[31] and the clinical and environmental factors, now we have a much broader view of the effect that all genes have on the development of wet AMD (Table S2). The strong genetic component in AMD is clearly demonstrated when only genes remain as strong predictors: in the logistic

Table 6 Multivariate model of AMD risk based on genotypes and clinical and environmental factors

Variables in the equation								
Variables	**B**	**SE**	**Wald**	***df***	***P*-value**	**OR**	**95% CI**	
							Lower	**Upper**
TLR_299	0.879	0.807	1.185	1	0.276	2.408	0.495	11.715
TLR_399	0.996	0.930	1.145	1	0.285	2.706	0.437	16.757
ARMS2	0.882	0.302	8.528	1	**0.003***	**2.417**	**1.337**	**4.370**
Smoking	0.605	0.335	3.253	1	0.071	1.830	0.949	3.531
Cataract	–0.495	0.357	1.927	1	0.165	0.609	0.303	1.226
Glaucoma	–1.125	0.513	4.815	1	0.028*	0.325	0.119	0.887
Hypertension	0.148	0.403	0.135	1	0.714	1.159	0.526	2.555
Diabetes	–0.902	0.346	6.790	1	**0.009***	**0.406**	**0.206**	**0.800**
Heart disease	–0.339	0.402	0.711	1	0.399	0.712	0.324	1.567
Constant	–2.819	1.116	6.383	1	0.012	0.060		

Notes: *Statistically significant. Bold values indicate statistically significant correlations.
Abbreviations: AMD, age-related macular degeneration; B, coefficient of the parameter to be analyzed in the logistic regression model; SE, standard error; Wald, Wald statistic; OR, odds ratio; CI, confidence interval.

regression model, the *ARMS2* gene shows the strongest association with the disease followed by *FCGR2A* – an innate immunity gene studied for the first time in AMD from our research team[31] and, finally, the *CFH* gene, another established factor. Similar study for the aforementioned genes must be replicated for the dry form of AMD as well. Our results from both studies highlight the role of innate immunity and it will be of interest to investigate the relationship between AMD and other genes that are involved in this inflammatory response in order to achieve a comprehensive picture of the genetic background.

So far, results from genetic analysis of patients with AMD have had limited clinical utility: *ARMS2* risk alleles show higher OR toward CNV progression compared to *CFH* risk alleles[37] whereas both SNPs are used in prediction models of progression to advanced AMD forms with or without body mass index and smoking.[38] Moreover, there have been numerous conflicting reports with regard to their use in selecting nutritional supplementation therapy based on the number of *ARMS2* and/or *CFH* risk alleles.[39,40]

Toward this direction of incorporating genetic findings in AMD clinical practice, the novel next-generation sequencing (NGS) approach will be useful, because this technique allows the targeted massive parallel sequencing of several genes in a large number of samples altogether in a cost-effective way, because each specimen can be identified by a unique "barcode". Genetically, AMD is highly heterogeneous and offers an ideal model in which to investigate its complexities with NGS. There are already several NGS studies in AMD that show promising results in this field, and it seems that this technique will increase the use of molecular diagnostics in AMD and the identification of rare causative variants.[41,42] Besides the analysis of genomic DNA, another approach that shows promise in AMD is the miRNA analysis in peripheral blood. miRNAs are small single-stranded non-coding RNA molecules containing 19–25 nucleotides that can recognize sequences in the 3′untranslated region of selected mRNAs and result in their translational repression or even cleavage. They can target multiple genes, and some of them are already implicated in ocular AMD.[43] Several groups have already identified profiles of miRNAs in plasma of peripheral blood of patients with AMD and can even differentiate dry from wet cases.[44,45] miRNAs in peripheral blood demonstrate stability and are not susceptible to RNAase degradation. Therefore, both DNA gene SNPs and miRNAs in peripheral blood could become very useful as biomarkers in the prediction or progression of AMD disease in the near future.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Table S1 Sequences of primers used in PCR-RFLP genotyping methods of SNPs in *ARMS2*, *CD14*, and *TLR4* genes

Primer	Sequence
Forward ARMS2_LOC1	5′-CAATGGTAGCCAGGACCCAT-3′
Reverse ARMS2_LOC2	5′-ATCCGTTAAGTCGGAAGGAG-3′
Forward CD14_F	5′-TTGGTGCCAACAGATGAGGTTCAC-3′
Reverse CD14_R	5′-TTCTTTCCTACACAGCGGCACCC-3′
Forward TLR4_1	5′-GATTAGCATACTTAGACTACTACCTCCATG-3′
Reverse TLR4_2	5′-GATCAACTTCTGAAAAAGCATTCCCAC-3′
Forward TLR4_3	5′-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3′
Reverse TLR4_4	5′-CCTGAAGACTGGAGAGTGAGTTAAATGCT-3′

Note: The underlined bases in both forward primers of *TLR4* gene (TLR4_1 and TLR4_3) denote an altered base that was introduced in order to create either an *Nco*I (Asp299Gly) or a *Hinf*I (Thr399Ile) restriction site.
Abbreviations: PCR-RFLP, polymerase chain reaction – restriction fragment length polymorphism; SNP, single-nucleotide polymorphism.

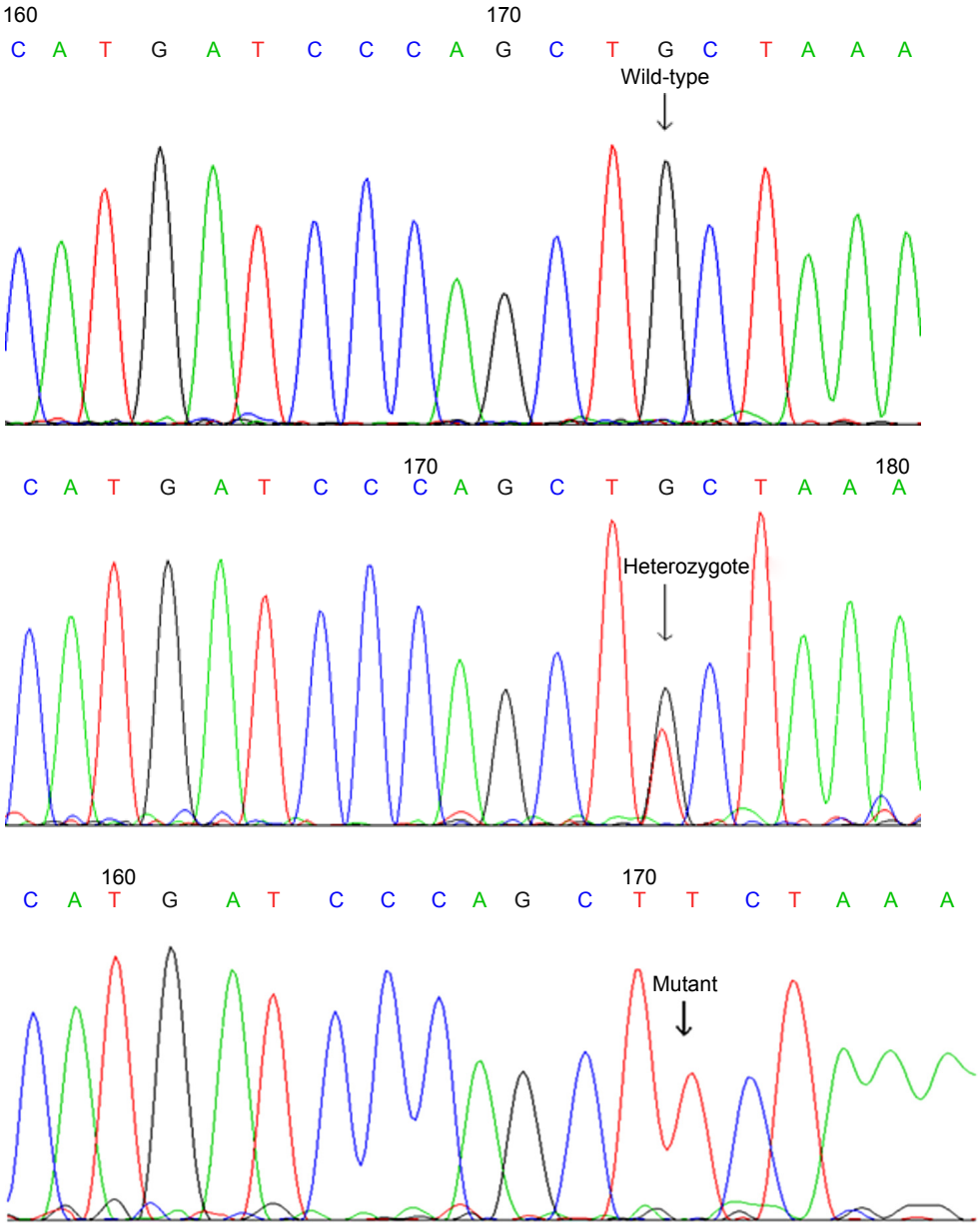

Figure S1 DNA sequencing analysis of a wild-type GG, a heterozygote G/T, and a mutant TT around the *ARMS2* A69S SNP area (forward primer).
Abbreviation: SNP, single-nucleotide polymorphism.

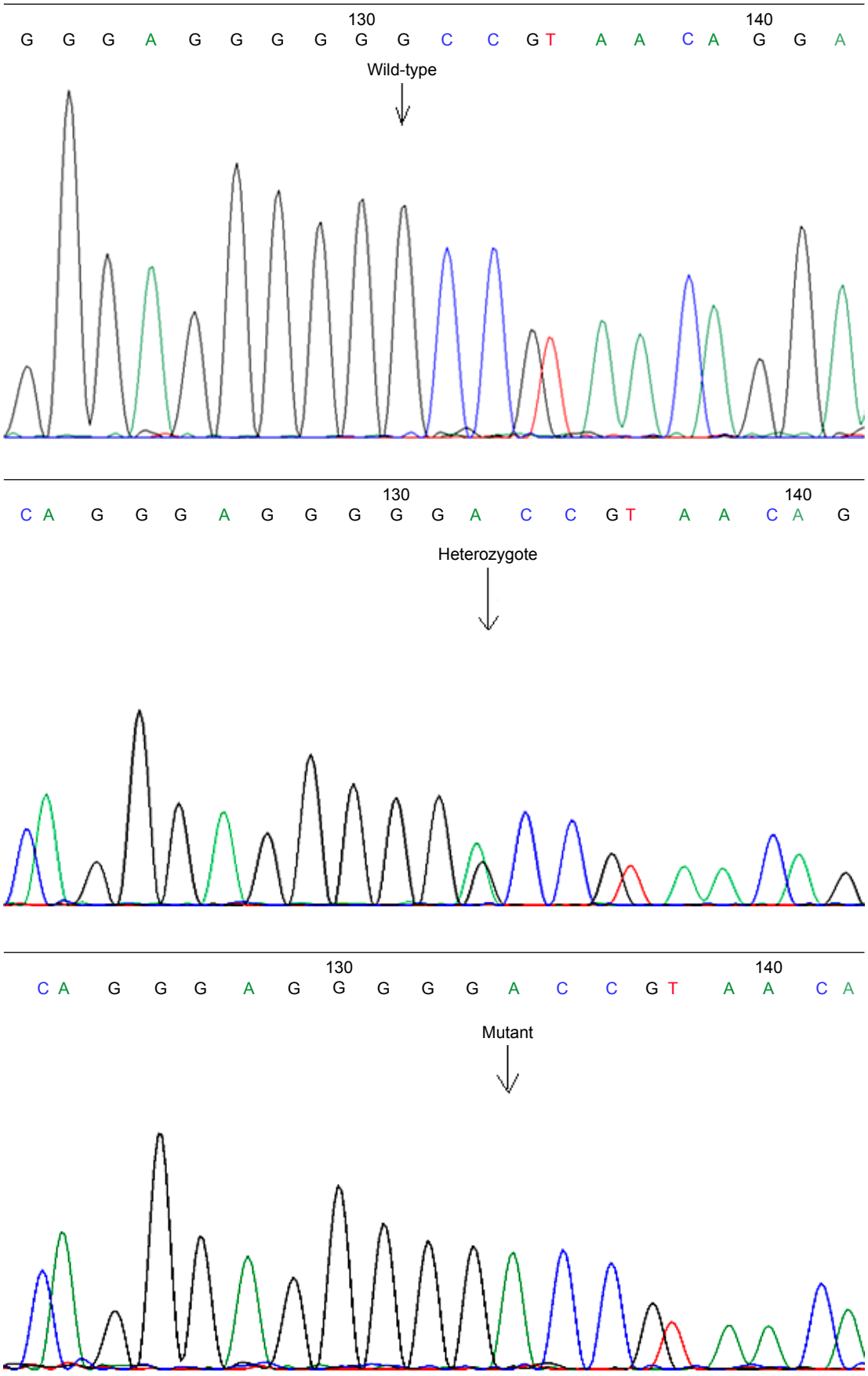

Figure S2 DNA sequencing analysis of a wild-type GG, a heterozygote G/T, and a mutant TT around the *CD14* C260T SNP area (reverse primer).
Abbreviation: SNP, single-nucleotide polymorphism.

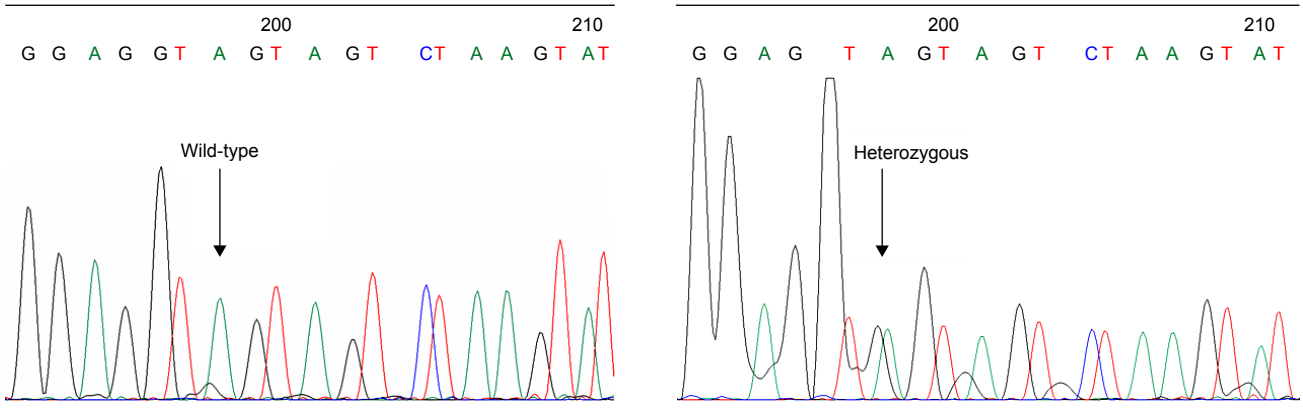

Figure S3 DNA sequencing analysis of a wild-type AA and a heterozygote A/G around the *TLR4* Asp299Gly SNP area (forward primer).
Abbreviation: SNP, single-nucleotide polymorphism.

Table S2 Multivariate model of AMD risk with all SNPs and all clinical variables

Variables	B	SE	Wald	*df*	*P*-value	OR	95% CI	
							Lower	Upper
TLR_299	0.704	0.849	0.687	1	0.407	2.021	0.383	10.669
TLR_399	1.084	0.972	1.244	1	0.265	2.957	0.440	19.876
CFH	0.542	0.221	6.027	1	**0.014***	**1.719**	**1.115**	**2.649**
FCGR2A	0.585	0.232	6.358	1	**0.012***	**1.795**	**1.139**	**2.829**
ARMS2	0.627	0.227	7.601	1	**0.006***	**1.872**	**1.199**	**2.923**
Age	–0.037	0.025	2.255	1	0.133	0.963	0.917	1.011
Sex	0.426	0.336	1.609	1	0.205	1.531	0.793	2.956
Smoking	0.428	0.367	1.360	1	0.244	1.534	0.747	3.150
Hypertension	0.270	0.423	0.406	1	0.524	1.310	0.571	3.003
Cataract	–0.490	0.385	1.622	1	0.203	0.613	0.288	1.302
Glaucoma	–0.933	0.546	2.918	1	0.088	0.393	0.135	1.148
Diabetes	–0.837	0.363	5.309	1	0.121	0.433	0.213	1.083
Heart disease	–0.271	0.413	0.430	1	0.512	0.763	0.340	1.714
Constant	1.511	1.996	0.573	1	0.449	4.529		

Notes: *Statistically significant. Bold values indicate statistically significant correlations.
Abbreviations: AMD, age-related macular degeneration; B, coefficient of the parameter to be analyzed in the logistic regression model; SE, standard error; Wald, Wald statistic; OR, odds ratio; CI, confidence interval; SNP, single-nucleotide polymorphism.