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

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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...
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 <10⁻⁶. 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. 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_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 AMD. 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 present and transfer it to the TLR4–MD2 complex in order to initiate the transduction of the NF-κB pathway. 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 cell 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. 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 chorioidal 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 (e.g., angioid streaks, high myopia, presumed ocular histoplasmosis syndrome, chorioidal 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).

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. 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 92°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 PvuII 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 uncleaved in the case

Table 1 Clinical characteristics of all 223 participants in the study

| Clinical characteristics | All, n (%) | Controls, n (%) | Patients, n (%) | 2^2 |
|-------------------------|------------|-----------------|----------------|-----|
| Sex                     |            |                 |                |     |
| Male                    | 110 (49.3) | 44 (42.7)       | 66 (55.0)      | 0.067|
| 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.
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 \textit{CD14} was digested with \textit{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 \textit{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 \textit{TLR4} amplicons after digestion with the \textit{Nco}I (Takara, Japan) restriction enzyme for the 299 residue and with \textit{Hinf}I (NEB, USA) for the 399 residue, fragment 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 \(\phi\chi\)174 \textit{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).
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 (Technelyssium 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 χ² tests were estimated with the G*Power 3.1.9.2 software.30 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 χ² 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 χ²-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 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) |         |        |
|                | T/T      | 16 (15.5%) | 20 (16.7%) | 1.09 (0.53–2.23) |         |        |
| 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.

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.
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 \( 7 \times 10^{-4} \) 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 genes31 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 5 TLR4 gene haplotype association with the disease

| Haplotype association with response (n=223) | TLR_299 | TLR_399 | Freq | OR (95% CI) | P-value |
|-------------------------------------------|---------|---------|------|-------------|---------|
| TLR_299 TLR_399                           | 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.

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

| Variables in the equation | B     | SE    | Wald | df  | P-value | OR         | 95% CI     |
|---------------------------|-------|-------|------|-----|---------|------------|------------|
| 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      | 0.118–0.887 |
| 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 team31 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 alleles37 whereas both SNPs are used in prediction models of progression to advanced AMD forms with or without body mass index and smoking.38,39 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′-CAATGGTAGCCAGGACCAT-3′                             |
| Reverse ARMS2 LOC2 | 5′-ATCCGTTAAGTCGGAGGAG-3′                             |
| Forward CD14_F   | 5′-TTGGTGCCAACAGATGAGTTACAC-3′                         |
| Reverse CD14_R   | 5′-TTTTTTTACACACAGGGTCACCC-3′                         |
| Forward TLR4_1   | 5′-GATTAGCATATTAGACTACTACCTGCAT-3′                    |
| Reverse TLR4_2   | 5′-GATCAACTTCTGAATAAGCATCCTCACC-3′                    |
| Forward TLR4_3   | 5′-GGTTGCATTCTCAGATGGAATTGGAGAA-3′                    |
| Reverse TLR4_4   | 5′-CCTGAAGACTGGAGATGTTAAATGCT-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 Ncol (Asp299Gly) or a HinfI (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 | 95% CI 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        |
| TLR_400    | −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        |

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.