Levels of the HtrA1 Protein in Serum and Vitreous Humor Are Independent of Genetic Risk for Age-Related Macular Degeneration at the 10q26 Locus

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Purpose. The purpose of this study was to determine if levels of the HtrA1 protein in serum or vitreous humor are influenced by genetic risk for age-related macular degeneration (AMD) at the 10q26 locus, age, sex, AMD status, and/or AMD disease severity, and, therefore, to determine the contribution of systemic and ocular HtrA1 to the AMD disease process.

Methods. A custom-made sandwich ELISA assay (SCTM ELISA) for detection of the HtrA1 protein was designed and compared with three commercial assays (R&D Systems, MyBiosource 1 and MyBiosource 2) using 65 serum samples. Concentrations of HtrA1 were thereafter determined in serum and vitreous samples collected from 248 individuals and 145 human donor eyes, respectively.

Results. The SCTM ELISA demonstrated high specificity, good recovery, and parallelism within its linear detection range and performed comparably to the R&D Systems assay. In contrast, we were unable to demonstrate the specificity of the two assays from MyBioSource using either recombinant or native HtrA1. Analyses of concentrations obtained using the validated SCTM assay revealed that genetic risk at the 10q26 locus, age, sex, or AMD status are not significantly associated with altered levels of the HtrA1 protein in serum or in vitreous humor ($P > 0.05$).

Conclusions. HtrA1 levels in serum and vitreous do not reflect the risk for AMD associated with the 10q26 locus or disease status. Localized alteration in *HTRA1* expression in the retinal pigment epithelium, rather than systemic changes in HtrA1, is the most likely driver of elevated risk for developing AMD among individuals with risk variants at the 10q26 locus.

Keywords: HtrA1, vitreous humor, serum, ELISA

Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss in the United States[1,2] and affects close to 200 million individuals worldwide.[3] Approximately 5% to 10% of these individuals suffer from an advanced form of the disease causing vision loss that may significantly degrade their quality of life.[4,5] Whereas increasing age is the strongest risk factor for developing AMD,[3] genetic factors explain a large part of the variation in initiation, progression, and clinical presentation of the disease.[6,7] The genetic variants most strongly associated with an elevated risk for AMD lie within the chromosome 10q26 region (Chr10 locus) encompassing the tightly linked age-related maculopathy susceptibility (*ARMS2)* and high temperature requirement A serine protease (*HTRA1*) genes.[8,9] Except for the 1q32 region (Chr1 locus), additional loci individually associated with AMD through genome-wide association studies (GWAS) have a marginal impact on the incidence of disease.[10,11] Elucidating the mechanisms driving AMD initiation and progression among individuals carrying risk variants at the Chr10 locus is therefore key to designing therapeutic strategies to effectively prevent or slow the progression of this blinding disease.

Among individuals with European ancestry, all genetic risk at the Chr10 locus is tagged by the rs10490924 variant (*ARMS2*) or single nucleotide polymorphisms (SNPs) in strong linkage disequilibrium with it.[12,13] Causality within the 10q26 locus has proven challenging to identify; however, recent work has narrowed the causal region to a block of SNPs overlapping *ARMS2* exon 1 and intron 1.[14,15] Analyses of allele-specific *HTRA1* mRNA expression in tissues from human donors have shown that this block includes a non-coding, cis-regulatory element that promotes RPE-specific expression of *HTRA1* mRNA in non-risk individuals. In carriers of the risk allele, this element is impaired, resulting in lower *HTRA1* expression by the RPE. Decreased expression is associated with reduced levels of the HtrA1

protein at the primary location of AMD-associated pathology, the RPE/Bruch's membrane (BM) interface. In contrast, an age-dependent increase in HtrA1 protein in the RPE/BM is observed among individuals with no risk alleles at the 10q26 locus.[15] HtrA1 functions as both a secreted serine protease and an extracellular chaperone capable of cleaving various extracellular matrix (ECM) proteins, proteoglycans, and growth factors.[16,17] Reduced *HTRA1* expression may therefore hinder the ability of the RPE to maintain the integrity of the RPE/BM interface with increasing age, leading to a higher risk for the formation and accumulation of basal laminar deposits, drusen, and/or pigment epithelium detachments characteristic of AMD.[18]

The observation that *HTRA1* mRNA and protein levels are reduced in RPE/choroid tissue but not the retina[15] supports the hypothesis that AMD risk at the Chr10 locus causes dysfunction that is specific to the RPE/BM/choroid interface. The level of the HtrA1 protein in aqueous humor of patients with naive choroidal neovascularization secondary to AMD was found to be higher than in healthy controls,[19] although the study did not determine if participants carried risk variants at the Chr10 locus. Additionally, elevated levels of the HtrA1 protein have been reported in the serum and plasma of patients with AMD carrying risk at the Chr10 locus when compared to those without risk.[20] It is unclear if variation in HtrA1 within sera reflects AMD severity or predicts progression to the late form of the disease. The goal of this study was to assess whether levels of HtrA1 in serum or vitreous are influenced by genetic risk at the 10q26 locus, AMD status and/or severity, and, therefore, to determine the potential contribution of systemic and ocular HtrA1 to the AMD disease process. Analysis of 248 samples collected from individuals with or without AMD using a validated in-house developed HtrA1 ELISA demonstrates that risk at the Chr10 locus, AMD status, or AMD severity are not significantly associated with altered levels of the HtrA1 protein in serum. Levels of HtrA1 in the vitreous humor of 145 human donor eyes is also independent of risk at the 10q26 locus or AMD status. This study therefore supports the hypothesis that the insult caused by risk at this locus in AMD is RPE-specific, and that serum or vitreous HtrA1 levels do not reflect disease onset or progression.

Methods

Human Serum Sample Selection and Processing

Subjects were recruited between 2009 and 2019 at the Sharon Eccles Steele Center for Translational Medicine (SCTM), John A. Moran Eye Center, University of Utah, United States and between 1999 and 2009 at the Cell and Molecular Biology Center, Department of Ophthalmology and Visual Sciences, University of Iowa, Iowa City, Iowa, United States, as part of a case/control study of the genetic etiology of AMD. These studies adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Boards of the University of Utah and University of Iowa. All participants provided informed written research consent and had self-reported European ancestry. All participants were screened for AMD (no AMD, early/intermediate AMD, or late AMD), with both eyes graded by the same two independent experienced observers at the time of recruitment. Screening and grading relied on multimodal imaging (fundus photographs, infra-red, and optical coherence tomography volume scans) collected at the time of enrollment. Grading was based on the international classification of mutually exclusive stages of age-related maculopathy introduced by the Rotterdam Group,[21] and was performed as previously described.[9] Venous blood was collected at the time of enrollment, processed, and stored at −80°C. Because freeze-thawing of serum samples may affect HtrA1 protein stability, serum samples were stored in single-use aliquots. Demographic data, including age, sex, ethnicity, smoking history, and medical history, were also collected at the time of recruitment. Samples included in this study were selected randomly based on heterozygous or homozygous risk or non-risk combinations of genotypes at rs10490924, time since blood collection, and age. The median time between blood collection and measurements of HtrA1 levels was 3 years (interquartile range [IQR] = 1 year). We observed no significant association between time since sample collection and serum HtrA1 levels (univariate linear regression, $P = 0.97$), indicating that the time between collection and testing did not significantly impact the levels of HtrA1 in serum.

Vitreous Protein Extraction

Human donor eyes were selected from a repository composed of more than 9000 pairs of human eyes processed, flash-frozen, or fixed within 5 hours of death held at the Sharon Eccles Steele Center for Translational Medicine, John A. Moran Eye Center, University of Utah. Studies have shown that there is minimal degradation of most proteins in tissues collected within 24 hours postmortem.[22] For each eye, AMD status (AMD or no AMD) was determined based on gross photographs of the posterior segments acquired during dissection, and, when available, recovered medical history. Donor eyes included in this study were selected randomly based on homozygosity for the risk or non-risk allele at rs10490924, time since blood collection, and age. Vitreous humor was recovered from each eye at the time of collection, flash-frozen, and stored at −80°C. Portions of vitreous humor from selected eyes were thawed on ice and weighed. They were then placed within an equal volume of Tissue Protein Extraction Reagent (T-PER, Cat. #78510; Thermo Fisher Scientific, United States) containing 1% Halt Protease and Phosphatase Inhibitor Cocktail. The mix was briefly sonicated using a probe sonicator and shaken on an Eppendorf tube shaker at 800 RPM every 20 seconds followed by no shaking for 20 seconds. This cycle was repeated for 18 hours at 4°C. The samples were then centrifuged at 21,000 × g for 30 minutes to pellet any remaining insoluble vitreous material. The soluble vitreous protein containing supernatant was thereafter collected, placed in clean Eppendorf tubes, and stored at −80°C. Samples used for analyses were thawed prior to protein quantification. Total protein concentration was determined using a 660 nm protein assay kit following the supplied protocol (Cat. #1861426; Thermo Fisher Scientific, United States). For ELISA assays, we found that a volume between 1 and 12.5 µL of vitreous resulted in the detection of HtrA1 within the linear range of the assay. Vitreous sample volume was brought to 100 µL with reagent diluent consisting of 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS).

Genotyping of Subjects and Donors

Genomic DNA was isolated from peripheral blood leukocytes with QIAamp DNA Blood Maxi kits (Qiagen, United

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States). Genotyping was performed by TaqMan assays (Applied Biosystems, United States) using 10 ng of template DNA in a 5 µL reaction. When available, predesigned assays were used. When predesigned assays were unavailable, custom assays were designed using the manufacturer's software. The thermal cycling conditions in the 384-well thermocycler (PTC-225; MJ Research) consisted of an initial hold at 95°C for 10 minutes, followed by 40 cycles of a 15-second 95°C denaturation step and a 1-minute 60°C annealing and extension step. Plates were read in the 7900HT Fast Real-Time PCR System (Applied Biosystems, United States). Data cleaning and quality control checks were performed using PLINK (version 1.9 and version 2.0).[23,24]

In-house (SCTM) HtrA1 ELISA

The level of the HtrA1 protein in plasma, serum, and vitreous humor lysate was measured using a custom sandwich ELISA that relied on HtrA1-specific NEP-2717 rabbit polyclonal antibody generated against full-length HtrA1 for capture and B18 mouse monoclonal anti-HtrA1 antibody for detection. Production and validation of these two antibodies for specificity and selectivity has been described previously.[15] The ELISA was also described in the same publication. Briefly, 96-well Maxisorp ELISA microplates (Thermo Fisher Scientific, United States) were coated with HtrA1-specific NEP-2717 polyclonal antibody at a dilution of 1:500 in 50 mM carbonate coating buffer (pH 9.6) and left overnight at 4°C. The coated plates were washed once with PBS containing 0.05% Tween-20 (PBST), then blocked for 2 hours at room temperature with SuperBlock (Cat. #37515; Thermo Fisher Scientific Pierce Protein Biology, United States) containing 0.05% Tween-20. Following three washes in PBST, serial dilutions of recombinant HtrA1 (Cat. #RP-77538; Thermo Fisher Scientific, United States) in PBS buffer were added to the plates as reference standards. After adding serum or vitreous samples diluted 10 to 25-fold in PBS, plates were incubated for 2 hours at room temperature and washed 3 times with PBST. The B18 monoclonal anti- HTRA1 antibody, diluted at 1:500 in PBST, was then added to the wells. After a 1-hour incubation at room temperature, plates were washed 5 times with PBST. A pre-absorbed affinity purified HRP-conjugated goat anti-mouse IgG antibody (Cat. #115-035-062; Jackson ImmunoResearch, United States) was diluted 1:10,000 in PBST and incubated in the plates for 30 minutes at room temperature. Wells were washed an additional 5 times before being treated with Super Signal West Dura (Cat. #34076; Thermo Fisher Scientific, United States) for 5 minutes. Luminescence was detected with a BioTek Synergy 4 multi-mode plate reader (Agilent Technologies, United States). For vitreous samples, levels of HtrA1 were normalized to total protein concentration (ng HtrA1 per mg of total protein, ng/mg) to control for differences in protein yields.

R&D HtrA1 ELISAs

We compared the SCTM ELISA to a previously developed and validated ELISA assay,[25] which used antibodies from R&D Systems (R&D assay). The protocol for the R&D ELISA was identical to that followed for the SCTM ELISA except that 1 µg/mL sheep polyclonal anti-HtrA1 antibody (Cat. #AF2916; R&D Systems, United States) was used for capture and 0.5 µg/mL mouse monoclonal anti-HtrA1 antibody (Cat. #MAB2916; R&D Systems, United States) was used for the detection.

HtrA1 Standards

The specificity and linearity of the SCTM and R&D HtrA1 ELISA assays were assessed using two recombinant HtrA1 protein standards, an Sf9-derived HtrA1-WT (Cat. #RP-77538; Thermo Fisher Scientific, United States) and a HEK293-derived catalytically inactive HtrA1-S328A (rHtrA1-S328A protein; Yurogen, United States), in addition to serum samples or serum depleted of HtrA1. Recombinant proteins and serum samples were diluted in PBS with 1% BSA diluent. Depletion of HtrA1 in serum was performed by first removing immunoglobulins by incubation with GammaBind Plus Sepharose (Cat. #17-0886-01; GE Healthcare, United States). The pre-cleared serum was then incubated with GammaBind Plus sepharose prebound with anti-HtrA1 derived from serum of immunized rabbits (exsanguination from mouse #8108) or with the pre-bleed serum from the same mouse. The unbound flow through from these depletions was then collected for ELISA.

Linearity and Parallelism of SCTM and R&D HtrA1 ELISA Assays

To verify the linearity and parallelism of the SCTM and R&D ELISA assays, increasing amounts of recombinant HtrA1 were spiked into reagent diluent (PBS + 1% BSA) containing 2% normal human serum (NHS, Cat. #NHS; Complement Technologies, United States). The amount of HtrA1 detected was then inferred using the standard curve for each assay and plotted against the known concentration of the recombinants. The optimal linear range of the assay was defined as the range of concentrations for which between 75% and 100% of inputted protein is recovered. Assay parallelism, defined as the ability of the assay to detect an analyte in highly diluted samples as compared to samples with little or no dilution, was assessed by measuring the concentration of HtrA1 in various dilutions of the protein in NHS. Any experiments measuring levels of HtrA1 in serum should be careful to use an amount of serum that falls within the dilutional parallelism of the assay.

MyBioSource HtrA1 ELISAs

We compared the SCTM HtrA1 ELISA to commercially available assays used in previous publications (MyBioSource Cat. #MBS2504576[19] and MyBioSource Cat. # MBS454847[20]; MBS1 and MBS2 assays, respectively; MyBioSource, United States). These ELISA kits contained sandwich ELISAs that use a colorimetric substrate of horseradish peroxidase along with HtrA1 reference standard and reagents. According to the manufacturer, the MBS1 ELISA has linear range of sensitivity of 2 to 20 ng/mL, whereas the MBS2 ELISA has a linear range of sensitivity of 0.2 to 2 ng/mL. Accordingly, to be in the linear range of each assay, serum samples were diluted 1:100 (MBS1 assay, low range) or 1:10 (MBS2 assay, high range). To compare ELISAs, we reconstituted the HtrA1 reference provided with the MBS1/MBS2 assays in their respective dilution buffers and tested it with both MBS1/MBS2 and SCTM ELISAs. In another set of experiments, we diluted Sf9-derived rHtrA1 or HtrA1-S328A in diluent used with the SCTM ELISA (PBS + 1% BSA) or with the MBS1/MBS2 assay diluents and assessed them using the 3 assays. Finally, because the MBS1/MBS2 manufacturer states that these ELISAs only recognize "native" HtrA1, we tested their ability to detect HtrA1 in the supernatant of HEK293

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cells expressing tet-inducible HtrA1 versus supernatant from HEK293 cells with an empty vector.

Statistical Analyses

Statistical analyses were performed using Prism version 10.0.0 (GraphPad Software LLC, USA) and R version 4.2.0 (R Core Team, USA).[26] The level of significance was set at 0.05 and all provided P values were 2-tailed. Associations between age and HtrA1 levels were assessed by extracting the slope (denoted β) and associated P -value from linear regressions in which age was the independent variable. The nonparametric Kruskal-Wallis test was used to identify associations between HtrA1 levels and sex, AMD status, AMD severity (no AMD, early/intermediate, or late AMD) or genotype at rs10490924. This test was performed for each variable individually. Significant tests were followed by post hoc pairwise comparisons using the Wilcoxon rank sum test when appropriate. All P-values were adjusted for multiple comparisons using Bonferroni corrections when appropriate.

Results

Validation of SCTM and R&D HtrA1 ELISA Assays

Validation and comparison of the SCTM and R&D ELISAs were performed using serum samples collected from 65 individuals (see Supplementary Table S1 for detailed demographics). Both assays display robust interassay reproducibility. Recovery of either Sf9-derived HtrA1-WT or rHtrA1-S328A with the SCTM ELISA ranges from 80% to 120% (Figs. 1A, 1B, Supplementary Figs. S1A, S1B). The optimal linear range of the assay lies between 1 and 20 ng/mL of protein input with either recombinant protein. In comparison, the R&D ELISA demonstrates a recovery of the rHtrA1-S328A ranging from 16% to 95%, with an optimal linear range between 2 and 40 ng/mL of protein input (Fig. 1C, Supplementary Fig. S1C). When the Sf9-derived rHtrA1-WT standard was used, the recovery exceeded input considerably (Fig. 1D, Supplementary Table S1D). Parallelism of each assay was tested with serum dilutions ranging from 2-fold to 128-fold (Fig. 1E, Supplementary Fig. S2). Both assays showed good parallelism with either standard within their respective linear range, with two-fold dilutions resulting in an approximately two-fold decrease in detected protein concentration. No significant differences in mean protein measured within the linear range of each assay were detected (Fig. 1F). Finally, concentrations of HtrA1 in human serum measured in the same samples using the SCTM and R&D assays correlated strongly ($r = 0.92$, $P < 0.001$; Supplementary Fig. S3A). Standard curves for the SCTM and R&D assays are shown in Supplementary Figs. S4A and S4B, respectively.

MBS1 and MBS2 Assays

The same 65 serum samples were used to compare the SCTM, MBS1, and MBS2 ELISAs (see Supplementary Table S1 for detailed demographic). Correlation between the concentration of HtrA1 in human serum measured with the SCTM or R&D ELISA and those measured in the same samples using the MBS1 or MBS2 assays correlated poorly ($r < 0.008$, $P > 0.05$; see Supplementary Figs. S3B–E). Surprisingly, there was no correlation between measurements made using the MBS1 and MBS2 ELISAs ($r = 0.16$, $P > 0.05$; see Supplementary Fig. S3F). The SCTM ELISA failed to recognize the HtrA1 standards from either MBS1 or MBS2 assays when diluted in the diluents provided with the respective kits. It did, however, recognize the rHtrA1-S328A standard when diluted in either MBS1 or MBS2 diluents (Supplementary Fig. S5). Conversely, neither MBS1 or MBS2 assays detected recombinant Sf9-derived HtrA1-WT regardless of the diluent used. We therefore proceeded to test whether the assays could recognize "native" HtrA1, which the manufacturer states is the only form of HtrA1 that these ELISAs recognize. To do so, we tested the ability of one of the assays (MBS2) to detect HtrA1 in the supernatant of HEK293 cells expressing tet-inducible HtrA1 and used supernatant from HEK293 cells with an empty vector as controls. The SCTM HtrA1 ELISA assay detected abundant amounts of native HtrA1 in the supernatants from HEK293 cells induced to express HtrA1. Additionally, the assay did not detect HtrA1 in the supernatants from HEK293 cells containing empty vector (Supplementary Fig. S6A). In contrast, the MBS2 ELISA failed to detect HtrA1 in supernatants from HEK293 cells, whether they were induced to express HtrA1 or not (Supplementary Fig. S6B). Depletion of HtrA1 from serum virtually eliminated the signal in the SCTM ELISA, indicating that this assay specifically detects HtrA1. In comparison, depletion of HtrA1 from serum resulted in a three-fold increase in the amount of HtrA1 detected in the MBS2 ELISA rather than eliminating the signal.

Subject and Donor Demographics for Serum and Vitreous Studies

Having validated the SCTM ELISA, we used an independent cohort of 248 individuals and 145 human donor eyes to assess associations between serum or vitreous HtrA1 concentrations and age, sex, AMD status, AMD severity, and genetic risk at the Chr10 locus (see the Table for detailed demographic). Out of the 248 individuals for the serum study, 145 carried no risk alleles at the Chr10 AMD locus (no risk group, GG at rs10490924), 32 had one risk allele (het risk group, GT at rs10490924), and 71 carried 2 risk alleles (homo risk group, TT at rs10490924). The median age at time of serum collection for all samples was 76, with an IQR of 10 years. The median age at time of collection did not differ significantly between genetic risk groups. There were no differences in the proportion of men and women within each group. Fifty-nine subjects (23.8%) did not present any clinical signs of AMD at the time of sample collection; 60 subjects (24.2%) had early or intermediate AMD in at least one eye, whereas 129 (52.0%) presented with neovascular AMD or geographic atrophy in at least one eye. Out of the 145 human donor eyes selected for the vitreous humor study, 125 (86.2%) carried no risk at the Chr10 AMD locus and 20 (13.8%) were homozygous for risk alleles at this locus. The median age at death for all donors was 78 (IQR = 14) and did not differ significantly between groups. The proportion of men and woman in each group did not differ significantly. Forty-six eyes (31.7%) had no AMD based on gross examination and recovered clinical history. Forty-three (30.0%) presented histopathological signs of AMD on gross examinations or were diagnosed with the disease by a clinician prior to death.

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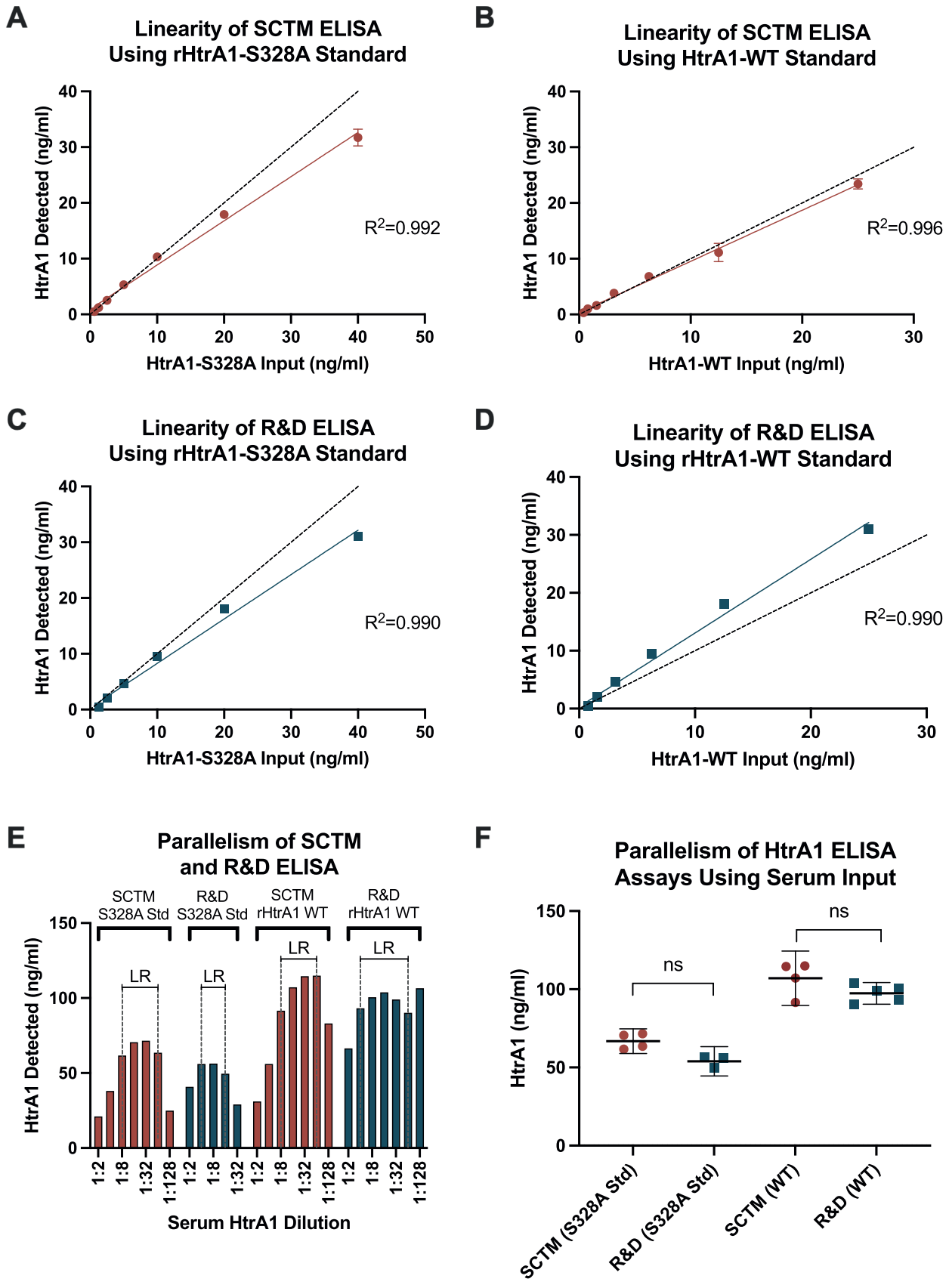

Figure 1. Linearity and parallelism of the SCTM and R&D ELISA assays using two recombinant HtrA1 standards. (**A–D**) Variation of HtrA1 detected as a function of HtrA1-S328A (**A, C**) and HtrA1-WT (**B, D**) standard input demonstrating linearity of the SCTM (**A, B**) and R&D (**C, D**) assays. (**E, F**) Parallelism of the two assays was demonstrated using serial dilutions of either recombinant HtrA1 protein in serum. (**F**) No significant differences in detected HtrA1 concentration were observed between the two assays within their respective linear range (LR).

Table. Demographic of Subjects and Donors Included in This Study

	GG at rs10490924 (No Risk)	GT at rs10490924 (Het Risk)	TT at rs10490924 (Homo Risk)	*P* Value for Comparison
Subjects				
N	145 (58.5%)	32 (12.9%)	71 (28.6%)	
Females	89 (61.4%)	19 (59.4%)	45 (63.4%)	0.9 (χ^2 = 0.16)
Smokers	47 (32.4%)	12 (37.5%)	39 (45.1%)	0.2 (χ^2 = 3.3)
Median age (IQR)	76 (11.0)	80 (8.5)	75 (10.5)	0.1 (χ^2 = 3.9)
No AMD	41 (28.3%)	9 (28.1%)	9 (12.7%)	
Early/intermediate AMD	40 (27.6%)	4 (12.5%)	16 (22.5%)	
Late AMD	64 (44.1%)	19 (59.4%)	46 (64.7%)	
Geographic atrophy	9	5	11	
Neovascular AMD	55	14	35	
Donors				
N	125 (86.2%)	–	20 (13.8%)	
Females	59 (47.2%)	–	11 (55.0%)	0.6 (χ^2 = 0.17)
Median age (IQR)	78.0 (15)		76.5 (11.5)	0.6 (χ^2 = 0.32)
No AMD	43 (34.4%)		3 (15.0%)	0.2 (χ^2 = 3.04)
AMD	36 (28.8%)		7 (35.0%)	
Indeterminate AMD status	46 (36.8%)		10 (50%)	

Variation of HtrA1 in Serum

The median concentration of HtrA1 in sera samples was 32.5 IQR = 11.9 ng.mL^{-1} (no risk 32.4 IQR = 12.5; het risk 33.7 IQR = 9.4; and homo risk 30.3 IQR = 10.2). No significant association between HtrA1 levels and age (β = −0.01 ng.mL^{-1} year^{-1}; $P = 0.9$) or sex ($P = 0.9$) were identified (Figs. 2A, 2B). Median HtrA1 levels did not differ significantly between smokers and nonsmokers ($P = 0.09$). No statistically significant differences were found between subjects with AMD (combined early/intermediate and late AMD, median HtrA1 31.9 IQR = 10.4 ng.mL^{-1}) and controls (median HtrA1 32.6 IQR = 11.8 ng.mL^{-1}, see Fig. 2C). Significant differences in HtrA1 levels were observed when stratifying samples based on AMD stage of severity (no AMD 31.9 IQR = 10.4 ng.mL^{-1}; early/intermediate AMD 30.5 IQR = 8.6 ng.mL^{-1}, or late AMD 33.7 IQR = 12.8 ng.mL^{-1}, $P = 0.03$). Pairwise comparison indicated that this difference was driven by higher HtrA1 levels in samples from patients with late AMD, as compared to individuals with early/intermediate AMD ($P = 0.03$ following Bonferroni correction, applied for comparison among the 3 independent groups with no AMD, early/intermediate AMD, or late AMD). This association was independent of Chr10 genotype (Supplementary Fig. S7). No significant interaction between genotype at the Chr10 locus and age ($P = 0.9$; Fig. 2D) or AMD status ($P = 0.3$, Fig. 2E) were identified. We found no significant associations between rs10490924 genotype and HtrA1 sera levels ($P = 0.08$; Fig. 2F).

Variation of HtrA1 in Vitreous Humor

Using the validated SCTM ELISA, we assessed associations between HtrA1 in vitreous humour (expressed per mg of total protein in the vitreous) and age, sex, AMD status, and genetic risk at the Chr10 locus among 145 samples collected from donor eyes (see the Table for detailed demographics). Vitreous humor samples were independent from the 248 serum samples used to investigate serum HtrA1 levels. The median level of HtrA1 in vitreous humour was 108.9 IQR = 112.1 ng.mg^{-1} (no risk 109.2 IQR = 113.2 and homo risk 107.1 IQR = 102.0). No significant association between HtrA1 levels and age (β = −0.1 ng.mg^{-1} year^{-1}; $P = 0.8$) or sex ($P = 0.2$) were identified (Figs. 3A, 3B). AMD status could only be ascertained for 79 eyes (63.2%) from the no risk group and 10 eyes (50%) from the homo risk group. Among these eyes, we observed no significant association between HtrA1 concentration and AMD status (no AMD 86.5 IQR = 114.2 ng.mg^{-1} and AMD 109.2 IQR = 131.1 ng.mg^{-1}; $P = 0.3$; see Fig. 3C). No significant interaction between genotype at the Chr10 locus and age was observed either (Fig. 3D). Overall, we found no significant associations between *ARMS2/HTRA1* risk status and HtrA1 in the vitreous humor (Fig. 3E).

Discussion

Using samples collected from 248 individuals and 145 human donor eyes, we showed that genetic risk at the 10q26 locus does not significantly influence the level of the HtrA1 protein in serum or in vitreous humor. The concentration of the protein in these two compartments is also independent of age, sex, and AMD status. Serum HtrA1 levels were found to be significantly higher in patients with late AMD, as compared to patients with early/intermediate AMD. However, this association was not driven by genotype at the Chr10 locus, and no significant differences were found when comparing subjects with no AMD to those with late AMD, or those with no AMD to patients with early/intermediate AMD. Altogether, these analyses demonstrate that serum and vitreous levels of HtrA1 do not reflect risk associated with the Chr10 locus or AMD onset or progression. They also support the hypothesis that alteration in *HTRA1* expression localized to the RPE, rather than systemic changes, is the main driver of elevated risk for developing AMD among individuals with risk variants at the 10q26 locus.

Few studies have assessed the effect of risk at the 10q26 locus on HtrA1 levels in serum. Consistent with our findings, no expression quantitative trait loci for *HTRA1* overlapping with AMD-associated genes have been identified in the liver.[27] Common protein expression quantitative trait loci identified for *HTRA1* in whole blood[28] are located outside of the region causal for AMD.[14,15] Elevated levels of the HtrA1 protein have been reported in the serum and plasma of patients with AMD with and without risk at the Chr10

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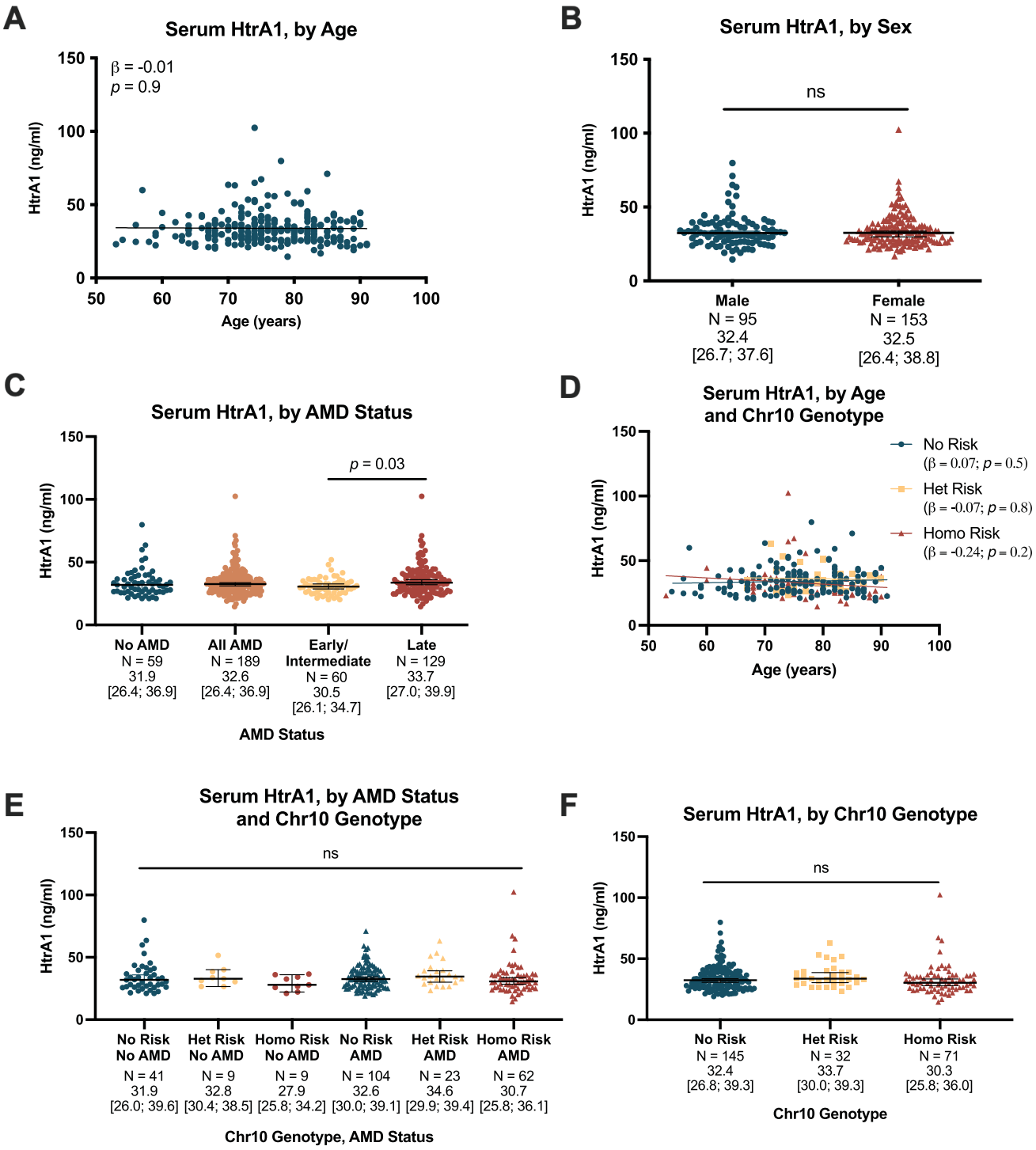

Figure 2. Association between levels of HtrA1 in serum and age, sex, AMD status, AMD severity and Chr10 genotype. In (**B, C, E, F**) sample size, median concentration, and range between the first and last quartiles are also indicated. (**A, B**) No significant associations were observed between serum HtrA1 levels and (**A**) age or (**B**) sex. (**C**) Serum HtrA1 was significantly higher in patients with late AMD as compared to subjects with early/intermediate AMD ($P = 0.03$, with Bonferroni correction applied for comparison between the three independent groups no AMD, early/intermediate AMD, and late AMD). This difference was independent from genotype at the Chr10 locus. (**D, E**) Age (**D**) or AMD status (**E**) do not significantly influence serum HtrA1 levels when stratified by genotype group. (**F**) No association between Chr10 genotype and HtrA1 level in serum was detected.

locus.[20] However, this study did not describe the validation of the assay that it relied upon (MBS2). The manufacturer (MyBioSource) provides no data to demonstrate the specificity of the MBS2 ELISA assay and our data would indicate that it is unable to detect either recombinant HtrA1 or a source of native HtrA1. It is therefore unclear which

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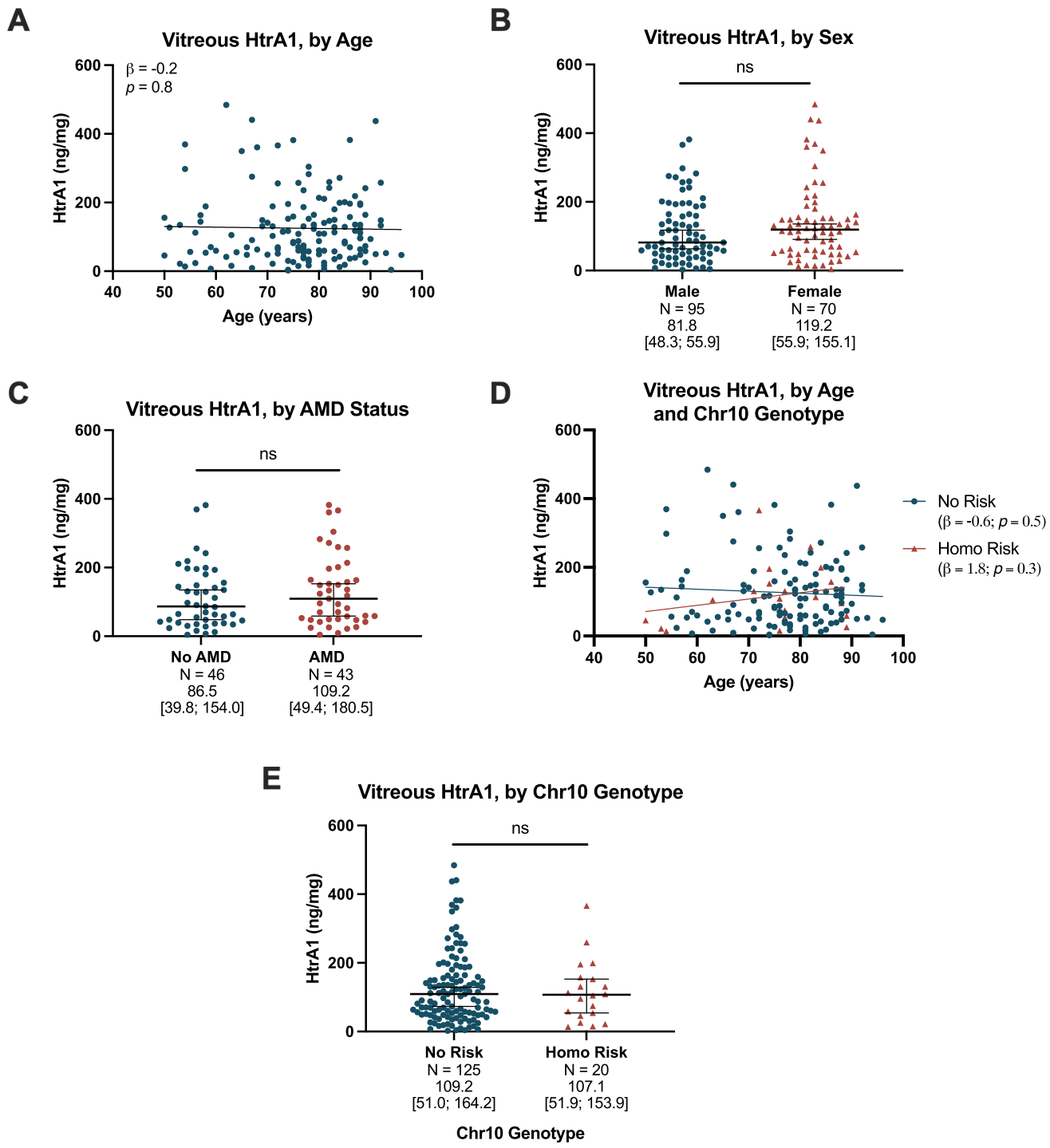

Figure 3. Association between levels of HtrA1 in vitreous and age, sex, AMD status, and Chr10 genotype. In (**B, C, E**) sample size, median concentration and range between the first and last quartiles are also indicated. (**A, B, C**) No significant associations were observed between HtrA1 level in vitreous and (**A**) age, (**B**) sex, or (**C**) AMD status. (**D**) Age does not significantly alter HtrA1 levels in vitreous humour. (**E**) No association between Chr10 genotype and HtrA1 level in vitreous was detected.

protein(s) the assay detects. As a consequence, the data reported by Pan et al. (2021)[20] should be re-evaluated with a validated HtrA1 ELISA assay, such as one using antibodies from R&D Systems, to allow for adequate comparison with our findings.

A close correlation between the concentration of proteins within the aqueous and vitreous humor has recently been demonstrated,[29] along with the existence of significant exchange between the two ocular compartments.[30] Proteomic profiling indicates that several vitreous and aqueous humor proteins are cell-specific, with an origin that can be traced to the retina or anterior segments.[30] Interestingly, vitreous humor protein levels display clear differences with serum concentrations, indicating that these two compart-

ments provide distinct biomarker information.[29] A previous report that HtrA1 protein in aqueous humor of patients with naïve choroidal neovascularization secondary to AMD is higher than in healthy controls,[19] did not determine if participants carried any risk variants at the Chr10 locus, making any comparison with our findings difficult. Additionally, our data indicate that the assay used to quantify HtrA1 (MBS1) in aqueous humor is unable to detect either of the recombinant HtrA1 proteins tested. Re-evaluation of these results with a validated HtrA1 ELISA is warranted. Whereas proteins produced by retinal cells may diffuse across the vitreous to reach the anterior chamber, proteomic profiling suggest that the HtrA1 detected in aqueous humor is likely derived from corneal stroma cells.[30] Our analysis indicates that, regardless of its origin, vitreous HtrA1 is not a suitable biomarker of risk associated with the Chr10 locus or AMD onset.

We have now shown that whereas the risk at the 10q26 locus drives lower *HTRA1* mRNA and protein in the RPE/choroid tissue, it does not influence the concentration of the protein in the retina,[15] vitreous humor, or serum. Together, these findings support the hypothesis that the dysfunction caused by risk at the Chr10 locus in AMD is localized to the RPE. The HtrA1 protein exists primarily as a trimer and is likely to be too large (approximately 150 kDa) to diffuse across BM, especially as the permeability of the membrane to large molecules decreases dramatically with age.[31] The lack of association between levels of the protein in serum and AMD susceptibility indicates that the contribution of serum HtrA1 to AMD pathobiology is marginal at best. We did observe that serum HtrA1 levels are significantly higher in patients with late AMD, as compared to patients with early/intermediate AMD; however, this association was not driven by genotype at the Chr10 locus. In addition, no differences were observed between individuals with no AMD and those with either early/intermediate or late AMD. Furthermore, we did not observe a significant difference in HtrA1 levels when accounting for both AMD severity and genotype at the Chr10 locus. A limitation of our study pertains to the comparatively smaller number of donor eyes carrying two risk alleles at the 10q26 locus. In addition, because our analyses were cross-sectional, we were unable to assess how serum or vitreous HtrA1 varies over time, or over the course of progression of AMD within the same individual. Past studies have shown that, in addition to susceptibility for developing AMD,[8,9] the risk at the 10q26 locus increases the likelihood of progressing to a late form of AMD significantly.[7,32] Longitudinal studies are necessary to fully assess the contribution of HtrA1 variation to the disease process in both serum and ocular compartments.

The 10q26 AMD locus also contains the *ARMS2* gene. Whereas evidence indicates that a *cis* regulatory region overlapping the *ARMS2* gene influences levels of *HTRA1* mRNA expression in the RPE-choroid,[15] assessing whether the Arms2 protein directly influences AMD pathogenesis has proven challenging. This is mainly due to an inability to reproducibly detect the native protein by mass spectrometry or immunoassay in serum or human eye tissues. We are therefore unable to determine whether its levels are influenced by age, gender, AMD status, or genetic risk at the 10q26 locus.

Overall, our findings suggest that therapies targeting HtrA1 to slow or prevent the progression of AMD need to act primarily on the RPE/BM interface. This study also demonstrates that systemic or ocular HtrA1 is unlikely to be a viable biomarker for activity, or indeed efficacy. Our findings may be useful to support future clinical trials targeting HtrA1. A trial exploring the potential of HtrA1 inhibition in slowing the progression of geographic atrophy was recently terminated due to high rates of intraocular inflammation and no benefits in preventing disease progression.[33] Future studies will generalize our approach to larger sample sizes across all stages of AMD severity and will seek to identify correlations in HtrA1 levels in multiple compartments in the hope of identifying surrogate markers consistent with genetic risk profile and disease onset and progression.

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