Prospective Study of Common Variants in the Retinoic Acid Receptor–Related Orphan Receptor α Gene and Risk of Neovascular Age-Related Macular Degeneration

Debra A. Schaumberg, ScD, OD, MPH; Daniel Chasman, PhD; Margaux A. Morrison, BS; Scott M. Adams, BS; Qun Guo, MS; David J. Hunter, MBBS, ScD; Susan E. Hankinson, ScD; Margaret M. DeAngelis, PhD

Objectives: The retinoic acid receptor (RAR)–related orphan receptor α gene (RORA) is implicated as a candidate for age-related macular degeneration (AMD) through a previous microarray expression study, linkage data, biological plausibility, and 2 clinic-based cross-sectional studies. We aimed to determine if common variants in RORA predict future risk of neovascular AMD.

Methods: We measured genotypes for 18 variants in intron 1 of the RORA gene among 164 cases who developed neovascular AMD and 485 age- and sex-matched controls in a prospective, nested, case-control study within the Nurses’ Health Study and the Health Professionals Follow-up Study. We determined the incidence rate ratios and 95% confidence intervals (CI) for neovascular AMD for each variant and examined interactions with other AMD-associated variants and modifiable risk factors.

Results: We identified one single-nucleotide polymorphism (rs12900948) that was significantly associated with increased incidence of neovascular AMD. Participants with 1 and 2 copies of the G allele were 1.73 (CI, 1.32-2.27) and 2.99 (CI, 1.74-5.14) times more likely to develop neovascular AMD. Individuals homozygous for both the G allele of rs12900948 and ARMS2 A69S had a 40.8-fold increased risk of neovascular AMD (CI, 10.1-164; P=.017). Cigarette smokers who carried 2 copies of the G allele had a 9.89-fold risk of neovascular AMD but the interaction was not significant (P=.08). We identified a significant AMD-associated haplotype block containing the single-nucleotide polymorphisms rs730754, rs8034864, and rs12900948, with P values for ACA=1.16×10^-9, ACG=5.85×10^-12, and GAA=.0001 when compared with all other haplotypes.

Conclusions: Common variants and haplotypes within the RORA gene appear to act synergistically with the ARMS2 A69S polymorphism to increase risk of neovascular AMD. These data add further evidence of a high level of complexity linking genetic and modifiable risk factors to AMD development and should help efforts at risk prediction.


The leading cause of blindness in white persons in the United States and other industrialized countries, age-related macular degeneration (AMD) has emerged as a paradigmatic example of a common complex disease caused by the interplay of genetic predisposition and exposure to modifiable risk factors. A large number of studies have established relationships between lifestyle factors such as diet, cigarette smoking, and obesity as well as common variants within a handful of genes and risk of AMD in multiple populations. Among the genetic risk factors, common variants in 2 genes, complement factor H (CFH) (1q32) and ARMS2/HTRA serine peptidase 1 (HTRA1) (10q26), have strong and consistent associations with risk of AMD and are estimated to contribute to a strikingly large proportion of AMD cases in the US population. Nonetheless, not every individual with a risk factor or set of risk factors will develop AMD, and efforts at developing risk prediction models based on currently understood risk factors are insufficient to reliably predict the development and progression of AMD in individuals. Refinements in exposure assessment for epidemiological risk factors such as cigarette smoking, diet, and obesity, as well as the identification of other disease-associated variants, may improve the predictive ability and lead to clinically relevant interventions to identify individuals that require closer follow-up and earlier or more targeted forms of intervention.

With such a goal in mind, the authors previously performed linkage analysis and gene expression microarray analysis on a family-based cohort that comprised extremely discordant sibling pairs (EDSP) (that is, pairs in which the unaffected sib-
lings had normal maculae at an age older [≥65 years] than that at which the index patient was first diagnosed with neovascular AMD). The EDSPs were used as a discovery cohort to identify novel candidate genes and pathways with biological relevance. Based on the results of these studies, the candidate gene RAR-related orphan receptor α (RORA; OMIM 600825) was chosen for further analysis. RORA is a retinoid-related orphan receptor and member of a distinct subfamily of nuclear receptors. RORA is known to be involved in a number of biological processes with potential relevance to AMD including immunity/inflammation, angiogenesis, and lipid and cholesterol metabolism. It is also located within a linkage peak identified previously by 2 independent studies. In an initial study, we identified common variants (rs4335725, rs12900948, and 2 haplotypes) within intron 1 of RORA that were associated with AMD in 2 independent, cross-sectional, clinic-based study populations.

In the present study, we investigated 18 common intron 1 variants of RORA in a prospective, nested, case-control study of neovascular AMD within the Nurses’ Health Study (NHS) and Health Professionals Follow-up Study (HPFS) cohorts. We aimed to replicate the association between the RORA gene and neovascular AMD and to further clarify the magnitude and interrelationships of this gene and other risk factors in a prospective study population.

METHODS

The NHS is an ongoing prospective study of 121,700 primarily white female registered nurses aged 30 to 55 years in the United States in 1976. The HPFS includes 51,529 largely white male health professionals in the United States who have been followed up prospectively since 1986. At that time, HPFS participants ranged in age from 40 to 75 years. From 1989 to 1990, we obtained blood samples from 32,826 NHS participants and between 1993 and 1995, from 18,162 HPFS participants, who form the study base. The institutional review board of Brigham and Women’s Hospital and the Harvard School of Public Health’s Human Subjects Committee approved the study protocol.

From the time of enrollment, NHS and HPFS participants completed a mailed questionnaire every 2 years, from which we obtained information on lifestyle factors including height and weight; we used this to calculate body mass index (calculated as weight in kilograms divided by height in meters squared), cigarette smoking history, and weight; we used this to calculate body mass index (calculated as weight in kilograms divided by height in meters squared), and weight; we used this to calculate body mass index (calculated as weight in kilograms divided by height in meters squared), diet assessed via validated semi-quantitative food frequency questionnaires. For the present study, participants on each biennial questionnaire were asked to provide a detailed family history of AMD.

ASCERTAINMENT OF AMD CASES AND CONTROL SELECTION

We used a validated 2-stage procedure to document incident cases of AMD. Briefly, we asked participants on each biennial study questionnaire about the diagnosis of AMD. When AMD was reported, we requested permission to review medical records. If permission was granted, we sent a letter to the participant’s ophthalmologist to obtain information on the date of AMD diagnosis, best-corrected visual acuity at the most recent examination, and the choroiditis lesions present (druzen; retinal pigment epithelium changes including atrophy, hypertrophy, and retinal pigment epithelium detachment; geographic atrophy; subretinal neovascular membrane; disciform scar), and other information. We classified cases as neovascular AMD if there was a retinal pigment epithelium detachment, subretinal neovascular membrane, or disciform scar not due to other factors (eg, histoplasmosis, choroidal rupture). Only participants in whom we confirmed the presence neovascular AMD with a visual acuity of 20/30 or worse attributable to AMD, who were first diagnosed after the date of receipt of the baseline blood specimen and were aged 50 years or older, were selected as cases for the present study. We classified participants based on the most severely affected eye.

We selected 3 controls for each case of neovascular AMD at random from study participants in the same cohort as the cases who were still at risk of AMD at the time the case was diagnosed; they were of the same age within 1 year and reported having an eye examination in the past 2 years. Multiple controls were used to increase study power. We previously demonstrated significant associations between the Y402H polymorphism in CFH as well as the A69S polymorphism in ARMS2 in the cases and controls.

GENOTYPING

We examined 18 single-nucleotide polymorphisms (SNPs) in intron 1 of the RORA gene. Seven of these SNPs (rs12916023, rs730754, rs8034864, rs12900948, rs12939194, rs17237514, and rs4335725) were shown to be significant, either individually or as part of a haplotype, in a previous study of 150 sibling pairs who were extremely discordant for AMD; that is, the index patient with the neovascular form of AMD who had an unaffected sibling with normal maculae who was older than 65 years (detailed information on the EDSP cohort is described elsewhere). These 7 significant SNPs were derived from an initial group of 148 SNPs chosen approximately every 3000 to 5000 base pairs (bp) to represent variation within the 730 kilobases of the RORA gene. For this analysis, we chose 11 additional tagging SNPs that surrounded the region encompassing the 7 significant SNPs using the HapMap (www.hapmap.org). In choosing the tagging SNPs, each SNP must have had (1) a minor allele frequency of 10% or greater, and (2) an r² value of at least 0.8. We extracted DNA from the buffy coat fraction of cenfuged blood specimens using the QiAmp Blood Kit (Qia-gen, Valencia, California). Sequenom SpectroDESIGNER software (version 3.0.0.3) (Sequenom, San Diego, California) was used to design Multiplex PCR (polymerase chain reaction) assays using a sequence that contained the SNP site and 100 bp of flanking sequence on either side of the SNP. Briefly, 10 ng of genomic DNA was amplified in a 5-μL reaction containing 1X HotStar Taq PCR buffer (Qiagen), 1.625 mM MgCl₂, 500 μM each of dNTP (deoxyribonucleotide triphosphate), 100 nM each of PCR primer, and 0.5 U of HotStar Taq (Qiagen). The reaction was incubated at 94°C for 15 minutes followed by 45 cycles of 94°C for 20 seconds, 56°C for 30 seconds, 72°C for 1 minute, and 3 minutes at 72°C. Excess dNTPs were then removed from the reaction by incubation with 0.3 U shrimp alkaline phosphatase (USB, Cleveland, Ohio) at 37°C for 40 minutes followed by 5 minutes at 85°C to deactivate the enzyme. Single-primer extension over the SNP was carried out in a final concentration of between 0.625 μM and 1.5 μM for each extension primer (depending on the mass of the probe), iPLEX termination mix (Sequenom, San Diego, California), and 1.35 U iPLEX enzyme (Sequenom) and pooled using a 2-step 200–short cycles program: 94°C for 30 seconds followed by 40 cycles of 94°C for 5 seconds, 5 cycles of 52°C for 5 seconds, 80°C for 5 seconds, and 72°C for 3 minutes. The reaction was then desalted by addition of 6 mg cation exchange resin followed by...
mixing and centrifugation to settle the contents of the tube. The extension product was then spotted onto a 384-well spectro-CHIP before being flown in a matrix-assisted laser desorption/ionization/time-of-flight mass spectrometer. Data were collected during real time, using SpectroTypER Analyzer 3.3.0.15, SpectraAQUIRE 3.3.1.1, and SpectroCALLER 3.3.0.14 (Sequenom). Genotypes for each subject were also checked manually as an additional quality control measure. All laboratory personnel were blinded to case/control status.

STATISTICAL ANALYSIS

We initially examined allele distributions and used χ² tests for Hardy-Weinberg equilibrium. We then compared genotype and allele frequencies between cases and controls using χ² tests, the Armitage trend test to examine evidence for an additive allele effect on AMD susceptibility, and the genotype case-control test to determine both additive and dominance (nonadditive) allelic effects.24

We then used logistic regression under additive, dominant, and recessive genetic models to estimate the incidence rate ratios and 95% confidence intervals (CI) for each genotype, adjusted for other risk factors. We first obtained separate estimates of the incident rate ratio in each cohort and tested for heterogeneity using the Cochran Q test. As there was no evidence of heterogeneity between cohorts (for each SNP, P ≥ 0.3), we present only pooled data from the 2 prospectively ascertained cohorts. Controlling for age and sex, we modeled the allelic effects using a multiplicative (ie, log-additive) coding scheme using a single variable for each SNP, coded 0 for subjects homozygous for the major allele (or, for the candidate SNPs, the allele previously found to be associated with the lowest risk of AMD), 1 for heterozygotes, and 2 for subjects homozygous for the minor allele (or for the candidate SNPs, the allele previously found to be associated with increased risk of AMD).

We next fit unconstrained models (ie, codominant) using separate indicator variables for subjects who were heterozygous and subjects who were homozygous for the risk allele. To arrive at the best-fitting model, we compared these alternative models using the Akaike Information Criteria.²⁵ As a rule of thumb, 2 models are statistically indistinguishable if the Akaike Information Criteria difference is less than ².

For SNPs that showed significant associations in the models controlling for age and sex, we extended the preferred models to control for potential confounding by other risk factors including cigarette smoking (current, yes/no), obesity (body mass index, ≥30 vs <30), regular aspirin use (yes/no), alcohol intake (continuous), consumption of fruits (continuous), and the ratio of ω-6 to ω-3 fatty acids in the diet (continuous). In the next step, we retained any significant risk factors and fit additional models controlling for the CFH Y402H and ARMS2/HTRA1 A69S variants, which were previously shown to be strongly associated with AMD in these cohorts.

For significant RORA SNPs, we additionally examined interactions between RORA and CFH Y402H, ARMS2 A69S, cigarette smoking, and obesity, and fit additional models to simultaneously estimate the stratum-specific incidence rate ratio (CI) for the joint effects of the RORA variant and the other risk factors.

We used a model-based method based on the observed data to calculate the attributable fraction in the population as a measure of the proportion of AMD cases to which each polymorphism contributes.²⁶²⁸

We estimated linkage disequilibrium (LD) (both r² and D’) between each pair of SNPs and constructed haplotype blocks in Haploview (http://www.broadinstitute.org/mpg/haploview) using the method proposed by Gabriel et al.²⁶ We inferred individual haplotypes and tested these for association with AMD in Haploview.

The study population included the 164 cases of incident neovascular AMD matched with 485 controls that were previously studied for association of CFH Y402H and ARMS2/HTRA1 A69S. The mean (SD) age at AMD diagnosis was 68.7 (6) years. Of the 19 RORA SNPs, genotype data were successfully obtained for each SNP in 98% or more of cases and controls, with the exception of rs7177611, which was successfully genotyped in 92% of cases and controls, and rs11071570, for which genotype data were available in 97% of cases and 94% of controls. We found no significant departures from Hardy-Weinberg equilibrium for any of the 18 SNPs in the control group (each P > .05).

The χ² tests for single-SNP analysis showed that 1 SNP, rs12900948, was significantly associated with increased risk of neovascular AMD (for genotype, P = .00018; for allelic trend, P = .00003). Table 1. This SNP was part of a haplotype block shown to be significant in a previous study of 150 EDSPs and in an unrelated case-control cohort from central Greece.²⁶ In addition, these analyses identified that participants with neovascular AMD were more likely to be heterozygous for 2 SNPs, rs730754 (for genotype, P = .038) and rs975501 (for genotype, P = .033).

In age- and sex-adjusted logistic regression models, we found that the G allele of SNP rs12900948 was significantly associated with increased risk of AMD under all 3 genetic models tested: additive (P = 2.2 × 10⁻⁵), dominant (P = .0013), and recessive (P = .0002) (Table 1). Odds ratio estimates from the additive model indicated a 1.76-fold increased risk of neovascular AMD for heterozygotes, and a 3.10-fold increased risk for participants who were homozygous for the G allele (Table 2). None of the other 17 RORA SNPs were significantly associated with risk of neovascular AMD under age- and sex-adjusted additive, dominant, or recessive models (Table 1).

We next fit an unconstrained model for SNP rs12900948 to distinguish between the log-additive model and a codominant model. Comparison of Akaike Information Criteria from these models indicated the log-additive model was the best fitting model for these data (Akaike Information Criteria difference, 2.0). Using log-additive models for the genetic effect, we extended the models for rs12900948 to control for additional risk factors for AMD. After controlling for cigarette smoking and obesity, the 2 strongest lifestyle risk factors for AMD in these cohorts, the odds ratios for rs12900948 were 1.73 (95% CI, 1.23–2.77) for 1 G allele, and 2.99 (95% CI, 1.74–5.14) for the with 2 copies of the G allele. Estimates for rs12900948 remained significantly associated with incidence of neovascular AMD in models that further controlled for other lifestyle risk factors (data not shown) or genetic factors CFH Y402H and ARMS2/HTRA1 A69S (Table 2). After controlling for these additional genetic risk factors, we observed an approximate 30% reduction in the magnitude of the odds ratio estimates for rs12900948, suggesting the possibility of shared biological pathways.

We tested for statistical interaction on the multiplicative scale between the rs12900948 SNP and both CFH
Table 1. χ² Tests and Age- and Sex-Adjusted Logistic Regression Estimates for Association Between 18 SNPs Within Intron 1 of the RORA Gene and Risk of Neovascular AMD in 164 Cases of Neovascular AMD and 485 Controls in the NHS and HPFS Cohorts

<table>
<thead>
<tr>
<th>Locus</th>
<th>No. (%)</th>
<th>Controls</th>
<th>Cases</th>
<th>Genotype, 2 df</th>
<th>Trend (1 df)</th>
<th>Additive Model</th>
<th>Dominant Model</th>
<th>Recessive Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>OR (95% CI)</td>
<td>P Value OR (95% CI)</td>
<td>P Value OR (95% CI)</td>
</tr>
<tr>
<td>rs12916023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.50</td>
<td>.98</td>
<td>1.00 (0.77-1.30)</td>
</tr>
<tr>
<td>rs4583176</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.20</td>
<td>.79</td>
<td>1.04 (0.74-1.40)</td>
</tr>
<tr>
<td>rs730754</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.038</td>
<td>.49</td>
<td>1.10 (0.84-1.43)</td>
</tr>
<tr>
<td>rs8034864</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.75</td>
<td>.69</td>
<td>1.07 (0.78-1.45)</td>
</tr>
<tr>
<td>rs975501</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.033</td>
<td>.52</td>
<td>1.09 (0.84-1.42)</td>
</tr>
<tr>
<td>rs12090948</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.00018</td>
<td>.00003</td>
<td>1.76 (1.36-2.29)</td>
</tr>
<tr>
<td>rs782925</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.66</td>
<td>.52</td>
<td>0.90 (0.65-1.24)</td>
</tr>
<tr>
<td>rs7177611</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.36</td>
<td>.21</td>
<td>1.21 (0.91-1.61)</td>
</tr>
<tr>
<td>rs10403737</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.56</td>
<td>.74</td>
<td>0.95 (0.72-1.26)</td>
</tr>
<tr>
<td>rs12591914</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.26</td>
<td>.11</td>
<td>1.26 (0.94-1.67)</td>
</tr>
<tr>
<td>rs16943429</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.41</td>
<td>.20</td>
<td>1.23 (0.89-1.69)</td>
</tr>
<tr>
<td>rs7495128</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.88</td>
<td>.92</td>
<td>0.99 (0.71-1.37)</td>
</tr>
<tr>
<td>rs4335725</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.98</td>
<td>.92</td>
<td>0.99 (0.71-1.37)</td>
</tr>
<tr>
<td>rs17237514</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.54</td>
<td>.25</td>
<td>1.17 (0.89-1.52)</td>
</tr>
<tr>
<td>rs2414687</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.80</td>
<td>.57</td>
<td>1.07 (0.83-1.39)</td>
</tr>
<tr>
<td>rs17270640</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.80</td>
<td>.57</td>
<td>1.07 (0.83-1.39)</td>
</tr>
<tr>
<td>rs11071570</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.80</td>
<td>.57</td>
<td>1.07 (0.83-1.39)</td>
</tr>
<tr>
<td>rs6494231</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.80</td>
<td>.57</td>
<td>1.07 (0.83-1.39)</td>
</tr>
</tbody>
</table>

Abbreviations: AMD, age-related macular degeneration; CI, confidence intervals; df, degrees of freedom; HPFS, Health Professionals’ Follow-up Study; NHS, Nurses’ Health Study; OR, odds ratio.
risk-associated alleles at rs12900948, there is a 4.6-fold increase in the risk of neovascular AMD compared with participants who never smoked and had no risk-associated alleles. Though statistically consistent with multiplicative effects, these estimates indicate that, combined with age and sex (Table 4), the estimated relative risk of neovascular AMD for participants who were current smokers and carried 2 G alleles was more than 40-fold higher among subjects who were homozygous for the risk-associated variant at both loci compared with subjects with no risk-associated alleles at either locus, although the confidence interval was wide (95% CI, 10.1-164).

Testing of multiplicative interaction terms demonstrated no statistically significant departure from multiplicative effects between rs12900948 and either cigarette smoking or obesity (P < .08; Table 4). We calculated the odds ratios for each genotype risk factor combination to identify whether any subgroups of subjects at particularly high risk of AMD could be identified (Table 4). Though statistically consistent with multiplicative effects, these estimates indicate that, compared with participants who never smoked and had no risk-associated alleles at rs12900948, there is a 4.6-fold increased incidence of neovascular AMD among non-smoking participants with 2 G alleles, whereas the risk is nearly 10-fold higher among participants who were current smokers and carried 2 G alleles.

Calculation of the population-attributable fractions using a model-based method showed an attributable fraction of 45% (95% CI, 32%-59%) for rs12900948 after controlling for CFH Y402H and ARMS2/HTRA1 A69S. The attributable fraction for all 3 SNPs (rs12900948 together with CFH Y402H and ARMS2/HTRA1 A69S) was 84% (95% CI, 80%-88%) for rs12900948.

Finally, we constructed linkage disequilibrium plots of the 18 SNPs using Haploview (Figure). Three haplotype blocks were constructed based on CIs using the method proposed by Gabriel et al. None of the 3 blocks...
contained the significant SNP, rs12900948. Association testing of these 3 haplotype blocks showed that 1 haplotype, haplotype 1 of block 2, was significantly associated with AMD (P = .03; Table 5). In an effort to replicate the haplotypes that were significant in the EDSP cohort, block 1 and block 4, as defined by Gabriel et al
Table 5. Haplotype Analysis of the NHS and HPFS Cohorts

<table>
<thead>
<tr>
<th>Block 2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>rs7495128</th>
<th>rs2414687</th>
<th>Overall Frequency, %&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Frequency Among Chromosomes Cases, No. (%)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Frequency Among Chromosomes Controls, No. (%)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>χ²</th>
<th>P Value&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>h1</td>
<td>G</td>
<td>G</td>
<td>0.17</td>
<td>184 (0.568)</td>
<td>478 (0.499)</td>
<td>4.55</td>
<td>.0329</td>
</tr>
<tr>
<td>h2</td>
<td>G</td>
<td>T</td>
<td>0.259</td>
<td>75 (0.231)</td>
<td>258 (0.269)</td>
<td>1.77</td>
<td>.1833</td>
</tr>
<tr>
<td>h3</td>
<td>A</td>
<td>G</td>
<td>0.224</td>
<td>65 (0.201)</td>
<td>222 (0.232)</td>
<td>1.341</td>
<td>.2469</td>
</tr>
</tbody>
</table>

Abbreviations: h1, h2, and h3; haplotypes 1, 2, and 3; HPFS, Health Professionals’ Follow-up Study; NHS, Nurses’ Health Study.

<sup>a</sup> Blocks were defined by the method outlined by Gabriel et al.<sup>29</sup>

<sup>b</sup> Overall frequency of each haplotype in the study population (percentages may not total exactly 100% owing to rounding).

<sup>c</sup> Frequency of each haplotype and the estimated number of chromosomes containing each haplotype among cases and controls, respectively. Percentages may not total exactly 100% owing to rounding.

<sup>d</sup> P value testing the significance of each haplotype vs all others.

Table 6. Replication of Risk Haplotypes Defined in an Earlier Study of Extremely Discordant Sibling Pairs<sup>5</sup> in the NHS and HPFS Cohorts for Block 1

<table>
<thead>
<tr>
<th>Block 1</th>
<th>rs730754</th>
<th>rs8034864</th>
<th>rs12900948</th>
<th>Overall Frequency&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Frequency Among Chromosomes for Cases, No. (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Frequency Among Chromosomes for Controls, No. (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>χ²</th>
<th>P Value&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>h1</td>
<td>A</td>
<td>C</td>
<td>A</td>
<td>0.42</td>
<td>90 (0.274)</td>
<td>443 (0.467)</td>
<td>37.036</td>
<td>1.16 × 10⁻³</td>
</tr>
<tr>
<td>h2</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>0.19</td>
<td>55 (0.166)</td>
<td>187 (0.197)</td>
<td>1.448</td>
<td>.23</td>
</tr>
<tr>
<td>h3</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>0.18</td>
<td>101 (0.308)</td>
<td>131 (0.138)</td>
<td>47.378</td>
<td>5.85 × 10⁻¹²</td>
</tr>
<tr>
<td>h4</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>0.17</td>
<td>54 (0.165)</td>
<td>159 (0.167)</td>
<td>0.013</td>
<td>.9092</td>
</tr>
<tr>
<td>h5</td>
<td>G</td>
<td>A</td>
<td>A</td>
<td>0.03</td>
<td>18 (0.056)</td>
<td>15 (0.016)</td>
<td>15.077</td>
<td>1.00 × 10⁻⁵</td>
</tr>
<tr>
<td>h6</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td>0.02</td>
<td>10 (0.030)</td>
<td>15 (0.015)</td>
<td>3.009</td>
<td>.0828</td>
</tr>
</tbody>
</table>

Abbreviations: HPFS, Health Professionals’ Follow-up Study; NHS, Nurses’ Health Study.

<sup>a</sup> Overall frequency of each haplotype in the study population (percentages may not total exactly 100% owing to rounding).

<sup>b</sup> Frequency of each haplotype and the estimated number of chromosomes containing each haplotype among cases and controls, respectively. Percentages may not total exactly 100% owing to rounding.

<sup>c</sup> P value testing the significance of each haplotype vs all others.

in the EDSP cohort, were constructed on the NHS/HPFS cohort using Haploview. In this analysis, block 1, which contained the SNPs rs730754, rs8034864, and rs12900948, was shown to be significantly associated with AMD in the NHS/HPFS cohort. Specifically, haplotypes 1 (P = 1.16 × 10⁻⁹), h3 (P = 5.85 × 10⁻¹²), and 5 (P = .0001) were all significantly associated with AMD. Block 4, which contained the SNPs rs17237514 and rs4335725, was not significantly associated with AMD in the NHS/HPFS cohorts (P > .8 for all; Table 6 and Table 7).

This prospective study of incident cases of neovascular AMD confirms and extends our recent findings of a significant association between the RORA gene and this leading cause of blindness in US adults. We identified a single SNP, rs12900948, that was associated with 3-fold increased incidence of neovascular AMD among carriers of 2 G alleles at this locus. This SNP was part of a haplotype (GCG) in a haplotype block that comprised SNPs rs730754, rs8034864, and rs12900948, which we previously found to be significantly associated with neovascular AMD in a cohort of EDSP as well as in 2 haplotypes (GAG and GCG) significantly associated with neovascular AMD in a separate, unrelated group of 139 prevalent cases of neovascular AMD and 121 controls from central Greece.<sup>3</sup> In the present study, haplotypes ACA, ACG, and GAA were significantly associated with neovascular AMD. We further observed a significant interaction between SNP rs12900948 and the ARMS2/HTRA1 A69S SNP. Based on the present study, individuals with 2 G alleles at rs12900948 as well as 2 copies of the ARMS2/HTRA1 A69S risk allele have an estimated 40-fold increase in incidence of neovascular AMD.

In exploring whether the effect of the rs12900948 variant is influenced by cigarette smoking or obesity, we found no statistical evidence for departures from multiplicative interaction between this SNP and these modifiable risk factors but statistical power was low and the P value for interaction with cigarette smoking was borderline at .08. Estimates of the joint effects of SNP rs12900948 and cigarette smoking showed that individuals with 2 G alleles have a nearly 10-fold increased incidence of neovascular AMD if they also smoke cigarettes compared with a 4.6-fold increased risk among those who never smoked.

Data from our controls indicates that this allele is common in the US population, with 73% of our US-based controls having at least 1 copy of the AMD-associated G allele. The high prevalence of this allele contributes to a strong attributable risk estimate of 45%. The rs12900948 variant and haplotypes associated with neovascular AMD in this and 2 prior cross-sectional study populations lie within a well-conserved region of the first intron of the RORA transcript (ENST00000333670). Based on the avail-
able evidence, we think it is most likely that the causal variation in this region (SNPs or insertion or deletion of copy number) has yet to be identified. In this regard, the SNP rs12900948 was not individually associated with neovascular AMD in the initial discovery population of EDSP, though it was significant as part of a haplotype. Further sequencing of the exons and the acceptor/donor splice sites adjacent to this region could help resolve the issue. Although there is no apparent functional change, it is possible that the rs12900948 or other undiscovered variants within intron 1 of the RORA gene could cause sequence changes, insertions, and/or deletions within modifying elements such as silencers and enhancers, or influence the splicing of the transcript, which could contribute to a functional effect.30-33

In the eye, RORA is involved in regulating the development of photoreceptors through the coordinated expression of several cone genes,34 and it is expressed in the ganglion cell layer and inner nuclear layer of the adult retina.35,36 More generally, RORA participates in several biological pathways including oxidative stress, inflammation, lipid metabolism, and angiogenesis, which have been implicated in the development of neovascular AMD.10-17

A nuclear receptor, RORA has been shown to regulate production of cytokines including interleukin-6, interleukin-8, and tumor necrosis factor α as well as the cellular adhesion molecules vascular cell adhesion molecule and intracellular adhesion molecule.37 It is also involved in regulation and homeostasis of lipoproteins such as high-density lipoprotein, serum amyloid A, and apolipoprotein A138 and is thought to be a key modulator of fat accumulation.16 In this context, it is interesting to hypothesize based on the present findings that RORA influences the development of neovascular AMD through the genes it regulates, or indirectly through the genes that regulate RORA.

As a complex disease, it is thought that the risk of AMD is altered through a combination of environmental effects plus effects of variants in several genes that lead to alterations in their interactions with each other as well as to alterations in their interactions with other genes and/or proteins.39 Because the genes involved in AMD have these pleiotropic effects, however, variants in the genes could also alter the clinical course or survival of individuals who carry certain alleles. Particularly, when the alleles are common, such effects could introduce selection bias when prevalent rather than incident cases are studied.40 The present study, using a validated prospective, nested, case-control methodology, therefore adds strength to our initial findings in 2 clinic-derived populations of prevalent AMD cases.

Although we cannot perform standardized clinical assessments of retinal status among participants of these large and geographically dispersed cohorts, we have demonstrated that our case ascertainment method has high specificity,22,41 which ensures minimal bias in a prospective study.16 Furthermore, the consistency of our previous findings linking CFH Y402H and ARMS2/HTRA1 A69S with AMD, as well as our prior work on modifiable risk factors for AMD,21,22,41,42 provide further reassurance of the validity of the present findings. The select nature of these cohorts of health professionals may limit generalizability but significant results have already been demonstrated in 2 other study populations including a group of cases and controls from central Greece.3 Further study of the possibility that variants in RORA may influence the development of earlier stages of AMD is also deserved, and these studies are currently under way in our prospective cohorts.

Whereas we observed a statistically significant interaction between rs12900948 and ARMS2/HTRA1 A69S, sparseness of data contributed to imprecision in stratum-specific effect estimates. This was a greater issue in terms of our ability to detect an interaction with cigarette smoking, which is less common in our cohorts of health professionals than in some other studies.43,44 Cigarette smoking and obesity are known to promote inflammatory activity,45 and previous studies have suggested that, in such situations, in which underlying levels of inflammatory activity are likely to be elevated, the effect of carrying AMD-associated risk alleles is magnified.2 Although not statistically significant, stratum-specific estimates showed an elevated incidence of neovascular AMD among homozygous carriers of the G allele that were current vs never-smokers. This coincides with evidence showing, for example, that homozygous staggerer mice that display decreased and dysfunctional RORA expression are more susceptible to (at least certain types of) inflammation.46

Regarding obesity, we hypothesized that obese individuals who carried the G allele might be more likely to develop AMD. In addition to possible shared pathways involving inflammation, our hypothesis was also based

Table 7. Replication of Risk Haplotype Defined in an Earlier Study of Extremely Discordant Sibling Pairs in the NHS and HPFS Cohorts for Block 4

<table>
<thead>
<tr>
<th>Block 4</th>
<th>rs17237514</th>
<th>rs4335725</th>
<th>Overall Frequencya</th>
<th>Frequency Among Chromosomes for Cases, No. (%)b</th>
<th>Frequency Among Chromosomes for Controls, No. (%)b</th>
<th>χ²</th>
<th>P Valuec</th>
</tr>
</thead>
<tbody>
<tr>
<td>h1</td>
<td>A</td>
<td>G</td>
<td>0.52</td>
<td>172 (0.523)</td>
<td>490 (0.516)</td>
<td>0.045</td>
<td>.8314</td>
</tr>
<tr>
<td>h2</td>
<td>A</td>
<td>A</td>
<td>0.31</td>
<td>102 (0.309)</td>
<td>300 (0.315)</td>
<td>0.038</td>
<td>.8452</td>
</tr>
<tr>
<td>h3</td>
<td>G</td>
<td>G</td>
<td>0.16</td>
<td>52 (0.159)</td>
<td>156 (0.164)</td>
<td>0.057</td>
<td>.8112</td>
</tr>
</tbody>
</table>

Abbreviations: h1, h2, and h3, haplotypes 1, 2, and 3; HPFS, Health Professionals’ Follow-up Study; NHS, Nurses’ Health Study.

a Overall frequency of each haplotype in the study population (percentages may not total exactly 100% owing to rounding).

b Frequency of each haplotype and the estimated number of chromosomes containing each haplotype among cases and controls, respectively. Percentages may not total exactly 100% owing to rounding.

c P value testing the significance of each haplotype vs all others.
on evidence showing specific interactions of obesity and RORA mediated through alterations in lipoprotein pathways. For example, homozygous staggerer mice with decreased and dysfunctional RORA expression are resistant to diet-induced obesity. Moreover, RORA appears to participate in regulating plasma cholesterol levels and positively regulates apolipoprotein A-I and apolipoprotein C-III gene expression, whereas its activity is also regulated by cholesterol. A balanced translocation in RORA has also been associated with severe obesity in humans. In spite of this evidence for biological interplay between RORA and obesity, however, estimates for association with AMD were similar for homozygous carriers of the G allele whether the individuals were obese or not. Such observations provide support for the argument that it is important to separate the concept of joint biological effects from the issue of statistical testing of interaction terms. Further study of interactions in prospective studies with larger sample sizes, as well as other types of studies to identify joint biological effects, will be necessary to address these complicated issues.

The identification of a number of prevalent AMD-associated polymorphisms has raised the question of the utility of population-based or targeted genetic testing. However, as others have pointed out, predictive models for AMD based on currently known risk factors are still inaccurate. This is an expected problem for a complex disease such as AMD in which multiple common genetic and nongenetic factors influence risk. Improvement of predictive models may be accomplished through enhanced measurement of exposures as well as identification of additional genetic and nongenetic risk factors. The utility of this approach will, of course, ultimately depend on the development of effective strategies for preservation of vision among individuals identified as having high risk of AMD.

In summary, common variants and haplotypes within the RORA gene appear to increase incidence of neovascular AMD. There is significant evidence of a multiplicative interaction between the RORA SNP rs12900948 with the ARMS2/HTRA1 A69S polymorphism in AMD. Cigarette smoking may also confer excess risk among individuals who carry 2 copies of the G allele at rs12900948, though further study of larger groups is needed to refine this estimate. These data identify another major gene associated with risk of neovascular AMD and add further evidence of the complex interplay among genetic and modifiable risk factors for AMD. Such information could lead to enhanced accuracy of risk prediction for neovascular AMD.

Submitted for Publication: December 7, 2009; final revision received March 10, 2010; accepted March 14, 2010.

Author Affiliations: Division of Preventive Medicine, Department of Medicine, Brigham and Women's Hospital (Drs Schaumberg and Chasan-Taber) and Channing Laboratory, Department of Medicine, Brigham and Women's Hospital (Drs Guo, Hankinson, and Hunter); the Ocular Molecular Genetics Institute and the Department of Ophthalmology, Massachusetts Eye and Ear Infirmary (Drs Schaumberg, Morrison, Adams, DeAngelis), Harvard Medical School, Boston, Massachusetts; and the Program in Molecular and Genetic Epidemiology (Drs Hunter and Departments of Epidemiology (Drs Schaumberg, Hankinson, Hunter) and Nutrition (Drs Hunter), Harvard School of Public Health, Boston, Massachusetts.

Correspondence: Debra A. Schaumberg, ScD, OD, MPH, Division of Preventive Medicine, 900 Commonwealth Ave E, Third Floor, Boston, MA 02215 (dschaumberg@rics .bwh.harvard.edu).

Financial Disclosure: None reported.

Funding/Support: This work was supported by National Institutes of Health grants EY017362, EY013834, EY009611, EY014458, CA87969, CA49449, and HL35464, and the Lincy Fund.

REFERENCES


