UNIVERSITY OF CALIFORNIA SAN DIEGO

CFH and HTRA1 exhibit an antagonistic effect on inhibiting oxidized phospholipids uptake in human retinal pigment epithelial cells

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by

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2019

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Table of Contents

Signature Page……..……..……iii

Table of Contents……iv

List of Abbreviations……..…… v

List of Figures……..…… vii

List of Schematics……viii

Acknowledgements……..……...……...…….....ix

Abstract of the Thesis…….…….…….…...x

Introduction……..……1

Results……..8

Discussion…….…….……18

Material and Methods……23

References……..29

List of Abbreviations

AMD	Age-related macular degeneration
ANOVA	Analysis of variance
AP	Alkaline phosphatase
ARPE 19	Acute retinal pigment epithelial-19
BM	Bruch's membrane
C3	Complement component 3
CD36	Cluster of differentiation 36
CFH	Complement factor H
CNV	Choroidal neovascularization
CRP	C-reactive protein
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
GA	Geographic atrophy
GWAS	Genome-wide association study
HEK 293	Human embryonic kidney 293 cells
HRP	Horseradish peroxidase
HTRA1	High temperature requirement serine protease A1
ml	Milliliter
ms	Millisecond
n.s.	Not significant
naLDL	Native low-density lipoprotein
ng	Nanogram

oxLDL	Oxidized low density lipoprotein
oxPL	Oxidized phospholipids
PC	Phosphatidylcholine
POVPC	1-palmitoyl-2-(5-oxovaleroyl)-phosphatidyl-choline
PR	Photoreceptor
RFU	Relative fluorescence unit
RLU	Relative light unit
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
SEM	Standard error of mean
SNPs	Single nucleotide polymorphism
TGF-B	Transforming growth factor beta 1
TIMP3	Tissue inhibitor of metalloproteinase
μg	microgram

List of Figures

Figure 1. Material validation ……10

Figure 2. Oil Red O staining of phagocytosed oxidized phospholipids……..…. 12
in ARPE19

Figure 3. ELISA analysis of HTRA1 binding affinity for LDL and CFH variants……..14

Figure 4: qPCR analysis of the effect of HTRA1 variants and oxidative stress ……..15
on CD36 mRNA expression

Figure 5. Immunostaining analysis of the effect of HTRA1 and CFH variants…….16
on CD36 protein expression

Figure 6. Western blot analysis of the effect of HTRA1 and CFH variants…….17
on CD36 protein expression

List of Schematics

Schematic 1. The structure of eye……..……..2
Schematic 2. The cross section of neural retina……...……3
Schematic 3. The AMD clinical features in cross section of neural retina……..…5

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Abstract of the Thesis

CFH and HTRA1 exhibit an antagonistic effect on inhibiting oxidized phospholipids uptake in human retinal pigment epithelial cells

by

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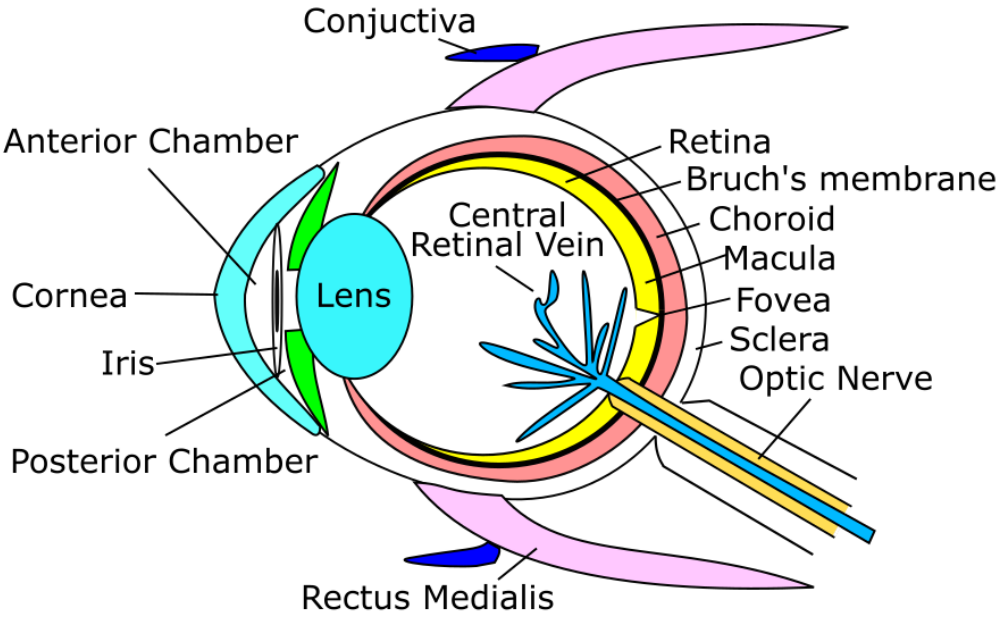
Age-related macular degeneration (AMD) is a major cause of central vision loss among the elderly. Genome-wide association studies (GWAS) have implicated polymorphisms in complement factor H (CFH) and high-temperature requirement A1 (HTRA1) to have key roles involved in the progression to advanced AMD. The complement factor H plays a key regulator role in the complement system of the innate immune defense. CFH is also found to have high affinity binding with oxidized phospholipids (oxPLs), which shields oxPLs from inducing inflammation in the eye. On the other hand, HTRA1 is a serine protease that is identified to be an

extracellular matrix homeostasis regulator and TGF-β1 pathway inhibitor. Despite the extensive studies on the two proteins' respective properties and involved pathways, the mechanism between the genetic risk factors and AMD disease pathology remains to be explored. My research project investigates the cellular interactions and molecular mechanisms of CFH and HTRA1 in an elevated oxidative stress environment. In this study, recombinant CFH and HTRA1 variants were produced for the experiments. The inhibitory effect of CFH and HTRA1 in modulating the uptake of oxidized low-density lipoprotein (oxLDL) into the retinal pigment epithelium cells (RPE) is demonstrated by Oil Red O staining. The CFH risk variant was identified to exhibit binding against HTRA1 interaction with lipoproteins *via* competitive ELISA. Furthermore, CD36 mRNA expression is found to be significantly heightened with the administration of HTRA1 or oxidative stress. However, the CD36 protein level analysis *via* immunocytochemistry and Western blot demonstrates a reduction of CD36 protein expression by proteolytically active HTRA1. We also found that CD36 protein expression is either preserved or enhanced with the addition of CFH variants. In light of this finding, we speculate CFH protects CD36 against proteolytic cleavage by HTRA1. This preliminary evidence suggests that CFH and HTRA1 have an antagonistic effect on the inhibition of oxidized phospholipids uptake in RPE cells. This finding provides a new aspect of the complement independent role of CFH in lipid metabolism which may be explored as a therapeutic treatment to AMD.

Introduction

Age-related macular degeneration (AMD) is a leading cause of visual impairment among elderly people over 60 years old worldwide [1]. As a result of an ever-aging population, AMD is a burgeoning public health problem in the United States [2, 3]. AMD is an ophthalmic disease that causes vision loss within the central part of the retina known as the macula, which is responsible for acute vision. The regions of retina affected by AMD include the retinal pigment epithelium (RPE), Bruch's membrane (BM), and photoreceptors (PR) [4]. The RPE is a monolayer of cuboidal cells that are positioned behind the PR [5]. The basal surface of RPE and BM form tight junctions that separate the retina and choroid (Schematic 1). This separation forms the blood-retina barrier, which regulates the passage of nutrients and waste between the choroid and RPE [6,7].

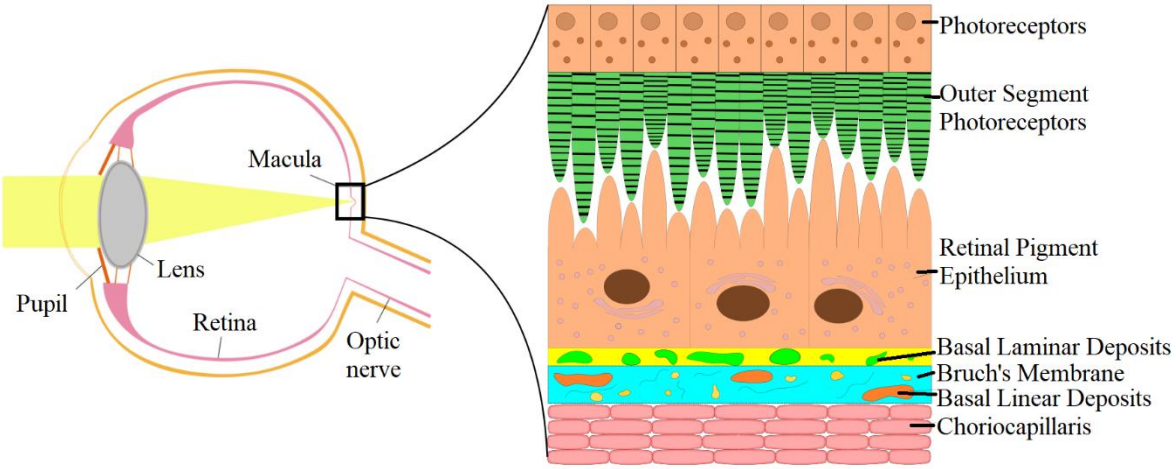


Schematic 1. This schematic displays the structure of the eye. The image is a cross section of the eye that displays the order of the retina, BM, and Choroid. Furthermore, the macula, the region most affected by AMD, is indicated.

The classification system for clinical progression of AMD has yet to be unified and established. There are two recently proposed classification systems for the progression of AMD known as the "Clinical Classification" system and the "Harmonized Three-Continent" system [8,9]. These classification systems evaluate the presence of abnormal pigmentary drusen,

diameter of drusen, area of drusen, choroidal neovascularization (CNV) and geographic atrophy (GA). Crucially, both classification systems identify the formation of large drusen to be strongly associated to the onset of advanced AMD.

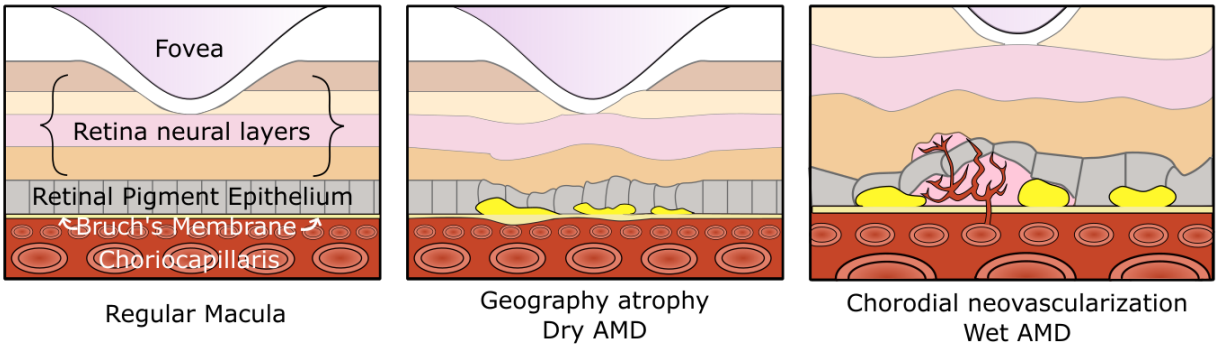
Drusen (singular: druse) are extracellular deposits that accumulate in the sub-RPE space between the RPE and BM (Schematic 2). The most common protein components of drusen are tissue metalloproteinase inhibitor 3 (TIMP3), clusterin, vitronectin, lipoproteins and serum albumin [10]. The protein components of drusen are involved in complement activation, acute inflammatory response, lipid metabolism, coagulation, fibrinolysis, and more [11]. Clinically, the three types of drusen are categorized as “hard” drusen, cuticular drusen, and “soft” drusen [12]. “Hard” and cuticular drusen are sub-RPE deposits less than 63um in diameter with well-defined edges. The cuticular drusen can group into clusters of 50 or more individual druse. “Soft” drusen are sub-RPE deposits that have less well-defined edges and are greater than 125um in diameter. The formation of these large soft drusen is strongly associated to the clinical progression of advanced AMD [13].



Schematic 2. Illustration of the retinal cross section that displays its different layers including the presence of drusen in the BM. The content of drusen forming deposits are from the oxidized cellular waste of the outer photoreceptors.

As lipid metabolism dysregulation is closely associated to drusen formation, the molecular interactions of CD36, a prominent lipid cell surface scavenger receptor, has a high possibility of impacting disease pathology. CD36 is a glycoprotein that consists of 472 amino acids and is expressed in cell types such as macrophages, platelets, microvascular endothelial cells, and adipocytes [14]. CD36 is a member of the class B scavenger receptor family that is found on the cell surface. The extracellular domain of CD36 can bind to oxidized LDL, thrombospondin, P. falciparum-infected erythrocytes, and hexarelin [15]. CD36's functional role is involved in the inhibition of neovascularization and the modulation of VEGF signaling, angiogenesis, lipid homeostasis, oxidized lipoprotein signaling, and apoptotic cell engulfment [16]. Furthermore, the internalization of oxLDL by CD36 leads to the formation of foam cells and proinflammatory reactions. The downstream cellular effects of CD36 activation with oxLDL engulfment includes the release of cytokines, macrophage migration inhibition, and transcription factor nuclear factor kB (NF-kB) pathway activation [15].

The two forms of advanced AMD are identified as "dry" and "wet". GA in the absence of CNV is the hallmark of dry AMD, which is characterized by cell death in the RPE layer and degeneration of overlying photoreceptors [17]. CNV in either the presence or absence of GA is the hallmark of wet AMD, which is characterized by the growth of blood vessels from the choroid into the avascular RPE and neural retina [18]. The poorly formed vasculature from CNV causes leakage of blood and fluid into retinal space, which leads to loss of vision (Schematic 3).



Schematic 3. Simplified illustration of the advanced AMD pathology. In dry AMD, the drusen deposits cause GA that induces localized cell death and visual loss. While wet AMD exhibits CNV caused by inflammation and angiogenesis at drusen sites crossing Bruch's Membrane into retinal space and leading to hemorrhages and loss of vision.

Progression to advanced AMD is associated with multiple environmental and genetic risk factors. The environmental risk factors associated with AMD include activities and surroundings that stimulate oxidative stress in the retina, such as cigarette smoking and sunlight exposure [19]. Since phototransduction is a metabolically demanding process, the retina's PR outer segment requires large amounts of energy. The energy demand is met by ATP production in mitochondria, which is fueled by oxygen metabolism. The high oxygen supply concentration from choroidal vasculature in conjunction with high ATP production by mitochondria results in the release of reactive oxygen species (ROS) into the retinal space. This increased production of ROS and free radicals due to heightened oxidative stress in the retina contributes to drusen formation or inflammation [20, 21]. The unsaturated phospholipids in PR outer segment are transformed into oxidized phospholipids (oxPLs) by ROS. Phosphatidylcholine (PC) is a common group of phospholipids. When oxidized, PC forms oxygen double bonds and transforms into 1-palmitoyl-2-(5-oxovaleroyl)-phosphatidyl-choline (POVPC) which may be identified as a foreign antigen by the immune system. As phospholipids have low aqueous solubility, in plasma they typically coexist with proteins in the form of lipoproteins. The oxPLs-carrying lipoproteins are transported by retinosomes to the RPE for intracellular metabolism or transfer to systemic circulation. However, with aging, the transportation between choroid and RPE reduces in

efficiency as the BM's permeability is decreased. This results in lipid and protein accumulation in the subretinal space, forming drusen [22]. The lipids and proteins that accumulate are likely to become further oxidized which promotes inflammation in the retina. Chronic inflammation in the retinal space further triggers signaling pathways leading to an increase in expression of pro-angiogenic factors, which fuels a cycle of degeneration within RPE and choroid that may induce CNV [23].

Numerous genetic risk factors have been associated with AMD, including fifty-two single-nucleotide polymorphisms (SNPs) at thirty-four susceptibility loci [24]. Two of the major susceptibility loci account for more than half of AMD's heritability: variants at chromosome 1q31 for complement factor H (CFH), and variants at chromosome 10q26 for HTRA1 [25]. These two gene products are the focus of our study.

HTRA1 is a member of the high temperature requirement factor family of chymotrypsin-like proteases [26]. HTRA1 is known to be a key regulator in the extracellular matrix (ECM) turnover by regulation of TGF-β1 and degradation of ECM proteoglycan [27-31]. However, the molecular mechanism by which HTRA1 influences AMD pathogenesis is still unclear. Overexpression of HTRA1 caused by SNPs within its promoter is a genetic risk factor for AMD. [32- 34]. HTRA1 variant SNP rs267598 has shown correlation to wet AMD with increased transcriptional activity of HTRA1 promoter [33]. The SNP rs11200638 results in a single nucleotide substitution from glycine to alanine within the promoter region that affects the HTRA1 serum response factor and transcription factor AP2a binding site [35]. The elevated expression of HTRA1 results in the degradation of BM proteins, which promotes choroidal blood vessel angiogenesis penetration of the more permeable BM into retinal space [25, 36]. The two high-risk SNPs of HTRA1 rs1049331 and rs2293870 are located within the coding region of

HTRA1 and produce variants with different enzyme activities. These genotypes increase the risk of developing wet AMD 10 times exceeding the wild-type genotype [37, 38]. As these HTRA1 SNPs are identified to be major risk factors of AMD, the functionality and quantity of HTRA1 protein is suggested to be significant in AMD pathogenesis.

CFH is a regulatory element of the complement pathway. CFH along with many other complement pathway related proteins such as C-reactive protein (CRP), complement component 3 (C3), complement regulatory proteins CFI are found densely in drusen [39-41]. SNPs in the CFH gene on chromosome 1q31 significantly increase risk for developing advanced AMD [24, 42- 44]. The CFH variant rs1061170 is a result of an SNP at the peptide position 402 with a tyrosine-to-histidine substitution [45]. Peptide position 402 on CFH is located at the C-reactive protein and glycosaminoglycan binding site. Changes at this position may alter ligand-binding properties of CFH [46]. The CFH 402H polymorphism is found to have reduced CFH binding to heparan sulfate in Bruch's membrane, oxidized phospholipids, or C-reactive protein [47]. Furthermore, it has been demonstrated that CFH binding affinity to oxLDL is correlated to decreased lipid uptake by cell [48].

Through patient studies, the joint effect of CFH and HTRA1 single-locus gene variants were found to be significant regarding wet AMD pathogenesis [49]. In cases of advanced AMD, CFH and HTRA1 are found to be colocalized densely in drusen [50]. HTRA1 protein expression is also upregulated in abnormal RPE cells surrounding drusen and within the choroidal neovascular structure in wet AMD [51]. The colocalization of these two AMD relevant proteins suggests possible molecular interactions that may play significant roles in AMD pathogenesis.

Results

Recombinant HTRA1 and CFH were produced for our experiments. HTRA1 wild-type and catalytically inactive S328A variants were produced in transformed DH5alpha *E. coli* cells. CFH wild-type and Y402H variants were produced in transfected HEK 293 cells. The protein sizes were confirmed *via* Western blot. The HTRA1 variants contain a polyhistidine tag, whereas the CFH variants contain an Alkaline phosphatase (AP) tag. The presence of a polyhistidine tag or AP tag on the recombinant proteins was confirmed *via* direct ELISA (Figure 1a-b). The presence of phosphoryl choline on oxidized phospholipids in oxLDL were affirmed with TEPC 15 *via* ELISA (Figure1c). Furthermore, the proteolytic activities of HTRA1 variants were quantified using protease activity assay, as indicated in (Figure 1d). Crucially, the HTRA1 S328A variant is demonstrated to have low proteolytic activity, whereas wild type HTRA1 is demonstrated to have high catalytic activity.

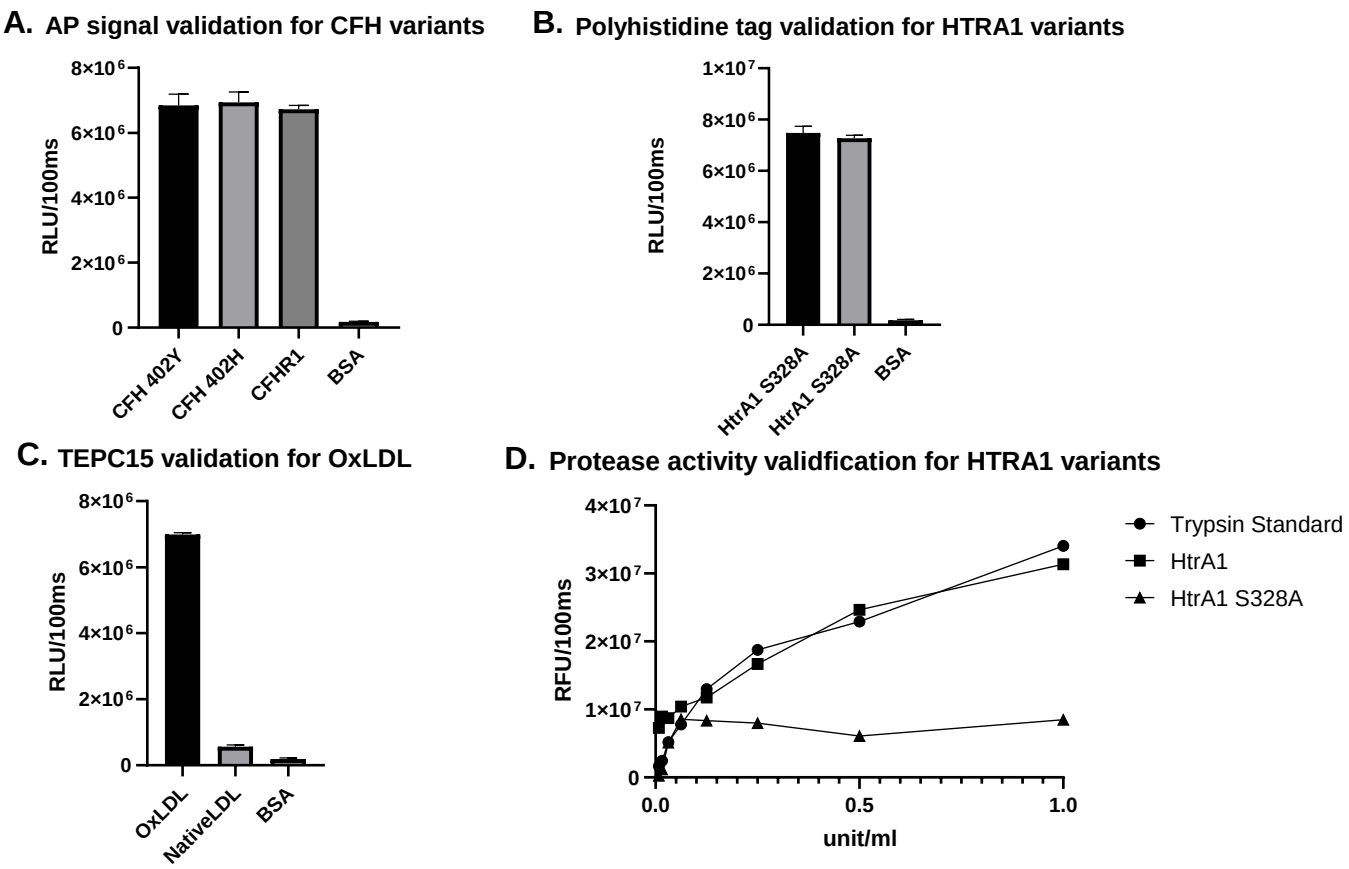


Figure1. Material validation of rCFH, rHTRA1 and oxLDL. The recombinant CFH variants were validated with AP signal detection via direct ELISA (A). The recombinant HTRA1 variants were validated with poly-histidine detection via direct ELISA (B). The oxLDL were validated with TEPC 15 detection of POVPC via direct ELISA (C). Furthermore, the protease activity of HTRA1 and HTRA1 S328A were assessed with protease activity assay (D). Detection signals were measured in RLU/100ms. Results are shown as mean ± SEM, N=3 for each sample group.

As phospholipid accumulation in drusen and lipid-mediated inflammatory responses are highly related in AMD, we investigated the effects of HTRA1 and/or CFH to lipids accumulation in cultured ARPE19 cells with *in vitro* incubation of native or oxLDL in combination with HTRA1 or CFH variants. The plate is seeded with ARPE19 cells and treated with a 25 μg/ml concentration of each sample combinations: naLDL, oxLDL alone, oxLDL + HTRA1 S328A, oxLDL + HTRA1, oxLDL + CFH 402H, oxLDL + CFH 402H + HTRA1, oxLDL + CFH 402Y, oxLDL + CFH 402Y + HTRA1. The average lipid droplet vesicles' pixel area size per cell were quantified after Oil red O staining. (Figure 2a). Compared to oxLDL alone treated control, the HTRA1, CFH variants alone, and HTRA1+CFH variant combination treated groups are demonstrated to significantly inhibit oxidized phospholipids uptake in ARPE cells. Further, CFH 402Y treated groups are demonstrated to have significantly stronger oxidized phospholipid uptake inhibition strength compared to CFH 402H treated groups. In addition, HTRA1 S328A is shown to not have a significant difference in oxidized phospholipid uptake in comparison to control. A representative Oil red O staining image for each sample group is also provided (Figure 2b-I).

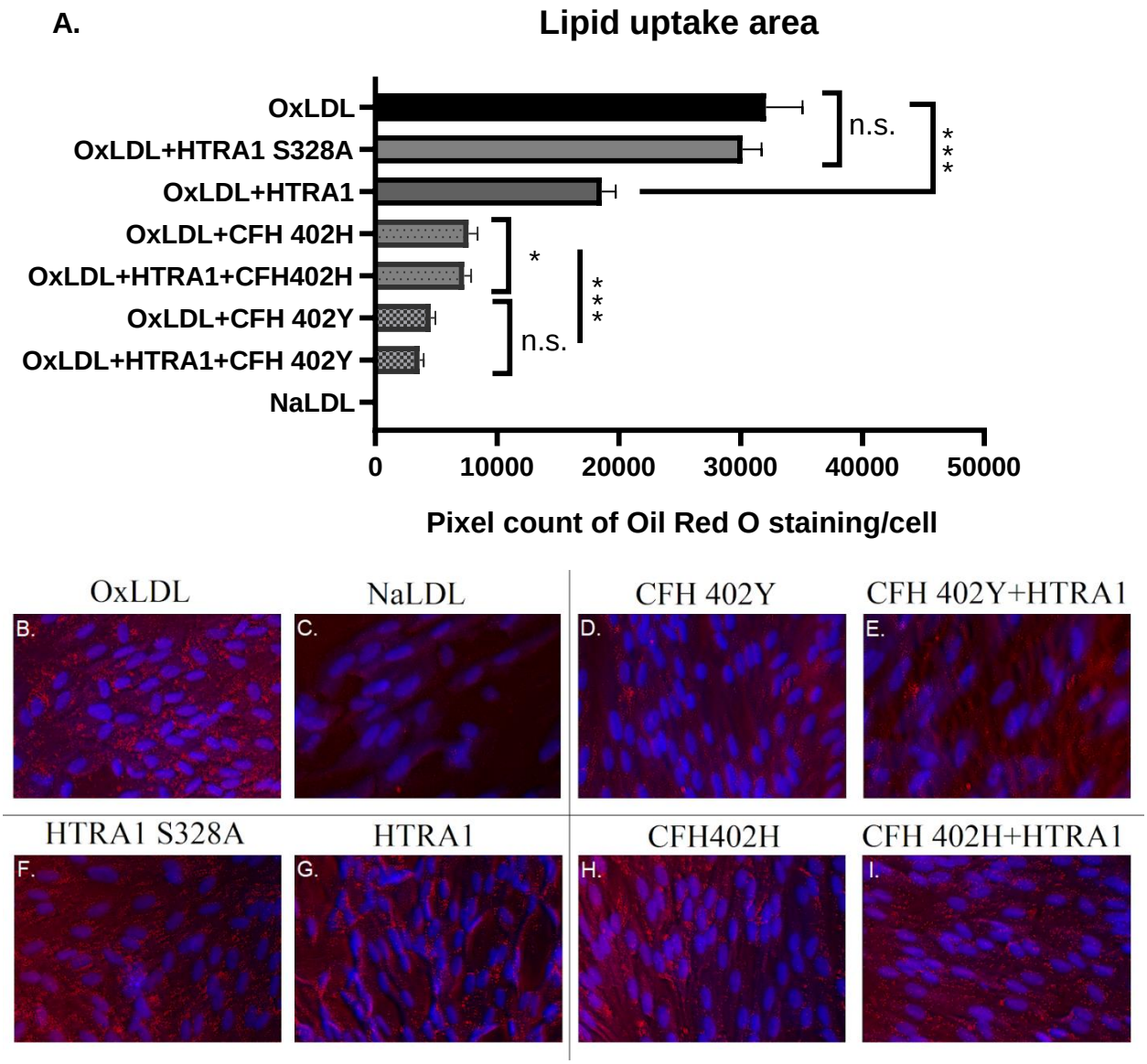


Figure 2. ARPE-19 cells were seeded and incubated for 48 hours with 25 µg/ml naLDL, or 25 µg/ml oxLDL alone, or oxLDL plus 25 µg/ml of HTRA1 S328A, or oxLDL plus 25 µg/ml of HTRA1, or oxLDL plus 25 µg/ml of CFH 402H, or oxLDL plus 25 µg/ml of CFH 402H and HTRA1, or oxLDL plus 25 µg/ml of CFH 402Y, or oxLDL plus 25 µg/ml of CFH 402Y and HTRA1. At the end of incubation, the cells were fixed and stained with Oil Red O. The red labeled lipid droplets were qualified for positive Oil Red O staining. Results are shown as pixel count of stained lipid droplets/cell (mean ± SEM, N=10 for each sample group, n.s.= non-significant, $^{*}P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$).

Competitive ELISA was performed to identify molecular interactions between CFH and HTRA1 variants in presence of oxLDL, naLDL, or BSA. In the oxLDL, naLDL, and BSA coated plates, HTRA1 binding affinity to coating antigen with CFH 402H competition binding is found to be significantly lower than HTRA1 only group (Figure 3a-c). In contrast, the competition of CFH 402Y has less impact on HTRA1 binding affinity to the coated antigens. The percent bound calculation is visualized in Figure 3d, where B/B0 calculation is drawn: B0 being the HTRA1 only group, B being the HTRA1 with CFH variant competition group. The percent bound of HTRA1 to oxLDL is shown to have a significance difference between the two CFH variants competition; CFH 402H variant competition with HTRA1 is shown to be having a significant inhibiting effect to HTRA1 in binding to both oxLDL and naLDL. To investigate the HTRA1 binding affinity to CFH variants, direct ELISA was performed to detect protein conjugation of HTRA1 to coated antigens (Figure 3e). HTRA1 found to have significantly higher binding affinity to oxLDL and naLDL. As to CFH variants, HTRA1 has a level of binding affinity similar to BSA.

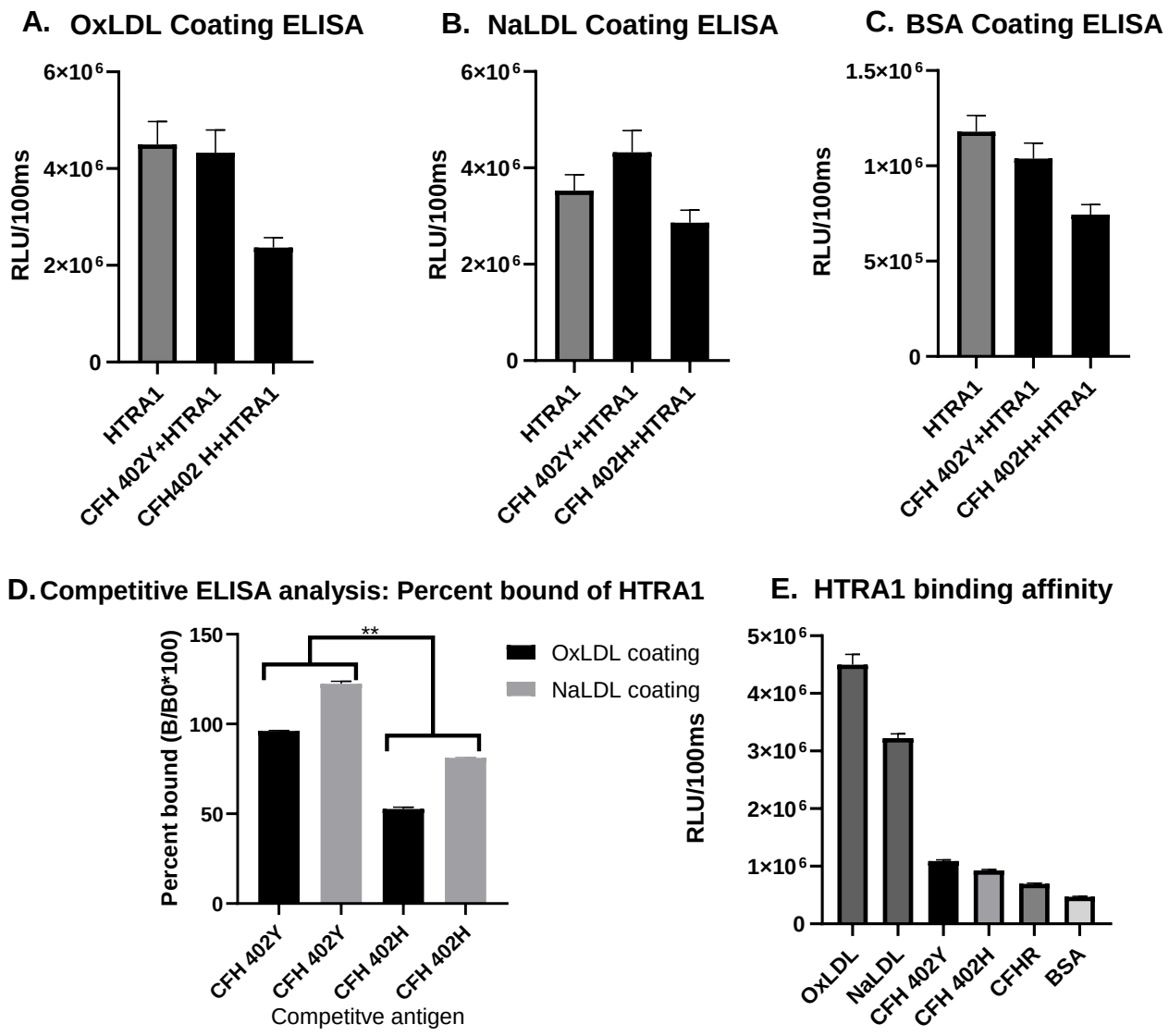


Figure 3. Relative binding property of HTRA1. The binding of recombinant protein HTRA1 to naLDL, oxLDL, or BSA antigens in competition with CFH 402Y or CFH 402H was measured with ELISA using RLU (A-C). Data are expressed in RLU and as mean ± SEM. N = 4 for each sample point. Relative binding property of HTRA1 to oxLDL, naLDL, CFH 402Y, CFH 402H, CFHR1, or BSA. The binding of recombinant protein HTRA1 to antigens was measured using RLU (D). Data are expressed in b/b0*100 percentage and as mean ± SEM. N = 4 for each sample point, $^{*}P < 0.05$ $^{**}P < 0.01$. The binding of recombinant protein HTRA1 to antigens was measured using RLU Relative bound percentage of HTRA1 to oxidized phospholipids in competition with rCFH 402Y or rCFH 402H (E). Data are expressed in RLU and as mean ± SEM. N = 3 for each sample point.

The gene expression assay was performed to identify the effects of oxidative stress or HTRA1 on CD36 mRNA expression (Figure 4). The naLDL and HTRA1 S328A treated group had no significant CD36 mRNA expression difference from the control group. On the other hand, oxLDL+HTRA1, oxLDL+HTRA1 S328A, oxLDL alone, and HTRA1 alone treated groups displayed significant increases in CD36 mRNA expression relative to control. Further, the oxLDL+HTRA1 group was analyzed to have significantly higher CD36 gene expression than all other groups.

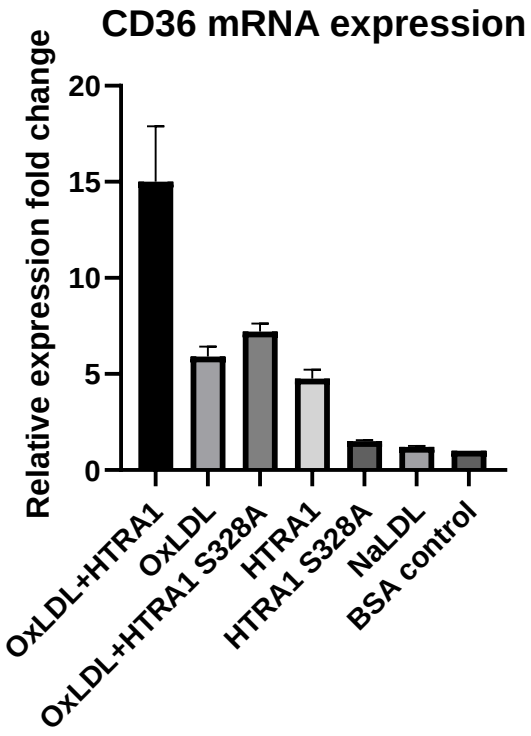


Figure 4. Gene expression was assayed by quantitative PCR on mRNA from ARPE19 cells treated with 50 μg/ml of naLDL, oxLDL or oxLDL pre-incubated with the 25μg/ml of recombined HTRA1, HTRA1 S328A for 18 hours, HTRA1 alone, or HTRA1 S328A alone. Relative mRNA levels of indicated genes were calculated by normalizing results with GAPDH and are expressed relative to untreated samples. Results are shown as mean ± SEM. N = 6.

The protein expression of CD36, following the treatments with HTRA1 variants alone or HTRA1 and CFH variants combination, was assessed with immunocytochemistry (Figure 5). Post-24 hours stimulation, each sample group was stained. The fluorescence signals from staining were quantified with a plate reader. Relative to the control, the wild-type HTRA1 treated group had significantly less CD36 protein expression. On the other hand, the HTRA1 S328A, HTRA1 + CFH 402H and HTRA1 + CFH 402Y treated groups were observed to have relatively similar CD36 protein expression level to the control.

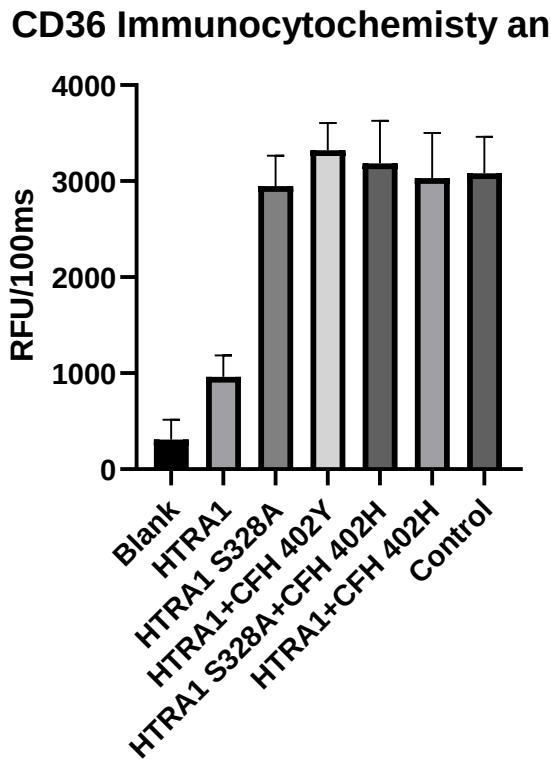


Figure 5. CD36 protein level was assayed by immunocytochemistry from ARPE19 cells treated with 50 μg/ml of naLDL, oxLDL or oxLDL pre-incubated with the 25μg/ml of recombined HTRA1, HTRA1 S328A for 18 hours, HTRA1 alone, or HTRA1 S328A alone. Data of relative protein expression level of CD36 were expressed in RFU and as mean ± SEM. N = 3 for each sample point.

To affirm the results from the immunocytochemistry, we performed a Western blot analysis to observe the *in vitro* CD36 protein expression following the treatments with HTRA1 variants alone or HTRA1 and CFH variants (Figure 6a). Relative to the control, the wild-type HTRA1 treated cells were observed to have less CD36 protein expression. In contrast, the HTRA1 S328A non-proteolytic activity variant treated group displayed a similar quantity of CD36 to the control. The HTRA1 + CFH 402H and HTRA1 + CFH 402Y treated groups are both observed to have increased fold of CD36 protein expression compared to control (Figure 6b).

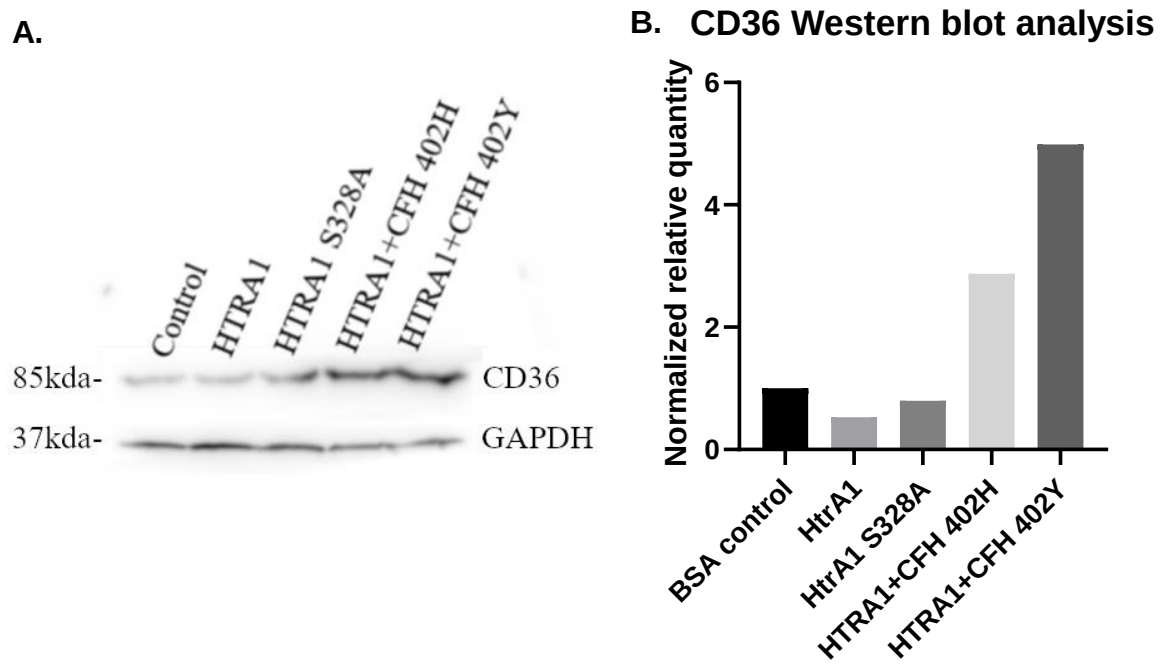


Figure 6. CD36 protein levels were assayed by Western blot from ARPE19 cells treated with 50 μg/ml of naLDL, oxLDL or oxLDL pre-incubated with the 25μg/ml of recombined HTRA1, HTRA1 S328A for 18 hours, HTRA1 alone, or HTRA1 S328A alone. Data of relative protein expression levels of CD36 were calculated by normalizing pixel density results with GAPDH and are expressed relative to untreated samples.

Discussion

AMD is a multifactorial chronic neurodegenerative disease that involves both genetic and environmental factors. The oxidative stress from the environment and the genetic SNP risk factors on susceptible loci contribute to the formation of drusen and the downstream inflammatory responses, which are both hallmarks of advanced AMD progression. In this study, we examined the interplay between oxidative stress and the two most significant genetic risk factors associated with AMD, HTRA1 and CFH.

With the recombinant HTRA1 and CFH variants made, we first examined the impact of the two proteins on lipid metabolism in human RPE cells. With administration of oxLDL with HTRA1 or CFH variants combination in ARPE 19 cells, we found that both HTRA1 and CFH variants can reduce oxidized phospholipid uptake. The contrast of the staining results in HTRA1 S328A treatment groups uncovered that the protease activity of HTRA1 is essential for the lipid uptake inhibition. Further, the genetic risk variant CFH 402H demonstrated weaker strength in oxPL uptake inhibition compared to the wild-type CFH 402Y. The data suggests both CFH and HTRA1 play a role in lipid uptake inhibition, which may regulate or mediate the lipid metabolism of the RPE cells surrounding drusen.

As the CFH risk variant reduces inhibition of oxLDL uptake compared to the non-risk variant, the inability of the cell to adequately process oxLDL is hypothesized to initiate the chronic inflammatory response in late AMD. Crucially, we have shown previously that uptake of oxLDL results in increased expression of IL-6, IL-8, MCP, CD36 and VEGF, which is a key mediator of choroidal neovascularization.

Next, competitive ELISA was conducted to assess the molecular interaction between HTRA1 and CFH variants in presence of oxidized phospholipids. While CFH 402Y demonstrated no significant competitive binding, CFH 402H is found to be inhibiting HTRA1

binding to oxLDL. HTRA1's binding affinity to CFH variants alone is found to be non-significant in comparison with control BSA. The CFH 402H's property in inhibiting HTRA1's binding to oxLDL may contribute to its weaker strength in oxPL uptake inhibition.

As lipid uptake is found to be reduced with the administration of HTRA1 and CFH variants, we further investigated CD36 in relation to HTRA1 and CFH. CD36 is a known transmembrane lipoprotein receptor that holds critical role in lipid metabolism. The mRNA and protein expression of CD36 in response to the HTRA1 or CFH variant treatment combinations were assayed. Through gene expression assay, HTRA1 was found to be stimulating CD36 mRNA expression. The combination of oxidative stress from oxLDL and HTRA1 treatment yielded a highly significant CD36 mRNA expression fold increase.

Through Western blot and immunocytochemistry, we observed decreased membrane expression of CD36 after treatment with HTRA1. This seemingly contradicted our mRNA expression data, which indicated *increased* expression of CD36 after treatment with HTRA1. We propose that this may result from HTRA1 cleaving the extracellular domain of CD36, which is recognized by the anti-CD36 <SR-B3> that we used for our blotting assays. Proteolytic cleavage may occur *anywhere* within the CD36 extracellular domain as long as cleavage results in a conformational change within the antibody-binding motif recognized by anti-CD36. If this is true, the decreased membrane expression of CD36 observed *via* Western blot and immunocytochemistry is an experimental artifact of proteolytic cleavage of the CD36 extracellular domain.

This hypothesis is indirectly supported by lipid uptake assays. We found that administration of oxLDL and HTRA1 results in less lipid uptake compared to administration of oxLDL alone. In conjunction with the Western blot and immunocytochemistry data, this suggests

that HTRA1 may inhibit lipid uptake *via* proteolytic cleavage of the CD36 extracellular domain, the same domain that is recognized by the anti-CD36. Interestingly, administration of oxLDL, HTRA1, and CFH results in less lipid uptake compared to administration of oxLDL and HTRA1. However, Western blotting and immunohistochemistry experiments indicate that there is *more* membrane expression of CD36 following administration of HTRA1 and CFH compared to administration of HTRA1 alone. More CD36 expression would presumably result in *more* lipid uptake, not less. We propose that there is less lipid uptake in the presence of CFH because CFH binds to the extracellular domain of CD36. This binding inhibits *both* proteolytic cleavage by HTRA1 *and* cellular uptake of oxLDL.

This two-pronged effect of CFH on the RPE-surface CD36 has important clinical implications. As people age, serum levels of CFH decreases [52]. Lower levels of serum CFH imply that less CFH is available not only to inhibit RPE uptake of oxidized products from the sub-RPE space, but also to protect CD36 from HTRA1-mediated proteolytic cleavage. Our *in vitro* experiments indicate that the *net* effect of decreasing levels of CFH is an *increase* in uptake of oxidized products. This effect persists even though CD36 de-protection (i.e. by less CFH binding) would presumably make the CD36 extracellular domain more accessible to HTRA1-mediated cleavage. Based on this observation, we propose that CD36 has a higher affinity for oxLDL compared to HTRA1.

This proposal also aligns with the known cellular effect of HTRA1 risk variant. As mentioned in the introduction, HTRA1 risk variant can result in 2-3 folds increase in HTRA1 expression [32]. It has been previously proposed that increased HTRA1 expression promotes progression to advanced AMD by cleaving the extracellular domain of RPE surface proteins and decreasing the integrity of Bruch's Membrane [25]. In addition to these effects, we propose a

regulatory effect of HTRA1 on AMD pathogenesis. As HTRA1 expression increases, the cleavage of RPE surface proteins is transduced to increase the transcriptional expression of CD36 *via* an unknown signaling mechanism. Increased expression of CD36 increases the capacity of RPE to uptake extracellular oxidized products. As sub-RPE oxidized deposits are phagocytosed into the RPE, apoptotic pathways are activated, resulting in cell death. Hence, decreased CD36 protection by CFH 402H and increased HTRA1 expression by risk haplotype would have the combined effect of increasing oxidized product uptake and promoting RPE death.

The biochemical mode by which HTRA1 may prevent lipid uptake is still under investigation. However, in this study, we present preliminary evidence of lipid cell surface receptor CD36 cleavage by HTRA1, thereby preventing lipid uptake into the RPE. Though CFH variants also contribute to the oxPL uptake inhibition, they present an antagonistic effect against HTRA1 in CD36 protein regulation. Additional investigation will be necessary to completely elucidate the role of the CFH - HTRA1 - oxLDL triad in extracellular lipid accumulation and association to AMD pathology. With further understanding of the lipid metabolism pathways and mechanisms in retinal space, the progression of AMD may be fully uncovered and possibly halted by the prevention or removal of oxPL deposits during drusen formation.

Material and Methods

Preparation of native and oxidized lipoprotein. Because phospholipids have low aqueous solubility, in plasma they typically coexist with proteins in the form of lipoproteins, such as ApoB in LDL. We thus chose LDL as a carrier for native phospholipids and oxidized phospholipids in this study. OxLDL was generated by incubating 1mg/ml human plasma low-density lipoprotein (Lee BioSolutions) with an oxidation agent (10 μM CuSO4) for 24h at 37 °C; native phospholipids on the surface of LDL were oxidized into oxPL. Native (unoxidized) phospholipids on naLDL were used as a control. After preparation, both naLDL and oxLDL were dialyzed with PBS containing 0.27 mM EDTA to prevent further oxidation.

Preparation of recombinant CFH. cDNA encoding alkaline phosphatase-ligand fusion human CFH402Y or CFH402H was subcloned into a pRK5 mammalian expression vector. The constructs then were transfected into HEK293 cells using lipofectamine 3000 (). The medium containing secreted CFH402Y or CFH402H protein was harvested and concentrated using Amicon Ultra (Millipore).

Preparation of HTRA1 variants. cDNA encoding Poly-histidine tagged human HtrA1 or HtrA1S328A was subcloned into pGEX bacterial expression vector. The constructs then were transformed into DH5alpha competent cells. Twelve hours post IPTG induction, the transformed DH5-alpha cell was centrifugally collected then lysed by sonication. A glutathione sepharose (Biovision) based affinity chromatography was performed on the lysate to purify and extract the recombinant HTRA1 protein variants which consists GST tag. The harvested medium containing HtrA1 or HtrA1S328A protein was purified using High Capacity Endotoxin Removal kit (Peirce).

Recombinant protein quantification. The recombinant proteins were quantified using micro BCA assay. The protein markers (alkaline phosphatase for CFH variants or poly-histidine

for HTRA1 variants) were affirmed by performing Direct ELISA. The molecular weight was confirmed with SDS-gel electrophoresis and Western blotting. Further, the protein catalytic functions of HtrA1 variants was confirmed with protease activity assay.

Protease activity assay. The protease activity of recombinant HTRA1 variants were assessed by Pierce™ Protease Activity Assay Kit 23266. Detailed protocol of the protease activity assay provided by Pierce™. We used the white plate and FRET plate reading to measure the relative fluorescence levels emitted from the protease activity. A trypsin standard was used as control.

Tissue Culture. ARPE-19, a spontaneously arising human RPE cell line that expresses the RPE-specific markers CRALBP and HEK293 human embryonic kidney cells, was obtained from the American Type Culture Collection (CRL-2302; ATCC, Manassas, VA). ARPE-19 and HEK293 cells were cultured in Dulbecco's modified Eagle's medium-F12 (DMEM/F12), supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified chamber with 5% CO2 to 80% confluency for stimulation.

Stimulation of RPE cells. Human ARPE-19 cells (ATCC CRL2302) were grown in DMEM/F12 (1:1) plus 10% FCS until visually confluent at 80%. After serum starvation in DMEM/F12 (1:1) plus 0.1% BSA for 24 h, cells were stimulated with naLDL, oxLDL, oxLDL plus HTRA1 S328A, oxLDL plus HTRA1, oxLDL plus CFH 402H, oxLDL plus CFH 402H and HTRA1, oxLDL plus CFH 402Y, or oxLDL plus CFH 402Y and HTRA1 for 48 hours (25 μg/ml for all material). The collected cell lysate was either further processed for gene expression assay or western blotting.

Oil-Red O staining. Lipid accumulation in ARPE-19 cells was assessed in vitro. ARPE-19 cells were resuspended into 0.5 ml of DMEM containing 20% FCS and seeded to each well of a 12-well culture plate. The following day, naLDL, oxLDL, or oxLDL plus HTRA1 S328A, oxLDL plus HTRA1, oxLDL plus CFH 402H, oxLDL plus CFH 402H and HTRA1, oxLDL plus CFH 402Y, or oxLDL CFH 402Y and HTRA1 was added to the medium and extended the incubation for another 48 hours (25 μg/ml for all material). After 48 hours of incubation, the cells were fixed and stained with Oil red O to assess lipids uptake [53]. Immediately after staining, the stained results were imaged and quantified for the pixel area of lipid droplets/cell was counted using ImageJ.

Real Time quantitative PCR. Total RNA was extracted from the cell lysates using Direct-zol™ RNA MiniPrep (ZYMO research, Irvine, CA). The RNA extract was converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem). The converted cDNA was used as template for qPCR experiments using Power SYBR Green qPCR Master Mix from Applied Biosystems (Foster City, CA) with primer sets for CD36. Relative mRNA levels were normalized with GAPDH. The relative gene expression was quantified from qPCR results using the 2^(−ΔΔCT) method with the following formula:

ΔCT = CT (a target gene) – CT (a reference gene).
$\Delta\Delta CT = \Delta CT$ (a target sample) – ΔCT (a reference sample)
Fold Change= $2^{-\Delta\Delta CT}$

Primer: Human CD36

FW CAGAGGCTGACAACTTCACAG
BW AGGGTACGGAACCAAACTCAA

Immunocytochemistry. ARPE-19 cells were resuspended into 0.5 ml of DMEM containing 20% FCS and seeded to each well of a 6-well culture plate. The following day, 25 μg/ml naLDL, 25 μg/ml oxLDL, 25 μg/ml of HTRA1 S328A, 25 μg/ml of HTRA1, 25 μg/ml of

CFH 402H and HTRA1, or 25 μg/ml of CFH 402Y and HTRA1 was added to the medium and extended the incubation for 24 hours. After 24 hours of incubation, the cells were fixed and stained with rabbit source anti-CD36 (Novus Bio) and anti-rabbit IgG Alexa flour 488 (Cell signaling).

Western blot. Cellular lysate was prepared using RIPA lysis buffer with phosphatase and protease inhibitors (Cell Signaling Tech.) and quantified for total protein by BCA assay with a standard curve generated using a BSA standard (Thermo Scientific). Same amount of protein samples was resolved by SDS-PAGE then transferred onto PVDF membrane, and probed using antibodies against polyhistidine for HTRA1 detection, or CD36. The HRP conjugated anti-Rabbit IgG was used as secondary antibody followed by SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific). Anti-GAPDH was used for an internal control. ImageQuant LAS 4000 (GE health) was used for signal quantification.

Direct ELISA. Direct Elisa was performed for material validation of CFH variants, HTRA1 variants and oxLDL. The proteins or lipoproteins (2 μg/ml) were coated in bicarbonate buffer on immunograde white plates at 4 °C overnight. then detected with responding antibody. Alkaline phosphatase substrate on CFH variants were detected by Lumiphos 530 (ThermoFisher Scientific). The poly histidine tag on HTRA1 variants were verified by HRP-linked anti-poly histidine (BD Bioscience). The POVPC in oxPL were verified by TEPC 15 (Signa-Aldrich). The HRP-linked anti rabbit IgG antibody (BioLegend) was used as secondary antibody. The HRP linked antibodies were excited with SuperSignal ELISA Pico Substrate at room temperature. Filter Max 3 plate reader was used to detect the luminescence signal expressed as RLU/100ms.

Competitive ELISA. For competitive ELISA, the lipoproteins (5 μg/ml) were coated in bicarbonate buffer on immunograde white plates (Thermal Fisher) at 4 °C overnight. After aspirating the coating solution, the plate was blocked in blocking buffer (PBS with 1% BSA) at 37°C for 2 hours followed by three washes with washing buffer (PBS, 0.05%). The HTRA1 or CFH variants combination samples of 10 μg/ml were prepared in dilution buffer (PBS, 0.05% 0.1% BSA). The diluted samples were then added to the wells and incubated in 37°C for 30 minutes. After three washes, the anti-rabbit HRP-linked anti poly-his antibody (BD Bioscience) of 2μg/ml in dilution buffer was added and incubated at room temperature for 2 hours. After four washes, the plate was incubated with SuperSignal ELISA Pico Substrate at room temperature and the luminescence was measured in relative light units using plate reader.

Statistical analysis. The data gathered from experiments were analyzed by analysis of variance (ANOVA) post-hoc t-test to identify the statistical significance between groups.

This thesis, in part is currently being prepared for submission for publication of the material. Lin, Victor; Shaw, Peter. The thesis author was the primary investigator and author of this material.

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