

Comprehensive Analysis of the Candidate Genes *CCL2*, *CCR2*, and *TLR4* in Age-Related Macular Degeneration

Dominiek D. G. Despriet,^{1,2,3} Arthur A. B. Bergen,^{1,4} Joanna E. Merriam,⁵ Jana Zernant,⁵ Gaetano R. Barile,⁵ R. Theodore Smith,⁵ Irene A. Barbazetto,⁵ Simone van Soest,¹ Arne Bakker,¹ Paulus T. V. M. de Jong,^{1,3,6} Rando Allikmets,^{5,7} and Caroline C. W. Klaver^{1,2,3}

PURPOSE. To determine whether variants in the candidate genes *TLR4*, *CCL2*, and *CCR2* are associated with age-related macular degeneration (AMD).

METHODS. This study was performed in two independent Caucasian populations that included 357 cases and 173 controls from the Netherlands and 368 cases and 368 controls from the United States. Exon 4 of the *TLR4* gene and the promoter, all exons, and flanking intronic regions of the *CCL2* and *CCR2* genes were analyzed in the Dutch study and common variants were validated in the U.S. study. Quantitative (q)PCR reactions were performed to evaluate expression of these genes in laser-dissected retinal pigment epithelium from 13 donor AMD and 13 control eyes.

RESULTS. Analysis of single nucleotide polymorphisms (SNPs) in the *TLR4* gene did not show a significant association between D299G or T399I and AMD, nor did haplotypes containing these variants. Univariate analyses of the SNPs in *CCL2* and *CCR2* did not demonstrate an association with AMD. For *CCR2*, haplotype frequencies were not significantly different between cases and controls. For *CCL2*, one haplotype containing the minor allele of C35C was significantly associated with AMD ($P =$

0.03), but this did not sustain after adjustment for multiple testing ($q = 0.30$). Expression analysis did not demonstrate altered RNA expression of *CCL2* and *CCR2* in the retinal pigment epithelium from AMD eyes (for *CCL2* $P = 0.62$; for *CCR2* $P = 0.97$).

CONCLUSIONS. No evidence was found of an association between *TLR4*, *CCR2*, and *CCL2* and AMD, which implies that the common genetic variation in these genes does not play a significant role in the etiology of AMD. (*Invest Ophthalmol Vis Sci.* 2008;49:364–371) DOI:10.1167/iovs.07-0656

Accumulating evidence demonstrates that dysregulation of the local inflammatory and immunologic response is an important causal pathway in age-related macular degeneration. Initial proof of this insight was provided by histopathology studies that showed that drusen contain complement components, complement regulators, immunoglobulins, and anaphylatoxins.¹ Recently, the role of inflammation in AMD was further established by multiple genetic studies. Genes involved in the complement pathway, such as the complement factor H (*CFH*) gene, the complement factor B (*FB*) gene, and the complement component 2 (*C2*) gene have repetitively been associated with AMD.^{2–8} The general hypothesis is that dysfunction of these genes may lead to an increase in complement activation and a high release of proinflammatory proteins, which results in an augmentation of the local inflammatory response. It is currently unclear whether inflammatory pathways other than complement regulation are involved in AMD pathogenesis.

The immune system detects and responds to infection mainly through a family of pattern recognition receptors called toll-like receptors (TLRs).⁹ These receptors recognize a wide range of microbial molecules (e.g., lipopolysaccharide, peptidoglycan, lipopeptide) and induce phagocytosis after binding. They are expressed by many immune cells, as well as by corneal cells and retinal pigment epithelium (RPE) cells.¹⁰ TLRs trigger a signal transduction cascade that results in activation of transcription factor NF- κ B, which leads to increased expression of proinflammatory genes.^{11,12} A significant association between a common single nucleotide polymorphism (SNP; rs4986790, D299G) in the *TLR4* gene and AMD was reported by Zarepari et al.¹³ This genetic variant alters the extracellular domain of the receptor, which interrupts the signaling transduction cascade¹⁴ and interferes with the expression of genes such as *TNF- α* , *IL-1*, *IL-6*, monocyte chemoattractant protein-1 (*MCP-1* or *CCL2*), and its cognate receptor *C-C* chemokine receptor-2 (*CCR2*).¹⁵ Although biologically plausible, the reported association between *TLR4* and AMD awaits confirmation.¹⁶

CCL2 and *CCR2* are key mediators in the infiltration of monocytes from blood into foci of inflammation. The *CCL2* protein is ubiquitously expressed and exerts its effect after binding to its receptor *CCR2*, which leads to actin rearrangement, shape change, and movement of monocytes.¹⁷ Evidence

From the ¹Department of Clinical and Molecular Ophthalmogenetics, Netherlands Institute for Neuroscience, Royal Netherlands Academy of Arts and Sciences (KNAW), Amsterdam, The Netherlands; the Departments of ²Ophthalmology and ³Epidemiology and Biostatistics, Erasmus Medical Center, Rotterdam, The Netherlands; the Departments of ⁴Clinical Genetics and ⁵Ophthalmology, Academic Medical Center, Amsterdam, The Netherlands; and the Departments of ⁶Ophthalmology and ⁷Pathology and Cell Biology, Columbia University, New York, New York.

Supported by Grant 60-60400-98-019 from the Netherlands Organization for Scientific Research (the Hague); the OOG Foundation (the Hague); Rotterdamse Blindenbelangen Association (Rotterdam); the General Netherlands Society for the Prevention of Blindness (Doorn); Prof. Dr. Henkesstichting (Rotterdam), and Swart van Essen (Rotterdam), the Netherlands; National Eye Institute Grants EY13435 and EY017404 (RA); the Macula Vision Research Foundation (RA); New York Community Trust (RTS); Wallach Foundation (RA, GRB); Elyachar Foundation (RA, GRB); Kaplen Foundation (RA, GRB); Wigdeon Point Charitable Foundation (RA, JEM); and an unrestricted grant to the Department of Ophthalmology, Columbia University, from Research to Prevent Blindness.

Submitted for publication June 2, 2007; revised August 16, 2007; accepted November 13, 2007.

Disclosure: D.D.G. Despriet, None; A.A.B. Bergen, None; J.E. Merriam, None; J. Zernant, None; G.R. Barile, None; R.T. Smith, None; I.A. Barbazetto, None; S. van Soest, None; A. Bakker, None; P.T.V.M. de Jong, None; R. Allikmets, None; C.C.W. Klaver, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Caroline C. W. Klaver, Department of Ophthalmology, Erasmus University Medical Centre Rotterdam, PO Box 2040, NL 3000 CA Rotterdam, The Netherlands; carolineklaver@yahoo.com.

TABLE 1. Baseline Characteristics of the Study Population

	U.S. Study			Netherlands Study		
	Cases ($n_{\text{tot}} = 357$)	Controls ($n_{\text{tot}} = 173$)	<i>P</i>	Cases ($n_{\text{tot}} = 368$)	Controls ($n_{\text{tot}} = 368$)	<i>P</i>
Diagnosis						
No AMD		173 (100.0)			368 (100.0)	
Early AMD	89 (24.9)			—		
Neovascular AMD	180 (50.4)			276 (75.0)		
Geographic atrophy	54 (15.1)			92 (25.0)		
Mixed AMD	34 (9.5)			—		
Age, y	78.2 (7.6)	74.1 (6.3)	<0.001	78.7 (6.9)	74.6 (5.8)	<0.001
<65	19 (5.3)	5 (2.9)		18 (4.9)	28 (7.6)	
65–74	88 (24.6)	98 (56.6)		70 (19.0)	150 (40.8)	
75–84	184 (51.5)	61 (35.3)		180 (48.9)	160 (43.5)	
≥85	66 (18.5)	9 (5.2)		100 (27.2)	30 (8.2)	
Sex			0.07			0.06
Men	143 (40.1)	83 (48.0)		139 (37.8)	164 (44.6)	
Women	214 (59.9)	90 (52.0)		229 (62.2)	204 (55.4)	

Data are unadjusted mean \pm SD for continuous variables and percentages for dichotomous variables. n_{tot} , total number of participants.

of a potential role of CCL2 and CCR2 in AMD was provided by Ambati et al.,¹⁸ who showed that aging mice deficient in these genes develop hallmarks of AMD (i.e., accumulation of drusen and lipofuscin, photoreceptor atrophy, and choroidal neovascularization). Similar to human AMD, complement-associated proteins such as C5, IgG, vitronectin, CD46, and serum amyloid P component were also present in the RPE of these mice. The occurrence of AMD-like disease in these knockout mice raises the question of whether CCL2 and CCR2 play a role in human AMD.

In this study, we assessed the association with the D299G allele of *TLR4* in independent case-control studies from the Netherlands and the United States. Furthermore, we performed a comprehensive genetic analysis of the *CCL2* and *CCR2* genes in the Dutch study and validated common variants of these genes in the U.S. study. We also performed quantitative (q)PCR experiments to investigate whether mRNA expression of these genes in the retinal pigment epithelium was different between individuals with AMD and healthy control subjects.

METHODS

Study Population

This study consisted of two independent populations of AMD cases and age-matched control subjects. The first set consisted of 357 unrelated patients with AMD and 173 unrelated control individuals from the Netherlands. Subjects were all Caucasian and were recruited from the Netherlands Institute of Neuroscience, Amsterdam, and Erasmus University Medical Center, Rotterdam, and through newsletters and patient organizations. Controls were 65 years of age and older and were mostly unaffected spouses or nonrelated acquaintances of cases or individuals who attended the ophthalmology department for reasons other than retinal disease.

The second set consisted of 368 unrelated individuals with AMD and 368 unrelated controls of American-European descent recruited at Columbia University as previously described.⁵ Cases and controls of both studies were examined by trained ophthalmologists before diagnosis (described later).

The study was approved by the Ethics Committee of Academic Medical Centre Amsterdam, and the Institutional Review Board of Columbia University and adhered to the tenets of the Declaration of Helsinki. All participants provided signed, informed consent for participation in the study, and for the publication of the data obtained, retrieval of medical records, and use of blood and DNA for AMD research.

Diagnosis of AMD

All participants underwent fundus photography after pharmacologic mydriasis. Fundus transparencies were subsequently graded according to a modification of the international classification and grading system for AMD under the supervision of senior retinal specialists (CCWK, PTVMdj, IAB, RTS, GRB).^{19,20} Grading criteria were identical for both studies. Cases were stratified according to the eye with the most severe disease: early AMD (soft indistinct drusen with or without pigmentary changes, or soft distinct drusen with pigmentary changes, i.e., stages 2 and 3) or end-stage AMD (stage 4). The latter was subclassified into geographic atrophy, neovascular macular degeneration, or a mixed type of end-stage AMD. Controls had no or only a few small hard drusen and no other macular disease (stage 0) in both eyes.

Genotyping

DNA was extracted from peripheral blood leukocytes after venous puncture. Exon 4 of the *TLR4* gene, as well as the promoter region, all exons and flanking intronic regions of *CCR2* and *CCL2*, and exon 9 of *CFH* were amplified by PCR. In the Dutch study, the samples were analyzed for sequence variations by using denaturing high-performance liquid chromatography (DHPLC) on an automated system (Wave; Transgenomic, Santa Clara, CA). For identification of homozygous variants in amplicons with frequent heterozygous SNPs, aliquots of a known wild-type sample were added to the DNA before the reannealing step. Variants on DHPLC were graded by two researchers and subsequently identified by direct sequencing (model 310; Applied Biosystems, Inc. [ABI], Foster City, CA). Discrepancies between DHPLC grading were also analyzed by using direct sequencing.

In the U.S. study, participants were genotyped for common sequence variations in the *CCR2*, *CCL2*, and *TLR4* genes (*Taqman* assay; ABI). Primer sequences are available on request.

Human Postmortem Eyes and Evaluation of RNA Expression

Human bulbi from 26 donors were provided by the Corneabank, Amsterdam. Histopathology was evaluated on 8- μ m sections of the maculae that were stained with the periodic-acid-Schiff reaction. Maculae with drusen and/or a continuous layer of basal laminar deposit were classified as cases (mean age, 76.31 \pm 2.72 [SD] years; $n = 13$); maculae with no disease were classified as age-matched controls (mean age, 75.43 \pm 2.07 years; $n = 7$) and young controls (mean age, 24.83 \pm 6.91 years; $n = 6$). We collected RPE cells from retinal sections with a

TABLE 2. Frequency of the Single Nucleotide Polymorphisms in the *TLR4* Gene
A. Netherlands Study

SNP	rs-Numbers	Nucleotide Change	Frequency		OR (95% CI)*	P
			Cases	Controls		
Genotype D299G	rs4986790	AA	0.903	0.893	1.00	0.61
		AG	0.094	0.107	0.85 (0.45–1.60)	
		GG	0.003	—	—	
K354K		AA	0.980	0.988	1.00	0.40
		AG	0.020	0.012	2.04 (0.39–10.71)	
		GG	—	—	—	
T3991	rs4986791	CC	0.897	0.877	1.00	0.29
		CT	0.100	0.123	0.72 (0.40–1.32)	
		TT	0.003	—	—	
					χ^2	P
<hr/>						
Allele						
D299G	rs4986790	G	0.050	0.053	0.05	0.83
K354K		G	0.010	0.006	0.47	0.49
T3991	rs4986791	T	0.050	0.061	0.57	0.45

*Adjusted for age and sex.

B. U.S. Study

SNP	rs-Numbers	Nucleotide Change	Frequency		OR (95% CI)	P
			Cases	Controls		
Genotype D299G	rs4986790	AA	0.885	0.907	1.00	0.44
		AG	0.107	0.090	1.21 (0.74–1.97)	
		GG	0.008	0.003	3.07 (0.32–29.71)	
					χ^2	P
Allele D299G	rs4986790	G	0.058	0.048	1.32	0.25

C. Both Studies Combined

SNP	rs-Numbers	Nucleotide Change	Frequency		OR (95% CI)	P
			Cases	Controls		
Genotype D299G	rs4986790	AA	0.895	0.903	1.00	0.74
		AG	0.101	0.096	1.07 (0.73–1.56)	
		GG	0.004	0.002	2.30 (0.24–22.13)	
					χ^2	P
Allele D299G	rs4986790	G	0.055	0.050	0.31	0.58

laser dissection microscope (P.A.L.M.; Microlaser Technologies AG, Bernried, Germany) and isolated and amplified RNA according to Agilent protocols (Agilent Technologies, Palo Alto, CA). Amplified RNA (200 ng) was transcribed into cDNA by reverse transcriptase (Superscript III; Invitrogen, Carlsbad, CA). We performed qPCR reactions and detected levels of amplified product by real-time monitoring of SYBR Green I dye fluorescence (Prism 7300; ABI), according to methods described by van Soest et al.²¹

Statistical Analysis

Baseline characteristics of cases and controls were compared by using analysis of covariance for continuous variables and logistic regression analysis for discrete variables and were adjusted for age and sex. Genotype distributions were tested for Hardy-Weinberg

equilibrium using the χ^2 test. Haploview software (<http://www.broad.mit.edu/mpg/haploview/>) provided in the public domain by The Broad Institute, Massachusetts Institute of Technology, Cambridge, MA) was used to estimate allele frequencies and allele-based risk of AMD. Logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for risk of AMD, adjusted for age and sex, with major alleles used as the reference. Haplotypes were estimated by using the expectation-maximization algorithm, and the risk of AMD for each haplotype was determined with Haplo.stats 1.2.2 (<http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm/>) provided in the public domain by Mayo Clinic, Rochester, MN). To account for multiple comparisons, we estimated the *q* statistic to determine the approximate false-discovery rate (FDR), which is defined as the proportion of statistical tests

TABLE 3. Frequency of the Single Nucleotide Polymorphisms in the *CCL2* Gene
A. Netherlands Study

SNP	rs-Number	Nucleotide Change	Frequency		OR (95% CI)*	P
			Cases	Controls		
Genotype						
-2578 A>G		AA	0.569	0.519	1.00	0.15
		AG	0.350	0.442	0.71 (0.45–1.13)	
		GG	0.081	0.039	1.96 (0.67–5.73)	
-2136 A>T		AA	0.663	0.589	1.00	0.09
		AT	0.287	0.397	0.67 (0.43–1.06)	
		TT	0.050	0.014	3.13 (0.67–14.57)	
IVS1 + 50 A>T	rs28730833	AA	0.975	0.992	1.00	0.15
		AT	0.008	0.025	0.25 (0.04–1.65)	
		TT	—	—	—	
C35C	rs4586	TT	0.388	0.451	1.00	0.27
		TC	0.473	0.459	1.31 (0.81–2.13)	
		CC	0.139	0.090	1.67 (0.75–3.73)	
A71T		GG	0.996	1.000	—	—
		GA	0.004	—	—	
		AA	—	—	—	
					χ^2	P
Allele						
-2578 A>G		G	0.256	0.221	0.059	0.81
-2136 A>T		T	0.193	0.213	0.544	0.46
IVS 1+50 A>T	rs28730833	T	0.004	0.012	1.704	0.19
C35C	rs4586	C	0.376	0.320	2.213	0.14
A71T		A	0.002	—	0.491	0.48

*Adjusted for age and sex.

B. U.S. Study

SNP	rs-Number	Nucleotide Change	Frequency		OR (95% CI)	P
			Cases	Controls		
Genotype						
C35C	rs4586	TT	0.343	0.367	1.00	0.70
		TC	0.480	0.481	1.07 (0.77–1.47)	
		CC	0.177	0.152	1.24 (0.81–1.92)	
					χ^2	P
Allele						
C35C	rs4586	C	0.417	0.393	0.90	0.34

C. Both Studies Combined

SNP	rs-Number	Nucleotide Change	Frequency		OR (95% CI)	P
			Cases	Controls		
Genotype						
C35C	rs4586	TT	0.361	0.388	1.00	0.57
		TC	0.477	0.476	1.08 (0.83–1.40)	
		CC	0.162	0.137	1.27 (0.88–1.83)	
					χ^2	P
Allele						
C35C	rs4586	C	0.400	0.374	1.53	0.22

called significant that are actually false positive.^{22,23} The *q* statistic, also known as FDR-adjusted probabilities, was calculated incorporating all probabilities from the 54 tests performed for SNPs and haplotypes in this study. Mean gene expression levels between cases and controls were compared by Mann-Whitney U test and were adjusted for expression of housekeeping genes (*RBLPO*, *CYCLOP*, and *EF1a*) to correct for differences in cDNA load.²⁴

RESULTS

Table 1 shows the characteristics of cases and controls. Cases were, on average, 4 years older than controls in both studies. The distribution of gender was not significantly different between cases and controls.

TABLE 4. Frequency of the Single Nucleotide Polymorphisms in the *CCR2* Gene
A. Netherlands Study

SNP	rs-Number	Nucleotide Change	Frequency		OR (95% CI)*	P
			Cases	Controls		
Genotype						
V52V	rs3918367	GG	0.980	0.975	1.00	0.67
		GT	0.020	0.025	0.73 (0.16–3.21)	
		TT	—	—	—	
V64I	rs1799864	GG	0.855	0.810	1.00	0.11
		GA	0.141	0.182	0.61 (0.33–1.13)	
		AA	0.004	0.008	0.38 (0.02–6.20)	
R233Q		GG	1.000	0.992	—	0.50
		GA	—	0.008	—	
		AA	—	—	—	
N260N	rs1799865	TT	0.453	0.492	1.00	0.86
		TC	0.449	0.443	1.04 (0.65–1.68)	
		CC	0.097	0.066	1.47 (0.60–3.61)	
IVS 1+103 G>A	rs3092960	GG	0.992	0.992	1.00	0.93
		GA	0.008	0.008	1.11 (0.10–12.96)	
		AA	—	—	—	
I318T		TT	0.992	1.000	—	
		TC	0.008	—	—	
		CC	—	—	—	
					χ^2	P
Allele						
V52V	rs3918367	T	0.010	0.012	0.08	0.78
V64I	rs1799864	A	0.153	0.099	1.26	0.26
R233Q		A	—	0.004	2.00	0.16
N260N	rs1799865	C	0.322	0.287	0.93	0.33
IVS 1+103 G>A	rs3092960	A	0.004	0.004	0	0.99
I318T		C	0.004	—	1.00	0.32

*Adjusted for age and sex.

B. U.S. Study

SNP	rs-Number	Nucleotide Change	Frequency		OR (95% CI)	P
			Cases	Controls		
Genotype						
V64I	rs1799864	GG	0.796	0.826	1.00	0.50
		GA	0.183	0.166	1.14 (0.78–1.67)	
		AA	0.022	0.008	2.77 (0.43–10.53)	
					χ^2	P
Allele						
V64I	rs1799864	A	0.113	0.091	1.90	0.17

C. Both Studies Combined

SNP	rs-Number	Nucleotide Change	Frequency		OR (95% CI)	P
			Cases	Controls		
Genotype						
V64I	rs1799864	GG	0.820	0.822	1.00	0.88
		GA	0.166	0.170	0.98 (0.71–1.34)	
		AA	0.015	0.008	1.79 (0.55–5.84)	
					χ^2	P
Allele						
V64I	rs1799864	A	0.097	0.093	0.11	0.74

SNP analysis in the *TLR4* gene did not show a significant association with D299G or T399I in the *TLR4* gene and AMD. We identified a previously unknown rare variant (i.e., K354K,

in the amplified region of exon 4; Table 2). Haplotype analysis of the three SNPs in *TLR4* did not convey a risk of AMD. We determined the potential additive effect of the hetero- and

TABLE 5. Haplotype Analyses in the Dutch Study
A. *TLR4* Gene

	D299G	K354K	T399IT	Freq. in Cases	Freq. in Controls	OR (95% CI)*	P
H1	1	1	2	0.009	0.013	0.61 (0.16–2.31)	0.47
H2	2	1	2	0.041	0.048	0.75 (0.38–1.46)	0.40
H3	1	1	1	0.939	0.924	Ref	

*Adjusted for age and sex; 1 = major allele; 2 = minor allele.

B. *CCL2* Gene

	–2518 A > G	–2076 A>T	IVS1+50 A>T	C35C	A71T	Freq. in Cases	Freq. in Controls	OR (95% CI)*	P
H1	1	2	1	1	1	0.190	0.197	1.11 (0.72–1.71)	0.65
H2	2	1	1	2	1	0.248	0.247	1.15 (0.76–1.72)	0.51
H3	1	1	1	2	1	0.125	0.072	1.99 (1.07–3.73)	0.03
H4	1	1	1	1	1	0.424	0.458	Ref	

*Adjusted for age and sex; 1 = major allele; 2 = minor allele.

C. *CCR2* Gene

	V52V	V64I	R233Q	N260N	IVS1+103 G>A	I308T	Freq. in Cases	Freq. in Controls	OR (95% CI)*	P
H1	1	2	1	1	1	1	0.073	0.099	0.61 (0.34–1.09)	0.10
H2	1	1	1	2	1	1	0.313	0.274	1.10 (0.75–1.61)	0.63
H3	1	1	1	1	1	1	0.597	0.606	Ref	

*Adjusted for age and sex; 1 = major allele; 2 = minor allele.

homozygous genotypes of D299G and T399I in *TLR4*, and did not find evidence for such an effect (D299G $P = 0.74$; T399I $P = 0.50$). The frequency of D299G was within the same range in the U.S. study, and no significant frequency differences between cases and controls were found. Pooling studies did not alter results (Table 2), nor did adjustment or stratification for the *CFH* Y402H allele (data not provided). Analysis of RNA expression of *TLR4* in the RPE was low and did not reveal any significant differences between cases and controls.

In the Netherlands study, we found five different variants in the *CCL2* gene: two localized in the promoter region (–2578 A>G; –2136 A>T), one intronic variant (IVS1 +50 A>T), one previously described synonymous SNP (C35C), and a newly identified missense variant in exon 3 (A71T). We observed six variants in the *CCR2* gene: two synonymous (V52V; N260N) and three nonsynonymous (V64I; R233Q; I318T) substitutions and one intronic SNP (IVS1 +103G>A). Genotype frequencies of all SNPs were in Hardy-Weinberg equilibrium. No statistically significant association was found between any of the sequence variations in these genes and AMD in the univariate analysis. The frequencies of C35C of *CCL2* and V64I of the *CCR2* gene were within the same range in the U.S. study, and did not show any significant differences between cases and controls. Pooling did not alter these results (Tables 3, 4), nor did stratification or adjustment for Y402H of *CFH* (data not provided). We generated haplotypes using all identified SNPs in the Dutch study. For *CCR2*, the estimated haplotype frequencies were not significantly different. For *CCL2*, one haplotype containing the minor allele of C35C and the major alleles of all other SNPs was significantly associated with AMD ($P = 0.03$). This difference did not remain significant after adjustment for multiple testing ($q = 0.30$; Table 5).

Results from the gene expression study did not reveal any significant differences between cases and controls. Gene expression levels of *CCL2* and *CCR2* in the human RPE decreased with age. The expression level of *CCL2* was, on average, 2.6 times lower in the old control eyes than in the young non-AMD

eyes ($P = 0.15$ for difference). The expression level of *CCR2* was on average 1.3 times lower ($P = 0.81$ for difference). Expression levels were highly variable in the entire group and showed no significant differences between the AMD and the old control eyes (*CCL2*: $P = 0.62$; *CCR2*: $P = 0.97$).

DISCUSSION

We could not confirm the association between the D299G variant of the *TLR4* gene and AMD in two large, independent case-control studies. In addition, we did not find a significant relationship with genetic variants in the coding region of the *CCR2* and *CCL2* genes. The qPCR experiments did not reveal any significant differences in expression levels in these genes. The lack of positive results implies that these genes do not play an important role in the etiology of AMD.

Strengths of our study include the use of two independent case-control studies, both employing the same method of diagnosis. Although the genetic approach was different, the studies had very similar findings. The Dutch study was designed to detect known and unknown variants by using DHPLC; the U.S. study validated known variants with a genotyping assay (*Taqman*; Invitrogen). A limitation was that the statistical power to establish significant associations of rare alleles was still relatively low. We could detect ORs of at least 1.47 with a power of 80% and a significance level of 0.05 for allele frequencies of 0.20, whereas we were able to detect odds ratios of 1.90 or higher for allele frequencies of 0.05. Therefore, we cannot exclude that infrequent alleles of these genes carry a low risk of AMD.

The association of *TLR4* with AMD was initially proposed by Zarepari et al.¹⁵ in a study of Caucasians consisting of 667 cases and 439 controls, showing an increased risk for those with the D299G allele (OR = 2.65, 95% CI 1.13–6.25). Kaur et al.¹⁶ could not replicate this finding in a study consisting of 100 cases and 120 controls from India; on the contrary, they found

a slightly lower risk of AMD for the haplotype containing D299G. Our Caucasian study from two continents consisted of 725 cases and 541 controls and yielded results in line with those of Kaur et al. The allele frequency of D299G was very similar in both our case groups (5%), which approached the frequency in the cases of Zarepari et al. (6%).¹³ However, we found a similar frequency in controls (5%), whereas Zarepari et al. found a frequency of 3% in the control group.

The *CCL2* and *CCR2* genes were initially proposed as candidate genes in animal studies.¹⁸ We analyzed the genetic variation of these genes in all exons and flanking intronic regions in the Dutch study, and validated common variants in the U.S. study. The allele frequencies were very similar in both study populations and were within the same range as reported in other Caucasian populations.²⁵ Our analyses revealed no significant associations with single SNPs. In particular, we did not find altered risks for the -2518 and -2076 alleles in the promoter of *CCL2*, which are known to increase the risk of coronary artery disease, HIV infection, and AIDS dementia.^{26,27} We also failed to detect an association with the V64I allele in *CCR2*, which reduces the risk of HIV progression and coronary artery disease.^{28,29} Contrary to the univariate analyses, haplotype analysis revealed one statistically significant haplotype in *CCL2*. However, this association did not remain significant after correction for false-discovery rate, suggesting a false-positive finding.

RNA expression of *CCL2* and *CCR2* showed high variation among individuals, but was within the same range in cases and controls. Thus, as opposed to mice, in which deficiency of the *CCL2* or *CCR2* genes leads to a prominent AMD-like phenotype, we did not find evidence of decreased RNA-expression of *CCL2* and *CCR2* in humans with AMD, nor did we find any association with genetic variants. The opposite appears to be true of the *CFH* gene: whereas genetic variations show high association with AMD in humans, *CFH*-deficient mice do not develop a significant AMD phenotype. Taken together, these data suggest a different pathogenesis in mice and humans, leading to similar pathologic features. What are the possible explanations? First, the sequences of these genes are not fully identical, which could lead to differences in protein function between mice and humans. Second, biological pathways generally contain many proteins with equivalent function, and this functional redundancy may differ across species.

In summary, the findings in our study do not support a role for common genetic variation in the *TLR4*, *CCL2*, and *CCR2* genes in the etiology of AMD. These results, however, do not exclude the possibility that immune response and/or inflammatory pathways other than the alternative complement cascade are involved in the disease. The broad spectrum of inflammatory proteins found in AMD eyes warrants further research in this domain.

Acknowledgments

The authors thank all clinicians from Rotterdam, Amsterdam, and New York who referred study participants and assisted in data collection; Anke Essing for performing the qPCR experiments; Tiia Falk and Mihai Busuioc for technical assistance; and Ada Hooghart for the grading of fundus transparencies.

References

- Hageman GS, Luthert PJ, Victor Chong NH, Johnson LV, Anderson DH, Mullins RF. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. *Prog Retin Eye Res.* 2001;20(6):705-732.
- Despriet DD, Klaver CC, Witteman JC, et al. Complement factor H polymorphism, complement activators, and risk of age-related macular degeneration. *JAMA.* 2006;296(3):301-309.
- Edwards AO, Ritter R 3rd, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. *Science.* 2005;308(5720):421-424.
- Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet.* 2006;38(4):458-462.
- Hageman GS, Anderson DH, Johnson LV, et al. 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(20):7227-7232.
- Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. *Science.* 2005;308(5720):419-421.
- Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science.* 2005;308(5720):385-389.
- Maller J, George S, Purcell S, et al. Common variation in three genes, including a noncoding variant in CFH, strongly influences risk of age-related macular degeneration. *Nat Genet.* 2006;38(9):1055-1059.
- Pasare C, Medzhitov R. Toll-like receptors: linking innate and adaptive immunity. *Microbes Infect.* 2004;6(15):1382-1387.
- Kindzelskii AL, Elnor VM, Elnor SG, Yang D, Hughes BA, Petty HR. Toll-like receptor 4 (TLR4) of retinal pigment epithelial cells participates in transmembrane signaling in response to photoreceptor outer segments. *J Gen Physiol.* 2004;124(2):139-149.
- Cook DN, Pisetsky DS, Schwartz DA. Toll-like receptors in the pathogenesis of human disease. *Nat Immunol.* 2004;5(10):975-979.
- Parker LC, Whyte MK, Vogel SN, Dower SK, Sabroe I. Toll-like receptor (TLR)2 and TLR4 agonists regulate CCR expression in human monocytic cells. *J Immunol.* 2004;172(8):4977-4986.
- Zarepari S, Buraczynska M, Branham KE, et al. Toll-like receptor 4 variant D299G is associated with susceptibility to age-related macular degeneration. *Hum Mol Genet.* 2005;14(11):1449-1455.
- Arbour NC, Lorenz E, Schutte BC, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet.* 2000;25(2):187-191.
- Michelsen KS, Wong MH, Shah PK, et al. Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. *Proc Natl Acad Sci U S A.* 2004;101(29):10679-10684.
- Kaur I, Hussain A, Hussain N, et al. Analysis of CFH, TLR4, and APOE polymorphism in India suggests the Tyr402His variant of CFH to be a global marker for age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 2006;47(9):3729-3735.
- Charo IF, Taubman MB. Chemokines in the pathogenesis of vascular disease. *Circ Res.* 2004;95(9):858-866.
- Ambati J, Anand A, Fernandez S, et al. An animal model of age-related macular degeneration in senescent Ccl-2- or Ccr-2-deficient mice. *Nat Med.* 2003;9(11):1390-1397.
- Bird AC, Bressler NM, Bressler SB, et al. An international classification and grading system for age-related maculopathy and age-related macular degeneration: the International ARM Epidemiological Study Group. *Surv Ophthalmol.* 1995;39(5):367-374.
- van Leeuwen R, Klaver CC, Vingerling JR, Hofman A, de Jong PT. The risk and natural course of age-related maculopathy: follow-up at 6 1/2 years in the Rotterdam study. *Arch Ophthalmol.* 2003;121(4):519-526.
- van Soest S, De Wit GM, Essing AH, et al. Comparison of human RPE gene expression in macula and periphery highlights potential topographic differences in Bruch's membrane. *Mol Vis.* 2007;13:1608-1617.
- Storey JD, Tibshirani R. Statistical significance for genome-wide studies. *Proc Natl Acad Sci USA.* 2003;100(16):9440-9445.
- Freimer NB, Sabatti C. Guidelines for association studies in human molecular genetics. *Hum Mol Genet.* 2005;14(17):2481-2483.
- Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 2002;3(7):RESEARCH0034.
- Philpott S, Burger H, Tarwater PM, et al. CCR2 genotype and disease progression in a treated population of HIV type 1-infected women. *Clin Infect Dis.* 2004;39(6):861-865.

26. Szalai C, Duba J, Prohaszka Z, et al. Involvement of polymorphisms in the chemokine system in the susceptibility for coronary artery disease (CAD): coincidence of elevated Lp(a) and MCP-1-2518 G/G genotype in CAD patients. *Atherosclerosis*. 2001;158(1):233-239.
27. Gonzalez E, Rovin BH, Sen L, et al. HIV-1 infection and AIDS dementia are influenced by a mutant MCP-1 allele linked to increased monocyte infiltration of tissues and MCP-1 levels. *Proc Natl Acad Sci U S A*. 2002;99(21):13795-13800.
28. Ioannidis JP, Rosenberg PS, Goedert JJ, et al. Effects of CCR5-Delta32, CCR2-64I, and SDF-1 3'A alleles on HIV-1 disease progression: international meta-analysis of individual-patient data. *Ann Intern Med*. 2001;135(9):782-795.
29. Valdes AM, Wolfe ML, O'Brien EJ, et al. Val64Ile polymorphism in the C-C chemokine receptor 2 is associated with reduced coronary artery calcification. *Arterioscler Thromb Vasc Biol*. 2002;22(11):1924-1928.