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The *HtrA1* promotor polymorphism, smoking, and age-related macular degeneration in multiple case-control samples

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Abstract

Purpose—To assess the association and combined effect on the risk of age-related macular degeneration (AMD) by the *HtrA1* and *complement factor H (CFH)* polymorphisms, smoking and serum cholesterol.

Design—Clinic-based and population-based case-control.

Participants—Eight hundred and five AMD cases and 921 controls from The Eye Clinic of National Eye Institute, Age-Related Eye Diseases Study (AREDS), Blue Mountain Eye Study Cohort, and Minnesota Lions Eye Bank.

Methods—DNA Samples were genotyped for polymorphisms of rs11200638 in *HtrA1* promotor and rs380390 in *CFH*. HtrA1 protein in ocular tissue was measured. Interactions of the *HtrA1* risk allele with the *CFH* risk variant, smoking status and cholesterol were assessed.

Main Outcome Measures—AMD was evaluated by retinal specialists and AMD subtypes (geographic atrophy and neovascularization) were determined.

Results—Strong associations of the *HtrA1* risk allele (A) with AMD were present in all sample sets. A similar magnitude of association was observed for central geographic atrophy and neovascular AMD. The combination of the *HtrA1* and *CFH* risk alleles increased AMD susceptibility, as did the combination of the *HtrA1* risk allele with smoking. No combined effect of *HtrA1* risk allele and cholesterol level was found. Enhanced expression of HtrA1 protein was detected in retina with AMD.

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Précis:
The genetic variant of *HtrA1* confers increased risk for developing age-related macular degeneration (AMD). AMD risk increases further when the risk alleles from *HtrA1* are combined with either *CFH* risk alleles or history of smoking.

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Conclusions—Findings from multiple samples support an AMD genetic variant harbored within *HtrA1*. The risk of advanced AMD increased when the presence of risk alleles from *HtrA1* was combined with either *CFH* risk alleles or history of smoking.

Age-related macular degeneration (AMD) is the leading cause of blindness in older Western populations.[1] Both environmental and genetic factors contribute to AMD susceptibility.[2-5] Cigarette smoking is an established factor for AMD, while the association of advanced AMD with cardiovascular disease has been less consistent.[6] Recent studies have identified strong genetic associations with advanced AMD,[2] and associations of AMD with certain single nucleotide polymorphisms (SNPs) have been documented. [5,7-22]

Chromosome 10q26 is a locus implicated in several AMD linkage studies.[23-27] These studies found significant association signals for the pleckstrin homology domain–containing, family A, member 1 (*PLEKHA1*) and *LOC387715* (*ARMS2*) within 10q26. [28-30] Recently, another gene in 10q26 has been reportedly associated with AMD.[31,32] This new candidate is the *HtrA1* (high temperature requirement factor A-1) gene. The *HtrA1* gene is associated with the expression of a protein that can act as a serine protease and is involved in protein quality control and cell fate.[33] The identified polymorphism (SNP), rs11200638, located in the promoter region of *HtrA1*, is believed to be a responsible genetic variant for AMD risk.[32,34] In another study, the *HtrA1* SNP was particularly associated with neovascular AMD.[31] Individuals with the *HtrA1* risk allele showed an increased expression of HtrA1 protein in the retinal pigment epithelium (RPE) and in drusen of eyes with AMD.[32] Recently a retrospective matched-pair case-control study confirmed that alleles in *HtrA1* gene alter the risk of neovascular AMD and that the association is independent of *complement factor H* (*CFH)* polymorphism and smoking history.[35] However, the causal effect of SNPs in 10q26 region is currently disputed.[36]

In this study, we aimed to refine the association between the *HtrA1* SNP and AMD in four different AMD sample sets from Caucasian populations: two clinic-based case-control studies from the U.S. National Eye Institute (NEI)[7,14] and Age-related Eye Diseases Study (AREDS),[37] one case-control sample nested in a population-based study of the Australian Blue Mountains Eye Study (BMES),[38] and autopsied ocular tissue samples from Minnesota Lions Eye Bank (MLEB).[39] We also aimed to assess the magnitude of association, the attributable risk for bearing disease susceptibility alleles at the *HtrA1* promotor, and possible combined effects of the *HtrA1* SNP with the risk alleles for *CFH* SNP, and other non-genetic AMD risk factors, including cigarette smoking and serum cholesterol level.

Materials and Methods

Patient Population

Each participant provided written informed consent according to protocols approved by the Institutional Review/Ethics Boards of NEI Institutional Review Board, AREDS clinical site, University of Minnesota, or University of Sydney, respectively. This research followed the tenets of the Helsinki Declaration.

The clinic-based NEI case-control study included diagnosed advanced AMD cases and controls from the greater Washington, D.C. area, who had been evaluated by AREDS ophthalmologists at the NEI. Venous whole blood (10 mL) was collected from NEI study subjects. Genomic DNA was extracted and isolated using a QIAamp DNA Blood Maxi kit (Qiagen, Valencia, CA).[7,14] In addition, DNA samples from the AREDS Genetic Repository were obtained and included as a second clinic-based sample in the study.[40] NEI and AREDS patients and controls were self-identified as Caucasians of non-Hispanic descent and were 55 years or older. AMD status was assessed according to AREDS study guidelines after retinal photographic grading. Patients with advanced AMD had either geographic atrophy at least 175 microns in diameter

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involving the macular center and/or macular choroidal neovascularization (CNV) defined as nondrusenoid retinal pigment epithelial (RPE) detachment, serous or hemorrhagic retinal detachment, subretinal or sub-RPE hemorrhage, or subretinal fibrosis with drusen in at least one eye or evidence of history of photocoagulation for CNV.[41] The normal controls were clinically and by fundus photography evaluated and found to have either no drusen or less than 5 small drusen (<63μm), no evidence of significant extra-macular drusen, and an absence of all other retinal diseases affecting the photoreceptors and/or outer retinal layers. Subjects with high myopia, retinal dystrophies, central serous retinopathy, retinal vein occlusion, diabetic retinopathy, uveitis, and other retinal diseases, were excluded. Retinal photographs were taken of all patients and the AREDS controls. Smoking history was obtained using interviewer-administered questionnaires.[42]

The collection and clinical evaluation of BMES subjects has previously been described.[43,44] Briefly, the BMES is a population-based cohort study of common eye diseases and health-related parameters among suburban residents aged 49 years or older in the Blue Mountains region of Australia, near Sydney. The area has a stable and ethnically homogenous population. In this study, retinal photographs of study participants were taken of one (99%), or both eyes (98%) during 1992−1994. During the second BMES survey (1997−2000), 2,334 of 3,654 baseline participants (75% of survivors), as well as an additional 1,174 participants (85% of eligible) who had newly moved to the study area or were then in the eligible age group, were examined and photographed at that time (n=3508). AMD was evaluated from fundus photographs while graders were masked to participant identity. All advanced AMD cases were adjudicated and confirmed by a retinal specialist (PM). Early AMD was defined in either eye by: (1) presence of large (125 μm or larger diameter), soft indistinct or reticular drusen within the macular area or (2) presence of both large, soft distinct drusen within the macula and retinal pigmentary abnormalities in the absence of late AMD. This closely resembled the definition of early AMD used in the Beaver Dam, Wisconsin population study.[40] Advanced, or late-stage, AMD was defined as the presence of neovascular or atrophic AMD.[44,45] Smoking history was obtained using an interviewer-administered questionnaire.[46] DNA samples of 852 subjects consisting of 284 cases (54 advanced and 230 early AMD) and 568 age-, sex- and smoking status-matched controls were included in this study.

Donor eyes obtained from the MLEB were maintained at 4° C in a moist chamber until dissection photography. All tissue was acquired with consent of the donor or family members for use in medical research.[7,14] Sclera and some surrounding muscle were snap frozen and used as the source for DNA extraction. DNA from ocular tissue was extracted after proteinase K digestion. Criteria established by the Minnesota Grading System (MGS) correspond to definitions used in the AREDS classification system with high-resolution, stereoscopic macular images to determine AMD stages.[37,39]

SNP typing

SNP typing of *HtrA1* promotor (G/A), rs11200638, was performed using the PCR- Restriction Fragment Length Polymorphism (RFLP) method.[31] The amplified DNA fragment (685 bp) containing the polymorphic site was flanked by the primers of 5'-ATG CCA CCC ACA ACA ACT TT-3' and 5'-CGC GTC CTT CAA ACT AAT GG-3', respectively. The PCR mixture included 1XJumpStart ReadyMix REDTaq (Sigma), 20 ng DNA, and 70 pmole of primer. The program was run for 2 min at 94°C, followed by 39 cycles of 30 seconds' denaturation at 94° C, 40 seconds' annealing at 52°C and 55 seconds' extension at 72°C. RFLP analysis was conducted by incubating 15 μL of PCR product with 0.5 μL of *Eag1* restriction enzyme which cuts the G allele at position 140bp. Fragments were separated on 15% TBE polyacrylamide gels and visualized after ethidium bromide staining. Among the 1,726 samples, 1,702 were successfully genotyped. The call rate was 98.6%. The typing of *CFH* intron (rs# 380390) typing

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was performed by Taqman SNP Genotyping Assay (Assay ID# C___2530286_20, Applied Biosystems, Foster City, CA).

Serum cholesterol assessment

Fasting serum cholesterol was evaluated in the NEI study, AREDS and BMES using the normal range defined as 100 to 200 mg/dL.[6,47]

Immunohistochemistry

The avidin-biotin-complex immunoperoxidase technique was utilized on the unstained, de-paraffinized slides of AMD and normal human eyes in which macular sections were available as described previously.[12] The primary antibody was rabbit anti-human HtrA1 polyclonal antibody (R&D System, Minneapolis, MN) or control rabbit IgG. The secondary antibody was biotin-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA). The substrate was avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA), and the chromogen was diaminobenzidine and nickel sulfate. Positive reaction resulted in the production of a blue-blackish color.

Statistical analysis

Logistic regression was performed using SAS (Release 9.1; SAS Institute, Cary, NC) to compare genotype and allele frequencies in cases and controls, as well as to estimate odds ratios (OR), adjusted for age and sex. Smoking history was coded into two groups, ever and never smoked. Interactions were tested using product terms in logistic regression models. Attributable risk (AR), as a percentage, and 95% confidence intervals (CI), were calculated.[48]

Results

Demographic information from the four study samples is summarized in Table 1.

The distribution of the *HtrA1* promotor SNP in the control groups showed no significant deviation from Hardy-Weinberg equilibrium ($p > 0.05$). Strong associations, after adjusting for age and sex, between the A allele of the SNP and AMD were present in all sample sets with the exception of MLEB, due to its smaller sample size and lower proportion of subjects diagnosed with advanced AMD (Table 2). An allele dosage-dependent effect was also evident in the three subject sample sets. The likelihood of having AMD was greater for individuals with two risk alleles than for those with one risk allele (Table 2). In both the NEI and the AREDS samples, homozygozity in alleles A was associated with a two- to three-fold higher OR for AMD than heterozygosity. Moreover, the OR magnitude correlated with the proportion of advanced AMD cases in each sample set. ORs of 2.12 and 2.19 were found for the risk allele in the NEI and AREDS samples, where advanced cases accounted for 75% and 90% of the total cases, respectively. However, an OR of 1.52 was found for the risk allele in the BMES sample, where advanced AMD accounted for only 19% of all cases. A low OR of 1.02 was calculated in the MLEB samples, which most likely resulted from the small sample size and lack of advanced AMD cases.

Analysis of allele frequency quantitatively showed a dosage-dependent association of the *HtrA1* risk allele with disease severity. The risk allele A was associated with 2.85 higher odds of advanced AMD while the risk for early AMD was 1.57-fold higher (Table 3) ($p=0.0001$). Even though the ORs of atrophic and neovascular AMD were 2.17 and 2.97 respectively in the magnitude of the *HtrA1* association, the difference did not reach the level of significance (Table 3)($p>0.05$).

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The *CFH* intron G/C SNP (rs380390) and *CFHY402H* are in complete linkage disequilibrium, and both SNPs have been shown to be significantly associated with AMD.[14,49] Logistic analysis of the combined contribution of the *HtrA1* and the *CFH* intron G/C SNP unveiled a strong synergic effect between the A allele of the *HtrA1* SNP and the C allele of the *CFH* SNP for AMD susceptibility (Table 4). Carrying 3 out of these 4 *HrtA1* and *CFH* risk alleles conferred a 2.5-fold higher OR for AMD than carrying two risk alleles (OR 5.36 with respect to 1.22 and 1.74 combined, p=0.0001; OR 5.07 with respect to 2.03 and 1.74 combined, p=0.0008). No participant carried all 4 *HtrA1* and *CFH* risk alleles in the 4 sample sets.

We found an interaction between smoking and the *HtrA1* SNP by analyzing all NEI, AREDS and BMES cases for which a smoking history was available (Table 5). In each genotype category, ever smokers had a higher OR for AMD than never smokers ($p=8.73\times10^{-4}$ in heterozygotes and $p=1.17\times10^{-3}$ in homozygotes). Individual heterozygotes for the risk allele who also had ever smoked had an OR for AMD of 3.54, slightly higher than the product of smoking OR (1.70) and G/A genotype (OR 1.80) alone. However, subjects who had ever smoked and were also homozygous for the risk allele had an AMD OR of 17.71, substantially higher than the product of smoking alone OR (1.70) and A/A genotype alone (OR=3.54). In contrast, subjects homozygous for the risk allele had similar OR for AMD risk at different serum cholesterol levels (Table 5). The impacts of *HtrA1* promotor A allele, *CFH* intron SNP C allele and smoking in this study on the attributable risk of AMD were 22.3% (95% CI: 18.9%, 25.5%), 31.5% (95% CI: 27.2%, 35.6%) and 18.2% (95% CI: 12.6%, 23.4%), respectively.

Immunoreactivity against HtrA1 was weakly detected in the retinal vascular endothelia, internal limiting membrane and RPE of age-matched eyes with normal retina. However, strong immunoreactivity against HtrA1 was observed in the macula of the AMD eyes, with either neovascular or geographic atrophic lesions (Figure 1). Intense staining highlighted choroidal neovascular structure as well as the abnormal RPE and some drusen. No visible changes in intensity, number, or staining pattern were found in the peripheral retina in AMD eyes compared with normal eyes. A trend for higher HtrA1 expression was noted in the eyes with *HtrA1* variant genotypes as reported in our recent study of 57 AMD cases and 16 age-matched controls with normal retinas.[50]

Discussion

These findings confirm the contribution of the *HtrA1* promotor SNP to AMD prevalence in Caucasian populations and support the previous Utah Caucasian report.[32,51] The current study included multiple Caucasian AMD samples from distinct geographic areas, including two well-known AMD studies, the AREDS and BMES. Moreover, we demonstrated the existence of a risk allele dosage effect on AMD risk, and showed that the magnitude of association with the *HtrA1* SNP was similar for the two advanced AMD subtypes. Multivariate modeling found interaction between smoking and the *HtrA1* hazard allele(s), especially those homozygous for the risk allele. A recent study from Hong Kong has also found *HtrA1*SNP-smoking additive effect. [34] Attributable risk based on our estimates, suggest that AMD cases could be attributed more to the proportion of *HtrA1* and *CFH* variants, than to cigarette smoking. Our results also indicated a lower significance in the p value of the *HtrA1* SNP than the *LOC387715* SNP from the same cohorts as we reported before.[29] However, we cannot conclude the causal contribution of those two SNPs by simply comparing the p values.[29,36] Although it is possible that only one locus in 10q26 is truly attributed to AMD,[36] and the locus is composed of a linkage disequilibrium block that spans both the *HtrA1* gene as well as the *LOC387715* (ARMS2), our data demonstrated enhanced HtrA1 protein expression in AMD lesions. In contrast, no ARMS2 protein has been found in human eyes with or without AMD. Furthermore, multiple loci, rather than a single locus in a particular chromosome can contribute to either an increased or decreased risk of AMD. For example, the *CFH* gene resides within the region of

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the complement activation gene cluster, which also includes 5 '*CFH*-related' genes. Deletion of *CFHR1* and *CFHR3* is associated with a lower risk of AMD.[52,53]

CFHY402H, another strong AMD-susceptibility genetic variant, has been confirmed in multiple Caucasian AMD samples, but not in most Japanese or Chinese samples.[54-57] Unlike *CFHY402H*, the *HtrA1* promoter SNP appears to confer a significant AMD association in all of these three ethnic groups.[31,58,59] This suggests that HtrA1 might represent an older ancestral genetic variant, and may thus be distributed more globally.

Most AMD genetic association studies have been conducted as clinic-based case-control studies. This design has inherent limitations. Frequency of the genetic variant distribution in the population may not be estimated precisely, and thus reported attributable risk may be either over- or under-estimated. Information and selection bias may lead to a deviated estimation of the effect of a risk factor. Our samples included a nested case-control from a population-based study, the BMES,[60,61] which has the advantage of minimizing sample selection bias. However, due to the relatively small number of advanced AMD cases in this sample of generally healthy, older persons, the BMES OR can be probably be interpreted as representing early or intermediate-stage AMD, as the majority of BMES AMD cases were at this AMD stage. On the other hand, similar findings from multiple sample sources in this study support a strong association between *HtrA1* variant and AMD.

The *HtrA1* promotor SNP coincides with previous fine-mapping findings in a region strongly linked to AMD, 10q26.[25] Both *in vitro* and *in vivo* data indicate that this promoter SNP upregulates *HtrA1* mRNA and protein expression.[31] *HtrA1* SNP is highly associated with classic chroidal neovascularization AMD in a French study.[62] Therefore, our finding of higher expression of HtrA1 protein in both neovascular and atrophic AMD lesions is expected. It remains unclear as how increased HtrA1expression would contribute to AMD risk, or how the *HtrA1* risk allele enhances its protein expression, leading to an increased risk of AMD. HtrA1 is a member of the heat shock serine protease group of proteins which control many physiological and pathological processes, such as vascular permeability and extracellular matrix remodeling.[63] HtrA1 is also highly expressed in normal vascular endothelia but has low expression rates in neuronal tissue.[64] Active HtrA1 induces cell death in a serine protease-dependent manner. This proapoptotic property could explain the enhanced expression of HtrA1 in AMD lesions. The over-expression of HtrA1 could indicate active neovascular AMD or large drusen deposits and RPE abnormalities in atrophic AMD.[65] Further studies should aim to identify conditions that activate HtrA1 in ocular tissue, either at the genetic or epigenetic level, and investigate the effect of its activation on RPE, photoreceptor, choroidal neovascular components, Bruch's membrane and drusen in animal models of AMD.

Cholesterol data were available from NEI, BMES, and part of AREDS sample sets. The relationship between the *HtrA1* SNP and serum cholesterol level is not clear. In all subgroups in terms of the *HtrA1* SNP genotype, ORs for AMD seemed slightly lower with higher serum cholesterol levels than with normal levels, though not significantly.

In summary, our findings from multiple independent samples of AMD cases in the U.S. and Australia confirmed that the *HtrA1* promotor SNP is a global genetic marker for AMD in Caucasian populations. This *HtrA1* SNP has a combined effect with a *CFH* intron rs380390 SNP that is in strong linkage disequilibrium with the *CFHY402H* SNP. The *HtrA1* SNP contributed indiscriminately to the two principal disease subtypes. A combined effect of the SNP and smoking on AMD was detected. In addition, AMD eyes with the *HtrA1* promotor SNP showed higher HtrA1 expression in macular lesions.

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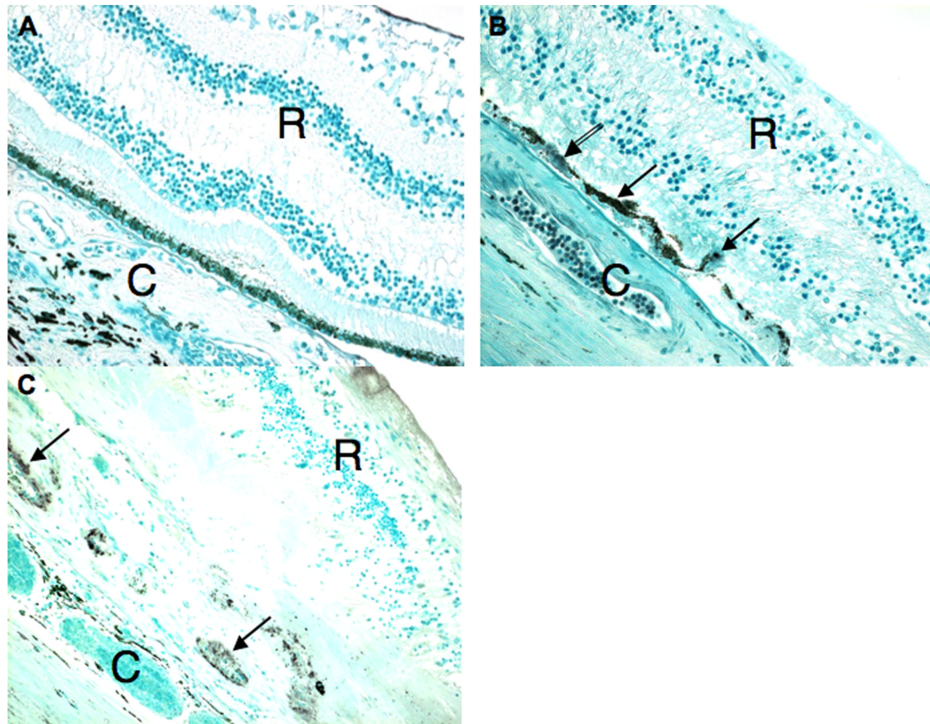

Figure 1.

HtrA1 protein expression (immunohistochemistry). (A) Lack of HtrA1 expression in the normal macula of a human eye; (B) Increased HtrA1 expression (black color) is noted in drusen (open arrow) and abnormal retinal pigment epithelium cells (arrows) of an eye with atrophic age-related macular degeneration; (C) choroidal neovascular vessel structure (arrows) of an eye with neovascular age-related macular degeneration. (R, retina; C, choroid; avidin-biotin complex immunoperoxidase)

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Table 1

Demographic Characteristics of Study Participants

Group	N	Age (mean±SD)	Female No. (%)	Ever Smokers No. (%)	Advanced AMD No. (%)
NEI Control	138	66 ± 11	79 (57)	66 (48)	0
NEI AMD	145	79 ± 7	76 (52)	85 (59)	109 (75)
AREDS Control	193	77 ± 4	105 (54)	94 (49)	0
AREDS AMD	330	79 ± 5	195 (59)	190 (58)	297 (90)
BMES Control	568	77 ± 8	344 (61)	54 (10)	0
BMES AMD	284	77 ± 8	172 (61)	27 (10)	54 (19)
MLEB Control	22	64 ±12	12 (55)	13 (59)	0
MLEB AMD	46	75 ± 14	27 (57)	19 (41)	12 (26)
Total Control	921	74 ± 9	540 (58)	227 (25)	0
Total AMD	805	78 ± 8	468 (58)	321 (40)*	472 (59)

AMD; age-related macular degeneration; SD: standard deviation; NEI: National Eye Institute sample set; AREDS: Age-related Eye Diseases Study sample set; BMES: Blue Maintain Eye Study sample set; MLEB: Minnesota Lions Eye Bank sample set

* Odds Ratio=2.11 (1.71, 2.61), $p=4.14\times10^{-12}$ in comparison with total control.

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Table 2

Odds Ratios (OR) and 95% Confidence Intervals (95% CI) of Age-related macular degeneration Cases Versus Unrelated Controls at the *HtrA1* Promotor SNP in Multiple Sample Sets

Sample set	SNP	Control	Case	Unadjusted		Age & Sex Adjusted	
				p	OR (95% CI)	p	OR (95% CI)
NEI	GG	74 (56.1%)	49 (34.5%)		1		1
	GA	51 (38.6%)	60 (42.3%)	0.03	1.78 (1.06, 2.99)	0.158571	1.56 (0.84, 2.88)
	AA	7 (5.3%)	33 (23.2%)	1.6×10^{-5}	7.12 (2.92, 17.37)	0.000876	6.09 (2.10, 17.64)
	GA+AA	58 (43.9%)	93 (65.5%)	3.8×10^{-4}	2.42 (1.49, 3.94)	0.016321	2.06 (1.14, 3.72)
	G	199 (75.4%)	158 (55.6%)		1		1
	A	65 (24.6%)	126 (44.4%)	2.0×10^{-6}	2.44 (1.70, 3.52)	0.000790	2.12 (1.37, 3.29)
AREDS	GG	106 (55.5%)	103 (31.2%)		1		1
	GA	73 (38.2%)	164 (49.7%)	2.2×10^{-5}	2.31 (1.57, 3.40)	0.000095	2.21 (1.48, 3.28)
	AA	12 (6.3%)	63 (19.1%)	1.0×10^{-6}	5.40 (2.75, 10.60)	0.000002	5.24 (2.63, 10.44)
	GA+AA	85 (44.5%)	227 (68.8%)	7.5×10^{-8}	2.75 (1.90, 3.97)	0.000001	2.58 (1.77, 3.77)
	G	285 (74.6%)	370 (56.1%)		1		1
	A	97 (25.4%)	290 (43.9%)	3.6×10^{-9}	2.30 (1.75, 3.04)	0.000000	2.19 (1.65, 2.91)
BMES	GG	349 (62.9%)	135 (49.6%)		1		1
	GA	186 (33.5%)	122 (44.9%)	6.3×10^{-4}	1.70 (1.25, 2.30)	0.000536	1.71 (1.26, 2.32)
	AA	20 (3.6%)	15 (5.5%)	0.06	1.94 (0.97, 3.90)	0.075062	1.89 (0.94, 3.80)
	GA+AA	206 (37.1%)	137 (50.4%)	3.0×10^{-4}	1.72 (1.28, 2.31)	0.000250	1.73 (1.29, 2.33)
	G	884 (79.6%)	392 (72.1%)		1		1
	A	226 (20.4%)	152 (27.9%)	5.9×10^{-4}	1.52 (1.20, 1.92)	0.000532	1.52 (1.20, 1.93)
MLEB	GG	15 (68.1%)	29 (63.0%)		1		1
	GA	6 (27.3%)	15 (32.6%)	0.66	1.29 (0.42, 4.02)	0.679225	1.30 (0.37, 4.60)
	AA	1 (4.6%)	2 (4.4%)	0.98	1.03 (0.09, 12.35)	0.636386	0.53 (0.04, 7.57)
	GA+AA	7 (31.8%)	17 (37.0%)	0.68	1.26 (0.43, 3.69)	0.787018	1.18 (0.35, 3.89)
	G	36 (81.8%)	73 (79.4%)		1		1
	A	8 (18.2%)	19 (20.7%)	0.74	1.17 (0.47, 2.93)	0.976067	1.02 (0.37, 2.77)

NEI: National Eye Institute sample set; AREDS: Age-related Eye Diseases Study sample set; BMES: Blue Maintain Eye Study sample set; MLEB: Minnesota Lions Eye Bank sample set

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Table 3

Odds Ratios (95% Confidence Intervals) of the *HtrA1* Promotor SNP in Age-related Macular Degeneration (AMD) Cases Stratified by Disease Stages and Subtypes after Pooling All Samples Sets

SNP	Stages		Subtypes	
	Early N =333	Advanced N=472	Geographic N=255	Neovascular N=249
A with respect to G	1.57 (1.14, 2.16)	2.85 (2.38, 3.40)*	2.17 (1.75, 2.68)	2.97(2.38, 3.40)**
	P=0.005	$P=1.90\times10^{-30}$	$P=8.12\times10^{-13}$	$P=1.66\times10^{-24}$

Non-AMD controls (n=899) as the references

*Advanced stage with respect to Early stage, P=0.0001

**Neovascular AMD with respect to geographic AMD, p>0.05

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Table 4

Combined Effect (Odds Ratios (OR), 95% Confidence Intervals (CI)) of the *HtrA1* Promotor SNP and the *CFH* SNP on Age-related Macular Degeneration Risk from All Pooled Samples from the Four Sample Sets

	CFH					
	Unadjusted OR (95% CI)			**Age, sex adjusted OR (95% CI),**		
HtrA1	**GG**	**GC**	**CC**	**GG**	**GC**	**CC**
GG	1	0.84 (0.56, 1.25)	1.98 (1.23, 3.21)	1	0.82 (0.54, 1.24)	2.03 (1.23, 3.35)
GA	1.00 (0.61, 1.66)	1.73 (1.15, 2.61)	5.17 (2.98, 8 97)	0.97 (0.58, 1.62)	1.74 (1.13, 2.67)	5.07** (2.87, 8.94)
AA	1.37 (0.65, 2.88)	6.07 (2.80, 13.14)	Not available	1.22 (0.56, 2.66)	5.36* (2.44, 11.81)	Not available

*SNP type AA GC with respect to SNP types AA GG & GA GC combined: p=0.0001

**SNP type GA CC with respect to SNP types GG CC & GA GC combined: p=0.0008

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Table 5

Combined Effect, (Odds Ratios, 95% Confidence Intervals) of *HtrA1* Promotor SNP and Smoking Status on the Risk of Age-related Macular Degeneration

		***HtrA1* Genotype**		
		GG	**GA**	**AA**
Smoking Status	Never Smoked	1.00	1.80 (1.40, 2.32)	3.54 (2.25, 5.56)
	Ever Smoked	1.70 (1.25, 2.30)	3.55 (2.61, 4.85)*	17.71(7.49, 41.88)**
Cholesterol	Normal	1.00	2.17 (1.41, 3.34)	6.89 (3.03, 15.66)
	High	1.23 (0.85, 1.76)	2.41 (1.65, 3.50)	4.86 (2.70, 8.74)

*$p=8.73\times10^{-4}$ in comparison with Never Smoked with SNP type GA

**$p=1.17\times10^{-3}$ in comparison with Never Smoked with SNP type AA