# HUMAN HtrA1 IN THE ARCHIVED EYES WITH AGE-RELATED MACULAR DEGENERATION

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## ABSTRACT

*Purpose:* HtrA1 belongs to the high temperature requirement factor A family of serine proteases, which are involved in protein quality control and cell fate. A single-nucleotide polymorphism (SNP), rs11200638, in the promoter of *HtrA1* at chromosome 10q26 is reported as a likely causal variant for age-related macular degeneration (AMD). The SNP is located in the regulatory region and increases production of HtrA1 protein. This study investigates HtrA1 expression and SNP genotypes in archived ocular slides with AMD.

*Methods*: Macular, nonretinal, and peripheral retinal cells were microdissected from archived slides from 57 eyes with AMD and 16 age-matched, non-AMD controls. *HtrA1* rs11200638 SNP genotyping was performed using polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis. *HtrA1* transcripts were measured using real-time reverse transcriptase–PCR. *HtrA1* protein expression was evaluated using avidin-biotin complex immunohistochemistry.

*Results: HtrA1* (G/A) SNP was successfully genotyped in 52 AMD cases and 13 non-AMD subjects. The frequencies of the risk allele (A) were 55 of 104 (52.9%) and 8 of 26 (30.8%) in AMD and control groups, respectively. *HtrA1* mRNA was detected in normal peripheral and macular retinas, higher in the periphery than maculae. *HtrA1* mRNA was much higher in the macula and a lot lower in the periphery of the AMD eyes as compared to control eyes. HtrA1 protein was expressed in normal retinal vascular endothelia and retinal pigment epithelia. Intense immunoreaction against HtrA1 was found in AMD lesions, slightly more in wet than dry AMD lesions.

*Conclusion:* This study successfully analyzes *HtrA1* SNP and transcript expression in microdissected cells from archived paraffin fixed slides. Up-regulation of HtrA1 is detected in the macular lesions of AMD eyes. The data further suggest that rs11200638 in *HtrA1* promoter is associated with AMD development.

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## INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly of the world and has a strong genetic component.[1,2] Within the past 2 years, researchers have begun identifying the genes underlying AMD.[3] Some associated genes are involved in inflammatory responses, which can cause tissue damage if not properly controlled.[4] The most documented association is between the *complement factor H (CFH)* polymorphism and AMD.[5-10] A meta-analysis of 8 studies assessing association between the *CFH* Y402H polymorphism and AMD indicates that this polymorphism plays a role in almost 60% of AMD at the population level.[11]

Recently, another gene has been reported to be associated with AMD development.[12,13] The new candidate is the *HtrA1* (*h*igh *t*emperature *r*equirement factor A-1) gene at chromosome 10q26.2, where strong association signals and high linkage disequilibrium have been identified at *pleckstrin homology domain–containing family A, member 1* (*PLEKHA1*), the hypothetical gene *LOC387715* age-related maculopathy susceptibility 2(AMDS2), and *HtrA serine peptidase 1* (*HtrA1*, alternate name: *PRSS11*), or *PLEKHA1/LOC387715/HtrA1*.[14] Later study has noted that a single-nucleotide polymorphism (SNP) in *LOC387715* is in near absolute linkage disequilibrium with a SNP in *HtrA1*.[15]

HtrA1, one of the three proteins in the HtrA family of serine proteases, is a secretory protein and an inhibitor of transforming growth factor (TGF)-β family member.[16] Ubiquitous expression of HtrA1 is found in various normal adult human tissues, such as epidermis, where expression is very high; vascular endothelia, where expression is high; and neuronal cells, where expression is very low.[17]

A SNP, rs11200638, in the promoter region of *HtrA1* is reported to be a causal variant for AMD risk at chromosome 10q26 with a population attributable risk of 49.3%.[12,13] The *HtrA1* SNP is associated with wet AMD.[13] HtrA1 expression has been shown to increase in the retinal pigment epithelium (RPE) and drusen of 4 AMD eyes with a *HtrA1* risk allele.[12] In this study, we genotyped rs11200638 and evaluated expression of HtrA1 in archived eyes with AMD and age-matched, non-AMD eyes to find any possible correlation between the HtrA1 and AMD phenotype.

## METHODS

### CASES

The National Eye Institute (NEI) institutional review board approved the study for human subjects. Archived, paraffin-embedded slides of 73 autopsied eyes from 73 subjects were collected from the NEI (41 cases) and Wilmer Ophthalmological Institute (Wilmer),

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**Bold** type indicates AOS member.

Johns Hopkins Hospital (32 cases). Among the 73 cases, 57 eyes (32 from Wilmer, 25 from the NEI) had a diagnosis of AMD and 16 (from NEI) showed normal retina and choroids and were called "non-AMD eyes." All eyes were serially sectioned via the macula through the pupillary–optic nerve head axis. For the NEI cases, molecular analyses (SNP and reverse transcriptase–polymerase chain reaction [RT-PCR]) and immunohistochemistry were performed in selected cases. For the Wilmer cases, only SNP analysis was performed, because only 1 or 2 slides per case were available.

### MICRODISSECTION

The archived paraffin-embedded sections were de-paraffinized with xylene, rehydrated with a series of ethanol solutions, and stained with hematoxylin-eosin according to the user guide of Paradise Sample Quality Assessment Kit (Molecular Devices Corp, Sunnyvale, California). These uncovered, stained slides were visualized under a light microscope. The nonretinal (corneal and/or iris), peripheral retinal, and macular retinal cells were carefully microdissected as described previously.[18] Approximately similar numbers of peripheral and macular retinal cells were obtained from each case.

### SNP ASSAY

The microdissected, nonretinal cells were immediately placed in proteinase K–enriched DNA extraction buffer (tromethamine hydrochloride, pH 8.0; 10 mM ethylenediamine tetraacetic acid, pH 8.0; 1% polyoxyethylene 20 sorbitan monolaurate; and 0.5 mg/mL proteinase K) and incubated at 37°C overnight. The incubation mixture was heated at 95°C for 10 minutes to inactivate proteinase K. The extracted DNA was used for whole genome amplification following the manufacturer's instruction (Amersham Biosciences, Piscataway, New Jersey). SNP typing of *HtrA1* promotor (G/A), rs11200638, was performed by PCR–restriction fragment length polymorphism (RFLP) method. The amplified DNA fragment (685 base pair [bp]) containing the polymorphic site was flanked by the following primers: 5′- atgccacccacaacaacttt-3′, and 5′- cgcgtccttcaaactaatgg -3′. The PCR mixture included 1XJumpStart ReadyMix REDTaq (Sigma-Aldrich Corp, St Louis, Missouri), 100 ng DNA, and 70 pmole of primers. The program was run as 2 minutes 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 that cuts g allele at position 140 bp. Fragments were separated on 15% TBE polyacrylamide gels and visualized after ethidium bromide staining.

### RT–POLYMERASE CHAIN REACTION

RNA was extracted from both peripheral and macular retinal cells to perform RT-PCR for *HtrA1* mRNA. Total RNA was extracted from the microdissected cells using Paradise Sample Quality Assessment Kit (Molecular Devices Corp, Sunnyvale, California). The isolated RNA was used for cDNA synthesis using reverse transcription reaction with Superscript II RNase $H^-$ Reverse Transcriptase (Invitrogen Corp, Grand Island, New York) and random hexamers (Promega Corp, Madison, Wisconsin). Real-time PCR was performed using a Stratagene Mx3000 Real-Time PCR System and Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, California). The primers for *HtrA1* were 5′-tggaatctcctttgcaatcc-3′ and 5′-ttcttgg tgatggcttttcc-3′. For the internal control purpose, *beta-actin* was amplified using primers 5′-cccagcacaatgaagatcaa-3′ and 5′-acatctgctggaaggtggac-3′. Reactions were performed in a final volume of 25 µL with 2 µL single-strand cDNA. The real-time PCR cycling conditions were as follows: 95°C for 10 minutes, followed by 45 cycles for 30 seconds at 95°C, 60 seconds at 55°C, and 60 seconds at 72°C followed by fluorescence measurement. Following PCR, a thermal melt profile was performed for amplicon identification. To determine the Ct, the threshold level of fluorescence was set manually in the early phase of the PCR amplification. The relative gene expression was presented as arbitrary units of the ratio of Ct of the gene over Ct of *beta-actin*.

### IMMUNOHISTOCHEMISTRY

The avidin-biotin-complex immunoperoxidase technique was utilized on the unstained, de-paraffinized slides of 41 NEI cases in which macular sections were available. The primary antibody was mouse anti-human HtrA1 monoclonal antibody (R&D Systems, Inc, Minneapolis, Minnesota) or control rabbit IgG. The secondary antibody was biotin-conjugated horse anti-mouse IgG (Vector Laboratories, Inc, Burlingame, California). The substrate was avidin-biotin-peroxidase complex (Vector Laboratories, Inc, Burlingame, California), and the chromogen was diaminobenzidine and nickel sulfate. Positive reaction results in the production of a blue-black color.

### STATISTICAL ANALYSIS

The chi-square test was performed in order to compare the allele frequencies reported for the cases and controls with 1 degree of freedom. A *P* value of <.05 was considered to be significant. Odds ratios were calculated based on the unmatched case-control design.

## RESULTS

### DEMOGRAPHY AND OCULAR PATHOLOGY

A total of 57 cases (eyes) had classic AMD and 16 cases (eyes) did not have any retinal diseases except some with pinguecula and cataracts (Table 1). The average ages were 83.1 ± 8.9 years for the AMD group and 72.7 ± 8.1 years for the control group. In the AMD group, 25 were female and 15 were male; gender was unknown in 17 cases. In the non-AMD group, 5 were female and 7 were male; gender was unknown in 4 cases.

Pinguecula was found in 26 cases (19 in the AMD group and 7 in the non-AMD group), and cataracts were found in 51 cases (40 in the AMD and 11 in the non-AMD group). All eyes in the AMD group demonstrated classic AMD lesions in the macula as defined in literature.[19,20] Subretinal choroidal neovascularization and photoreceptor loss with or without disciform scars were found in 36 (63.2%) of the 57 eyes (Table 1). These eyes were diagnosed with neovascular (wet) AMD. The remaining 21 eyes were diagnosed with AMD showing areolar (geographic or dry) atrophy without neovascularization (Table 1). These cases were characterized by a loss of photoreceptors, alteration or loss of RPE cells, drusen formation, and/or calcification in the macula.

### *HtrA1* SNP GENOTYPE

**TABLE 1. DEMOGRAPHIC AND PATHOLOGIC DATA FOR THE ARCHIVED CASES: AMD AND NON-AMD GROUPS**

| GROUP | NO. OF CASES | MEAN AGE | GENDER | | | PINGUECULA | CATARACT | AMD CATEGORY | |
|---|---|---|---|---|---|---|---|---|---|
| | | | MALE | FEMALE | NA | % | % | DRY | WET |
| AMD | 57 | 83.11 ± 8.90 | 15 (26.3%) | 25 (43.9%) | 17 (29.8%) | 19/51* (37.3%) | 40/50* (80%) | 21 (36.8%) | 36 (63.2%) |
| Non-AMD | 16 | 72.67 ± 8.09 | 7 (43.7%) | 5 (31.3%) | 4 (25.0%) | 7/16 (43.8%) | 11/16 (68.8%) | … | … |

AMD, age-related macular degeneration; NA, not available.
*Eyes of 51, not 57, were examined for pinguecula. Eyes of 50, not 57, were examined for cataracts.

SNP of *HtrA1* promotor (G/A), rs11200638, was successfully obtained in 65 of the total 73 studied cases (Figure 1). The SNP allele frequency analysis showed a significant difference between the AMD and non-AMD groups ($P$ = .04, OR = 2.53; Table 2). The A (*HtrA1* SNP) allele frequencies were 52.9% for the AMD group and 30.8% for the non-AMD group. Furthermore, the genotypes and allele frequencies are almost equally distributed in wet (51.6%) and dry (50.0%) AMD (Table 2).

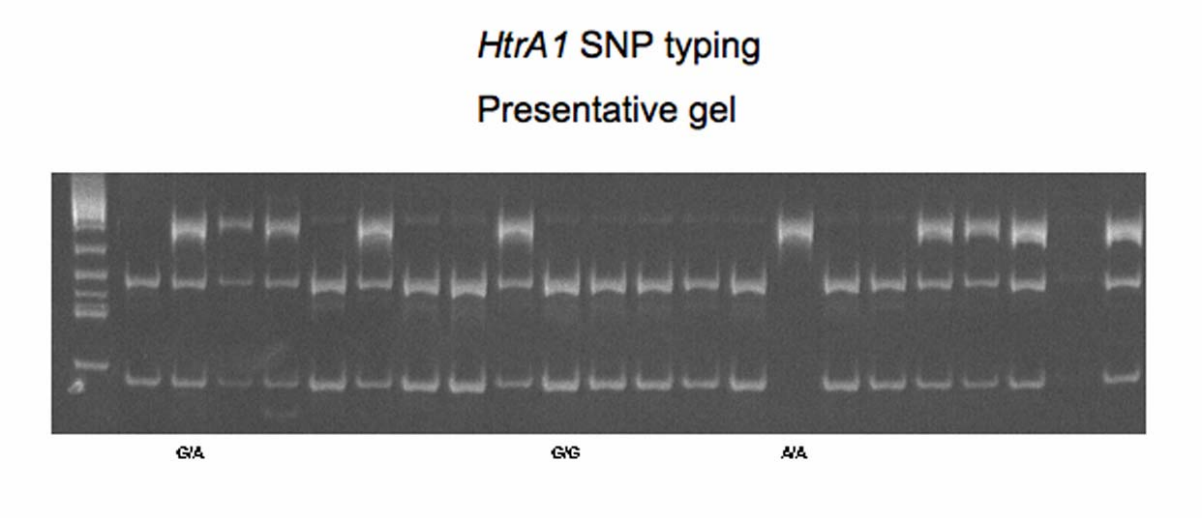


**FIGURE 1**

Representative gel images of *HtrA1* SNP. Three different patterns are identified representing G/A (685 bp, 545 bp, and 140 bp), G/G (545 bp and 140 bp), and A/A (685 bp) on the gel.

### HIGH HtrA1 EXPRESSION IN THE EYES WITH AMD

*HtrA1* transcripts were successfully recovered in a total of 10 cases that had *HtrA1* SNP information; among them, 6 were AMD cases (3 wet and 3 dry AMD) and 4 were age-matched, non-AMD controls (Figure 2). In the non-AMD eyes, *HtrA1* mRNA was much more highly expressed in the periphery than maculae. In contrast, the pattern of *HtrA1* mRNA expression was reversed in the AMD eyes: much higher in the maculae than peripheral retina. Interestingly, the eye with homozygous *HtrA1* SNP (AA) showed the highest *HtrA1* mRNA in its maculae. No significant differences were noted between wet and dry AMD eyes.

**TABLE 2. DISTRIBUTION OF HTRA1 RS11200638 SNP TYPES AMONG AMD CASES AND CONTROLS***

| GENOTYPE | CONTROL (N=13) | AMD (N=52) | | | | $\chi^2$ (*P* VALUE) | OR |
|---|---|---|---|---|---|---|---|
| | | DRY n=18 | WET n=31 | UNKNOWN n=3 | ALL N=52 | | |
| GG | 5 | 4 | 7 | 0 | 11 | | |
| GA | 8 | 10 | 16 | 1 | 27 | | |
| AA | 0 | 4 | 8 | 2 | 14 | | |
| GA+AA | 8 (61.5) | 14 (77.8) | 24 (77.4) | 3 | 41 (78.8) | | |
| G allele | 18 | 18 | 30 | 1 | 49 | Reference | Reference |
| A allele | 8(30.8) | 18 (50.0) | 32 (51.6) | 5 | 55 (52.9) | 4.07 (0.04) | 2.53 |

AMD, age-related macular degeneration; OR, odds ratio; SNP, single-nucleotide polymorphism.
*The numbers in parentheses are percentages.

Immunoreactivity against HtrA1 was detected weakly in the retinal vascular endothelia, internal limiting membrane, and RPE of the control eyes with normal retina. In general, positive HtrA1 staining was observed in the macula of the AMD eyes, with either wet or dry types (Figure 3). Intense staining highlighted both choroidal neovascular structure and drusen. However, few AMD lesions did not depict reactivity to HtrA1. No visible changes in intensity, number, or staining pattern were found in the peripheral retina in the eyes with AMD as compared to the normal eyes.

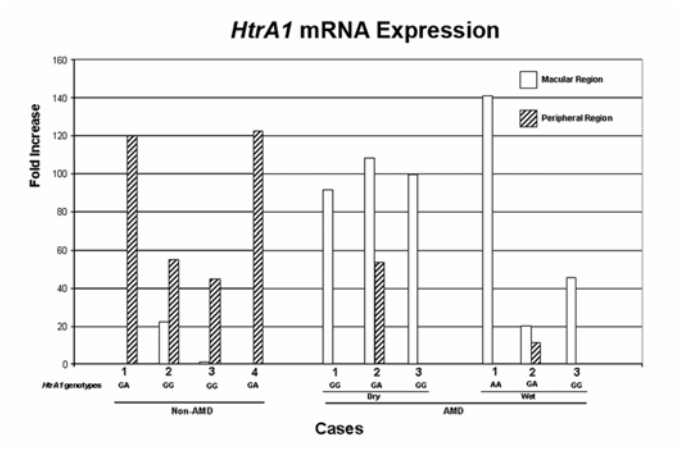


**FIGURE 2**

*HtrA1* mRNA expression (real-time RT-PCR). *HtrA1* transcripts are higher in the peripheral retina than macular region of the normal eyes. In contrast, *HtrA1* transcripts in the macula are much higher than in the periphery of the eyes with age-related macular degeneration (AMD). The highest *HtrA* mRNA is expressed in a wet AMD eye with *HtrA1* AA genotype.

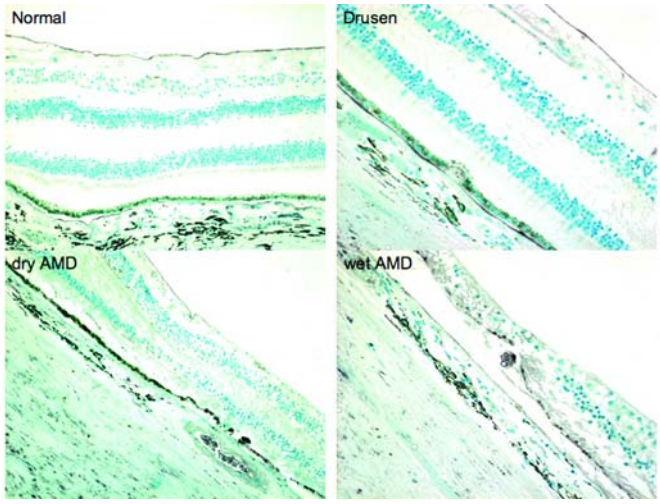


**FIGURE 3**

HtrA1 protein expression (immunohistochemistry). Increase of HtrA1 expression (blackish color) is seen in a small hard drusen, abnormal retinal pigment epithelia of eye with dry age-related macular degeneration (AMD), and a choroidal neovascular structure and degenerated photoreceptors of eye with wet AMD (avidin-biotin immunoperoxidase, original magnification, ×100).

## DISCUSSION

Similar to our previous studies on CX3CR1 and ERCC6 in AMD,[4,10,18] we have successfully applied a combination of microdissection and PCR-RFLP to analyze different genetic variants using archived, paraffin-embedded slides; this time, *HtrA1* promoter rs11200638 SNPs were investigated. Furthermore, we have also detected HtrA1 transcripts and protein expression in these ocular sections using microdissection combined with quantitative RT-PCR and immunohistochemistry.

Since the first report of the *LOC387715* variant being a major risk factor for AMD,[14] several studies have confirmed the finding.[21-25] Maller and colleagues[15] have suggested that the true AMD susceptibility SNP was a S69A polymorphism (rs10490924) in *LOC387715*, which exits in near absolute linkage disequilibrium with SNPs in the *HtrA1* gene. Two recent studies[12,13] discovered a highly significant association between AMD and *HtrA1*, which may be the true gene at this locus.

The current study demonstrates an association between the *HtrA1* promoter polymorphism (rs11200638) and AMD on archived histopathologic sections. A higher frequency of the A allele is found in the AMD cases (52.9%) as compared to the frequency found in the controls (30.8%). Yang and colleagues[12] have recently reported the frequencies of 40.3% in 442 AMD patients and 25.2% in 309 controls in a Caucasian cohort in Utah. Our findings are also compatible with our recent data of two case-control cohorts and population-based case-control studies, in which the same *HtrA1* polymorphism is consistently found to be associated with AMD (C. M. Bojanowski, ARVO meeting, 2007. Abstract 3653). Two recent articles[12,13] document a variant in the regulatory region of *HtrA1* rs11200638, which appears to be the true SNP association at this locus.

Our findings of higher expression of HtrA1 protein and transcript in the peripheral retinas as compared to the macula in normal eyes are not surprising, since HtrA1 is reported in vascular endothelia and low expression is found in neuronal tissue.[17] Anatomically there are more retinal vessels in the periphery than macular region in the normal eye. De Luca and colleagues[17] have demonstrated high to medium HtrA1 expression in mature layers of epidermis, secretory breast epithelium, proliferative endometrium, liver, and kidney tubules of normal human tissues, and HtrA1 expression seems in concordance with cellular secretory properties. Our findings of enhanced expression of HtrA1 in AMD eyes suggest possible active neovascularization in the macular lesions with wet AMD or large drusen deposits (secretory deposits in Bruch's membrane) resulting from abnormal RPE cells with dry AMD.

*HtrA1* genes are highly conserved among mammalian species: the amino acid sequences encoded by *HtrA1* cDNA clones from cow, rabbit, and guinea pig are 98% identical to human.[26] In *Escherichia coli*, a functional *HtrA1* gene product is required for cell survival after heat shock or oxidative stress; its role appears to be the degradation of denatured protein.[27] Human *HtrA1* gene is up-regulated during aging.[28] Indeed, HtrA1 is a member of the heat shock serine protease and is up-regulated by cellular stress.[27,29,30] Active HtrA1 induces cell death in a serine protease–dependent manner. Serine protease activity in HtrA1 is important for its pro-apoptotic property and the degradation of extracellular matrix proteins.[31] This functional property of HtrA1 could explain the enhanced expression of this protein found in both wet and dry AMD lesions.

The higher-risk allele frequency of *HtrA1*, rs11200638 in AMD cases, as compared to non-AMD controls, and the increasing expression of HtrA1 documented both at mRNA and protein levels by RT-PCR and immunohistochemistry in AMD lesions are consistent with a possible role of this protein in both wet and dry AMD, possibly via its known inhibitory effects on TGF-β proteins.[13] The mechanism of transcriptional activation of HtrA1 is currently unknown, but activation of protease activity by peptides binding to its PDZ domain has been reported.[32] Collagen C-propeptide is a specific physiological regulator of HtrA1. Further studies should aim at identifying conditions that activate HtrA1 in ocular tissue, on either the genetic or the epigenetic level, and investigating the effect of its activation on RPE, photoreceptor, choroidal neovascular components, and drusen in our animal model of AMD.[33]

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## PEER DISCUSSION

DR SHALESH KAUSHAL: High temperature requirement factor (HtrA1) is a mammalian serine protease that is homologous to a class of proteins found in *Escherichia coli*. Its members are up-regulated by heat shock and endoplasmic reticulum stress as part of the unfolded protein response. It also has been implicated in arthritis, specifically in cleaving fibronectin and the activation of matrix metalloproteinases. Clearly, it has a role in extracellular matrix function and remodeling, like many other gene products associated with macular disease.

This current paper focuses on the role of an *HtrA1* risk allele (rs11200638) in age-related macular degeneration (AMD). Recent population studies implicate this single-nucleotide polymorphism in AMD with a population attributable risk of 49.3%. Here, the authors examine archived eye tissue from normal and AMD patients for the presence of the mutation, levels of *HtrA1* mRNA, and the histological localization of the HtrA1 protein. Their genotyping results strongly support the earlier population studies, arguing that the *HtrA1* risk allele is associated with AMD. Their analysis of *HtrA1* mRNA and protein localization suggests there are differences in HtrA1 expression in AMD patients, consistent with a role of the protein in the disease.

There several issues that should be addressed:

1. The major concern has to do with inconsistencies in the molecular analysis. *HtrA1* transcripts are detected in the peripheral retina of normal patients, and at much lower levels in the maculae (see Figure 2). In contrast, *HtrA1* mRNA in AMD patients is apparent in the macular retina with much lower levels in the periphery. It is surprising that very little mRNA was detected in the peripheral retina of AMD patients, especially since the protein was detected histologically in the peripheral retina of both AMD and normal eyes. In part, the mRNA measurements are hard to understand because the y-axis in Figure

2 is labeled "Fold Increase"; does this mean the Ct HtrA1 to Ct beta-actin ratio, or is it normalized in some other way? The authors should discuss the inconsistency.

2. There does not appear to be a good correlation between the presence of the *HtrA1* risk allele and the level of *HtrA1* mRNA (see Figure 2). In the earlier work by Yang and coworkers,[1] the correlation was greater. In that study, a 2.7-fold increase in lymphocyte *HtrA1* mRNA levels and a 1.7-fold difference in HtrA1 protein levels in the RPE were noted in patients homozygous for the risk allele compared to normal individuals. By contrast, the mRNA levels shown in Figure 2 correlate poorly with the risk allele. The authors should offer some explanations for this discrepancy.
3. In a related point, it would be helpful to mention why retina was examined as opposed to RPE, which showed the greatest changes in HtrA1 in the earlier work. Examining the peripheral versus macular retina seems like a clever idea; it should be presented with a bit more fanfare.

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## REFERENCE

1. Yang Z, Camp NJ, Sun H, et al. A variant of the HTRA1 gene increases susceptibility to age-related macular degeneration. *Science* 2006;314:992-993.

DR. CHI-CHAO CHAN: We appreciate Dr. Kaushal's pertinent and helpful comments, which were sent to me last month. His major concern has to do with inconsistencies in the molecular analysis. *HtrA1* transcripts are detected in the peripheral retina of normal subjects, and at much lower levels in the maculae as was shown in Figure 2. In contrast, *HtrA1* mRNA in age-related macular degeneration (AMD) patients is detected in the macular retina with much lower levels in the periphery. It is surprising that very little mRNA was detected in the peripheral retina of AMD patients, especially since the protein was detected histologically in the peripheral retina of both AMD and normal eyes. In part, the mRNA measurements are difficult to understand because the y-axis in Figure 2 was labeled as "Fold increase". Dr Kaushal asks if this means the Ct *HtrA1* to Ct *beta-actin* ratio, or if this value was normalized in some other way. We are sorry for the confusion and have revised Figure 2 with a more readable image to compare *HtrA1* transcripts in different genotypes and phenotypes. The "fold increase" of *HtrA1* mRNA, as shown on the y-axis, is the result of a comparison between a particular region of the AMD eyes to that region of the non-AMD eyes with a normal retina. The *HtrA1* mRNA of the normal retinal cells is the reference. Therefore, there were general decreases of *HtrA1* mRNA in the periphery of AMD eyes compared to normal peripheral retina. These findings do not imply the absence of HtrA1 protein in the peripheral retina of the AMD eyes, and we can still detect the presence of this protein. We do not find an inconsistency.

Dr. Kaushal further comments that there does not appear to be a good correlation between the presence of the *HtrA1* risk allele and the level of *HtrA1* mRNA as illustrated in Figure 2. He notes that in the earlier work by Yang et al 2006 the correlation was greater and, in that study, a 2.7-fold increase in lymphocyte *HtrA1* mRNA levels and a 1.7-fold difference in HtrA1 protein levels in the RPE were noted in patients who were homozygous for the risk allele compared to normal individuals. He commented that by contrast, the mRNA levels shown in our Figure 2 correlated poorly with the risk allele. We believe, although it may not be a perfect correlation between the presence of the *HtrA1* risk allele and the level of *HtrA1* mRNA, that the level of *HtrA1* mRNA definitely increases in AMD macula, with the highest levels in homozygous (AA), followed by heterozygous (GA) and then wild-type (GG). We do not know the significance of HtrA1 in the peripheral retina. In a related point Dr. Kaushal mentions that it would be helpful to discuss why the retina was examined as opposed to retinal pigment epithelium (RPE), which showed the greatest changes in HtrA1 in the earlier work. He commented that examination of the peripheral versus macular retina seemed like a clever idea and should be presented with emphasis. In our opinion, because the archived AMD eyes were usually in the late stage of the disease and most RPE cells had been destroyed, we were limited to examining the retinas, which were also affected by AMD. We thank him for encouraging us to examine the peripheral versus macular retina and, indeed, several publications have indicated that both the macula and peripheral retina are affected by AMD. In a proteomic study of central and peripheral retina with progressive AMD, approximately 60% of the proteins exhibited changes that are specific to either the macula or periphery, with the remaining 40% demonstrating changes in both regions (Ethen, et al, Invest Ophthalmol Vis Sci 2006;47:2280-90). In another study, a significantly lower level of thrombospodin-1 (TSP-1) was detected in the far periphery than in the equator and submacular regions of AMD eyes (Uno, et al, Br J Ophthalmol 2006;90:48-54). Feigl and coworkers recorded the rod-mediated multifocal ERG and demonstrated a functional loss of rod response in early AMD patients, which implies functional abnormality in peripheral retina (Eye 2005;19:431-41).