The Regulation of AMD Pathobiology by Complement Factor H

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Cell Biology in the Graduate School of Duke University

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ABSTRACT

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Abstract

Complement factor H (CFH) is a major susceptibility gene for age-related macular degeneration (AMD); however, its impact on AMD pathobiology is unresolved. Here, the role of CFH in the development of AMD pathology in vivo was interrogated by analyzing aged Cfh+/- and Cfh-/- mice fed a high fat, cholesterol-enriched diet. Strikingly, decreased levels of CFH led to increased sub-retinal pigmented epithelium (RPE) deposit formation, specifically basal laminar deposits, following high fat diet. Mechanistically, our data show that deposits are due to CFH competition for lipoprotein binding sites in Bruch’s membrane. Interestingly and despite sub-RPE deposit formation occurring in both Cfh+/- and Cfh-/- mice, RPE damage accompanied by loss of vision occurred only in old Cfh+/- mice. We demonstrate that such pathology is a function of excess complement activation and C5a production, associated with monocyte recruitment, in Cfh+/- mice versus complement deficiency in Cfh-/- animals. Due to the CFH dependent increase in sub-RPE deposit height we interrogated the potential of CFH as a novel regulator of Bruch’s membrane lipoprotein binding and show, using human Bruch’s membrane explants, that CFH removes endogenous human lipoproteins in aged donors. Although the CFH H402 variant shows altered binding to BrM, this does not affect its ability to remove endogenous lipoproteins. This new understanding of the complicated interactions of CFH in AMD-like pathology provides an improved foundation for the development of targeted therapies for AMD.
3.2 Results ..............................................................................................................................42

3.3 Discussion ........................................................................................................................ 54

3.4 Materials and Methods ..................................................................................................56

4. CFH regulates sub-RPE lipoprotein accumulation ............................................................63

4.1 Introduction .....................................................................................................................63

4.2 Results ..............................................................................................................................64

4.3 Discussion ........................................................................................................................71

4.4 Materials and Methods ..................................................................................................74

Conclusions ..................................................................................................................................79

References ....................................................................................................................................80

Biography .....................................................................................................................................95
List of Figures

Figure 1: Posterior eye ocular anatomy. (A) Cross-section schematic of a human eye showing major structures. (B) Color fundus photograph from an elderly patient covering the area indicated in (A) showing a healthy macula, fovea, and optic nerve head (photograph courtesy of Eleonora Lad). (C) Histochemical cross-section of the foveal region indicated in (B), foveola (indicated by *), which contains only cone photoreceptors and no rods, is located at the center of the foveal pit. Photoreceptor layer (PR), RPE, BrM, and choroid, are indicated (fovea image courtesy of Christine Curcio) [8].

Figure 2: Drusen in early AMD. (A) Normal fundoscopic image. (B) Fundoscopic image of a patient with early AMD with significant macular drusen (yellow lesions in macula). (C) Cartoon of RPE/choroid interface where sub-RPE drusen forms showing retina, RPE and choroid (courtesy of Greg Hageman).

Figure 3: Schematic of RPE and Bruch’s membrane (BrM) in normal (A) and early AMD (B) eye. (A) BrM layers: 1 Basal lamina of the RPE, 2 Inner Collagenous Layer, 3 Elastic Layer, 4 Outer Collagenous Layer and 5 Basal Lamina of the Choriocapillary endothelium. (B) Sub-RPE deposits. Note that these form between RPE basal lamina and elastic layer. (Adapted from [20]).

Figure 4: RPE-derived Lipoprotein-like Particles. (A) Transmission electron micrograph showing lipoprotein-like particles as vesicles (white arrows) beneath the RPE basal lamina (black arrows) with osmium post-fixations and (B) Quick freeze/deep etch electron microscopy showing modified lipoproteins in BrM. (Adapted from [33]).

Figure 5: Association analysis of CFH haplotypes. Shown above is a set of the eight most informative SNPs in the CFH gene with all of the haplotypes with a frequency of >3%. The odds ratios (OR) for the comparison of cases and controls were calculated as shown. The major risk haplotype (H1) is shown in dark shading, and the protective haplotypes are shown in light shading. SNPs exclusively found in these risk and protective haplotypes are boxed. Note that IVS10 is a non-coding SNP but also associates exclusively with the H1 haplotype. (Adapted from [69]).

Figure 6: CFH H402 relative binding affinities on polystyrene surface. Cell culture treated plastic plates were incubated overnight with either CFH binding partners: MDA, CRP, intact C3, C3b, amyloid-beta or BSA. Plates were washed and CFH Y402 or H402 variants were added overnight. ELISA detection was performed with goat anti-CFH.
antibody (Quidel) and anti-goat IgG HRP. Note that CFH H402 variant causes a similar decrease in binding on a plastic surface for all binding partners (unpublished).

Figure 7: Fluid phase complement dysregulation in old Cfh+/− mice versus age-matched Cfh−/− mice, which are deficient in key activators of the complement cascade. (A-B) Densitometric analysis of CFH immunoblots from plasma (A) and lysates of RPE/choroid (B) across genotype and diet. Note, Cfh+/− mice have approximately half as much CFH as wild type (B6) mice. (C-D) Densitometric analysis of C3/C3b (C) and FB (D) immunoblots from plasma of B6, Cfh+/− and Cfh−/− mice fed a normal (ND) or high fat, cholesterol-enriched (HFC) diet. The dose effect of CFH on circulating C3 in plasma shows fluid phase complement dysregulation in Cfh+/− mice, compared to B6 mice and the absence of circulating C3 and FB in Cfh−/− mice. (E) Red blood cell (RBC) hemolysis assay in each genotype, Cfh+/− mice had approximately half of the intact C3 compared to wild type B6 mice, confirming fluid phase complement dysregulation. In contrast, as expected, Cfh−/− mice were unable to lyse antibody primed sheep RBCs due to the lack of reserve intact C3. (F) In order to establish the C3 deficiency as the primary mechanism for the inability of Cfh−/− plasma to lyse RBCs 2 µg of C3 and/or CFH were added to the Cfh−/− plasma samples. Only addition of C3 restored the hemolytic activity of the Cfh−/− plasma. Data is presented as mean ± SE. N=5-8 per group. Albumin (Alb) served as a loading control in A, C and D. GAPDH served as loading control in B.

Figure 8: Decreased CFH increases sub-RPE basal deposit load. (A) Transmission electron micrograph images of basal deposits along Bruch’s membrane (BrM). Large (>4 µm height) deposits were often seen in the Cfh+/−HFC and Cfh−/−HFC BrM. Vesicular structures with an electron dense shell of 80-160nm across were also frequently observed (A, Cfh+/−HFC, arrows) within a primarily amorphous deposit. Arrowhead, elastic lamina of BrM. Scale bars = 1 µm. (B) ANOVA quantitative analysis was performed on the mean basal deposit height (N=84-124 images per mouse) of each animal where the graph represents the mean ± SE across genotype and diet. Statistically significant increases in basal deposits were observed in the Cfh+/−HFC and Cfh−/−HFC mice. Asterisks indicate post-hoc Tukey test for a p<0.05 following a statistically significant genotype by diet interaction by ANOVA. N=6-8 mice per group. (C and D) Distribution of basal deposits for the three mouse genotypes fed a ND (C) or switched to a HFC diet (D) are represented by cumulative frequency curves showing the frequency of sub-RPE deposit heights (left panels) and mean spatial distribution of sub-RPE deposit heights (right panels). For the cumulative frequency plots the genotype with the least number of deposits (B6, black trace) is represented by the curve that is furthest left (closest to the y axis). Vertical bars at the top of the cumulative frequency traces indicate the maximum deposit size for each genotype and diet combination. The cumulative
frequency curves reveal a modest accumulation of basal deposits in the Cfh-/--ND, compared to the other genotypes on ND, but strikingly significant basal deposit accumulation occurs in both the Cfh-/- and Cfh+/- HFC fed animals and not in B6-HFC animals. Spatial distribution of sub-RPE deposits shows a central > peripheral decrease in sub-RPE deposit height following HFC diet (D, right panels).

Figure 9: Significant vision loss in response to the HFC diet is only detected in Cfh+/- mice. Scotopic electroretinogram (ERG) flash responses in wild type B6 (A), Cfh+/- (B) and Cfh-/- mice (C) fed a ND or HFC diet. Stimulus response curves of b-wave amplitudes. Data is expressed as mean and ± SE of the stimulus response curve overlaid with $\text{B} = (\text{Bmax1}*I/I+I1)/(\text{Bmax2}*I/I+I2)$ comparing ND (black) to HFC (green) with B6-ND overlaid (gray in Cfh+/- and Cfh-/- graphs) for genotype comparisons. Cfh-/-ND mice were slightly worse at baseline compared to B6-ND and Cfh+/-ND mice; however this failed to reach statistical significance by ANOVA. (A and C) B6 and Cfh-/- mice showed no statistically significant depression of ERG amplitude with HFC diet. (B) Cfh+/-HFC mice showed a marked decrease in b-wave amplitude (middle graph). * indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype by diet interaction by ANOVA. N=6-10 mice per group.

Figure 10: RPE damage in response to the HFC diet is only detected in Cfh+/- mice. (Left) Representative confocal fluorescence images of flat mounts of the central RPE from >90 week old mice Cfh+/- and Cfh-/- mice fed a ND or HFC diet that were stained with Hoechst 33342 (blue, nuclei) and anti-ZO-1 (green) and imaged RPE apical side up with the neural retina removed. In Cfh+/-HFC mice there are many more enlarged, multinucleate cells. (Right) Quantification of multinucleate (nuclei ≥ 3) RPE cells per field view demonstrating that Cfh+/-HFC RPE flat mounts have the largest number of multinucleate cells per field view of all the groups. Data are presented as mean ± SE. * indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype by diet interaction by ANOVA. # indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype interaction by ANOVA. N=6-8 per group.

Figure 11: Fluid phase complement dysregulation in old APOE4, Cfh+/- mice. (A) Densitometric analysis of CFH immunoblots from plasma across genotype and diet. Note, APOE4, Cfh+/- mice have approximately half as much CFH in the plasma as APOE4 mice. (B) Densitometric analysis of C3/C3b immunoblots from plasma of APOE4, APOE4, Cfh+/- and APOE4, Cfh-/- mice fed a normal (ND) or high fat, cholesterol-enriched (HFC) diet. The dose effect of CFH on circulating C3 in plasma shows fluid phase complement dysregulation in APOE4, Cfh+/- mice, compared to APOE4 mice and the absence of circulating intact C3 in APOE4, Cfh-/- mice.
Figure 12: Sub-RPE deposit formation in APOE4,Cfh+/-~HFC mice is decreased compared to Cfh+/-~HFC mice. (A) Transmission electron micrograph (25,000x, scale = 0.5 µm) of normal retinal pigment epithelium (RPE), Bruch’s membrane (BrM), and choriocapillaris (CC) in an aged APOE4 ND mouse. (B-C) Representative images of sub-RPE deposits (arrows) observed in an aged APOE4,Cfh+/-~HFC mouse (B) and Cfh+/-~HFC mouse (C). Significant, basal deposits were also observed in the APOE4,Cfh+/-~HFC mouse, and preliminary data shows a similar trend observed in non-APOE4 transgenic mice is also seen but to a lesser degree in APOE4 mice (data not shown). Scale bar = 0.5 µm.

Figure 13: APOE4, Cfh+/-~HFC mice show RPE damage in response to the HFC. (A) Representative confocal fluorescence images of flat mounts of the central RPE from >90 week old APOE4, APOE4, Cfh+/- and APOE4, Cfh-/~ mice fed a ND or HFC diet that were stained with Hoechst 33342 (blue, nuclei) and anti-ZO-1 (green) and imaged RPE apical side up with the neural retina removed. In APOE4, Cfh+/-~HFC mice there are many more enlarged, multinucleate cells. (B) Quantification of multinucleate (nuclei ≥ 3) RPE cells per field view demonstrating that APOE4, Cfh+/-~HFC RPE flat mounts have the largest number of multinucleate cells per field view of all the groups, however, the effect does not appear exacerbated compared to Cfh+/-~HFC mice. Data are presented as mean ± SE. * indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype by diet interaction by ANOVA. # indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype interaction by ANOVA. N=6-8 per group.

Figure 14: Significant Retinal thinning in APOE4, Cfh+/-~HFC mice. (A) Representative light microscopic images of toluidine blue-stained sections from eyes of aged APOE4, APOE4, Cfh+/- and APOE4, Cfh-/~ mice fed a HFC diet. OS, outer segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. (B) Photoreceptor degeneration was quantified by using the mean and ± SE of total ONL area. APOE4, Cfh+/-~HFC mice have decreased ONL thickness. (C) Significant INL thinning was also observed in APOE4, Cfh+/-~HFC mice. (D) INL and ONL thickness was measured at 200 µm intervals from the optic nerve to the ora serrata. Data are expressed as mean and ± SE of ONL thickness versus distance from optic nerve comparing mice fed ND (black) to HFC (green) for each genotype. * indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype by diet interaction by ANOVA. # indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype interaction by ANOVA. N=6-8 per group.

Figure 15: Complement activation in Cfh+/-~HFC mice versus local deficiency of key complement components in Cfh-/~ mice. (A-B) Densitometric analysis of C3 (A) and FB
(B) Immunoblots in the RPE/choroid across genotype and diet. $C_{fh}^{-/-}$ mice have almost no detectable levels of intact C3, only minimal detectable C3b/iC3b and no detectable levels of FB in the RPE/choroid lysates, whereas, $C_{fh}^{+/+}$ mice have significant intact C3 and show a statistically significant decrease in intact C3 following HFC diet ($p<0.05$), which is not seen in wild type B6 or $C_{fh}^{-/-}$ mice. Data is presented as mean ± SE. N=6-8 per group. (C) Anti-C3 (green) immunohistochemistry with Hoechst 33342 (blue, nuclei) staining of posterior eyecups. 100x images at the RPE/BrM interface show no detectable C3 can be localized to BrM, where sub-RPE deposit formation occurs in $C_{fh}^{-/-}$HFC mice, whereas in $C_{fh}^{+/+}$HFC mice significant amounts of C3 can be detected in BrM. N=3 mice per group. Scale bars = 5 μm.

Figure 16: C5a levels and CD11b+ myeloid in $C_{fh}^{+/+}$HFC mice. (A) C5a ELISA was performed on mouse plasma from $C_{fh}^{+/+}$ and $C_{fh}^{-/-}$ mice fed a ND or switched to a HFC diet. $C_{fh}^{+/+}$HFC mice have significantly increased C5a levels compared to $C_{fh}^{-/-}$HFC mice. Data is presented as mean ± SE. N=7-12 per group. (B) Anti-CD11b (green) with Hoechst 33342 (blue, nuclei) staining of posterior eye cups of $C_{fh}^{+/+}$HFC mice shows myeloid cells in and around the RPE/choroid and RPE/BrM.

Figure 17: Monocytosis in $C_{fh}^{+/+}$HFC mice versus local deficiency of key complement components in $C_{fh}^{-/-}$ mice. Classical and non-classical monocyte populations were identified by Ly6C and CD43 gating [98]. A statistically significant 5.3-fold increase ($p<0.05$) in classical monocytes was detected in $C_{fh}^{+/+}$HFC mice in response to diet, in contrast $C_{fh}^{-/-}$ mice have a baseline increase in classical monocytes but no change was observed in $C_{fh}^{-/-}$HFC mice, while B6 mice show a 1.5 fold increase ($p<0.05$). Non-classical monocytes showed a distinct, statistically significant, 2.5-fold increase ($p<0.05$) in $C_{fh}^{+/+}$HFC mice which was not observed in $C_{fh}^{-/-}$HFC mice, but was also observed to in B6 mice ($p<0.05$). Note that $C_{fh}^{+/+}$HFC mice represent the largest population of classical (12.8%) and non-classical (9.8%) monocytes in all genotypes.

Figure 18: In order to assess RPE/choroid monocyte populations, intravascular flow cytometry analysis was performed [98]. (A, top panel) MNP were increased in $C_{fh}^{+/+}$ mice following HFC diet but were unchanged in $C_{fh}^{-/-}$ mice. (A, bottom panel) Three clear MNP populations were evident in the extravascular space a Ly6C$^\text{hi}$, a Ly6C$^\text{lo}$ and a CD64$^+$ population. (B) Note that the CD64$^+$ subpopulation in $C_{fh}^{+/+}$HFC animals represents the largest myeloid cell population (CD64+, Ly6Chi, Ly6Clo and polymorphic neutrophils (PMN)) across all genotypes and diets within the study. The statistical significance of the MNP population changes was determined by using a Chi-squared test for $p<0.05$. * indicates statistical significant diet interaction. Data shown represents a pooled analysis of 6 mice per group.
Figure 19: 4C9 anti-C5a antibody characterization and in vivo dose response. (A) C5aR cells cultured in the presence of Calcium 3 dye given 0.4 nM mC5a agonist with increasing concentration of antibody and Calcium efflux was measured over a period of 2 minutes by fluorescence imaging plate reader (FLIPR). An IC50 of 0.12 nM was determined for 4C9 against 0.4 nM mC5a. (B) To determine the in vivo dose response of anti-C5a (4C9) in the context of posterior eye inflammation 25 mg/kg of sodium iodate was injected into the intravitreal space with a single injection of increasing concentrations of intraperitoneal anti-C5a (3, 10, 20, 30 and 60 mg/kg) or 60 mg/kg of isotype control (Iso 60 mg/kg). Electretinogram b-wave amplitudes are presented to increasing flash intensity of light (B). The therapeutic dose was 30 mg/kg and sub-therapeutic dose was 10 mg/kg.

Figure 20: Anti-C5a blocks the monocytosis and increased RPE/choroid extravascular CD64+ MNP in Cfh+/--HFC model. (A) Classical and non-classical monocyte populations were determined by Ly6C and CD43 gating as shown previously described (Toomey et al). A statistically significant 6-fold increase (p<0.05) in classical monocytes and 1.8 fold increase (p<0.05) in non-classical monocytes was detected in Cfh+/--HFC mice in response to diet. 30mg/kg of anti-C5a therapy was able to prevent this classical and non-classical monocytosis, whereas sub-therapeutic dosing at 10mg/kg was not. (B and C) A 2.8 fold increase (p<0.05) in RPE/choroid CD64+ MNP was seen in Cfh+/-- mice on HFC diet. 30mg/kg of anti-C5a therapy was able to prevent this increase in CD64+ MNPs, whereas sub-therapeutic dosing at 10mg/kg was not.

Figure 21: Anti-C5a protects RPE cells from damage in spite of significant sub-RPE deposit formation. (A) Transmission electron micrograph images of basal deposits along Bruch’s membrane (BrM). Large (>2μm) deposits were often seen in the Cfh+/--HFC and Cfh+/--HFC mice treated with 30mg/kg of anti-C5a, while only minimal sub-RPE deposit could be detected in aged-matched Cfh+/-- fed a normal diet. N=3 mice per group. (B) Confocal fluorescence images of flat mounts of the central RPE from >90 week old mice Cfh+/-- mice fed a ND or HFC diet treated with 0 or 30 mg/kg of anti-C5a that were stained with Hoechst 33342 (blue, nuclei) and anti-ZO-1 (green) and imaged RPE apical side up with the neural retina removed. In Cfh+/--HFC mice there are many more enlarged, multinucleate cells following HFC diet, whereas RPE cells were protected from damage in Cfh+/--HFC mice treated with 30mg/kg of anti-C5a. (C) Quantification of multinucleated (nuclei ≥ 3) RPE cells per field view demonstrating that Cfh+/--HFC mice treated with 30mg/kg of anti-C5a are protected. Data are presented as mean ± SE. * indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype by diet interaction by ANOVA. # indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype interaction by ANOVA. N=6-8 per group.
Figure 22: Anti-C5a ameliorates visual function attenuation in Cfh+/--HFC mice. Scotopic electoretinogram (ERG) flash responses in wild type B6, Cfh+/ and Cfh-/ mice fed a ND or HFC diet. Stimulus response curves of b-wave amplitudes. Data are expressed as mean and ± SE of the stimulus response curve overlaid with B = (Bmax1*I/I+I1)/(Bmax2*I/I+I2) comparing ND (black), HFC (green) and HFC 30mg/kg of anti-C5a (pink). Cfh+/--HFC mice were significantly worse following HFC diet, which was partially ameliorated with anti-C5a therapy. N=6-12 mice per group. ....................... 54

Figure 23: CFH competes with lipoproteins for binding to heparin-sepharose beads. (A-B) Heparin sepharose columns were pre-incubated with doubling concentrations of CFH or albumin, after washing off excess protein, 2µl of a lipoprotein preparation was added to all the columns. (A-B) SDS-PAGE gels showing Coomassie blue staining of separated proteins following incubation with increasing concentrations of CFH and elution with 1M NaCl. The identity of the bands was determined by MALDI-TOF mass spectrometry. Western blot was used to identify ApoE eluted from heparin column following increasing concentrations of CFH or albumin. The density of each band was measured to determine the relative concentration changes of each apolipoprotein with increasing CFH (A, bottom panel) or albumin (B, bottom panel). .......................................................... 65

Figure 24: CFH levels regulate lipoprotein binding in porcine BrM. Six mm porcine RPE/BrM tissue explants were incubated with doubling concentrations of CFH (62 nM-4 μM) in the presence of a 200 μM excess of albumin. (A) The amount of CFH bound to the tissue explant was determined by Western blot of the tissue lysate to determine EC50 binding. (B) Similar porcine tissue explants were incubated 2 μl of lipoprotein and increasing concentrations of CFH (0, 60, 120 nM) and lipoprotein binding to the explants was assessed by Western blot analysis for ApoB-100 and ApoE. Data are expressed as mean ±SE. * indicates p<0.05 for ApoE and ApoB-100 by ANOVA compared to 0 nM CFH. Data presented are a representative of experiment of three independent experiments (N=12-15). Actin served as a loading control in A-B. ......................................................... 67

Figure 25: CFH levels regulate lipoprotein binding and remove endogenous lipoproteins human in BrM. (A) Since aged human BrM contains endogenous sub-RPE lipoproteins we tested the ability of CFH to remove these accumulated lipoproteins ex vivo. Anti-CFH (green) immunohistochemistry of aged human BrM donor tissue before (upper panel) and after (lower panel) overnight incubation with 1 μM exogenous CFH, shows accumulation of CFH in BrM. Scale bar = 5 μm. (B) FPLC fractionation of human BrM lysates shows endogenous lipoproteins present in aged BrM tissue are removed with the addition of CFH (1 μM CFH, green trace). * indicates p<0.05 for total cholesterol in each
fraction comparing 0 μM CFH to 1 μM CFH. Three independent experiments each with an N=3 were performed to confirm these results of human BrM FPLC experiments.

**Figure 26:** CFH H402 variant shows equal accumulation in BrM but binds to different epitopes. (A) Human BrM explants from aged (73-79 years old) donors were incubated with 1 μM CFH Y402 or CFH H402. Bound CFH was quantified by western blot using CFH standard curves with of goat anti-CFH established to recognized CFH variants equally [87]. Equal binding was noted and type II error was calculated to establish statistical significance of the null hypothesis (β<0.05) (B) Porcine BrM explants were incubated with increasing concentrations of full-length CFH Y402 or CFH H402 to establish EC50 values for CFH Y402 (130 nM) and H402 (160 nM). Maximum binding was achieved at 0.5 mM with CFH Y402 and CFH H402. Addition of 0.5 mM CFH Y402 and 0.5 mM CFH H402 showed increased binding compared to 1 mM of CFH variants in isolation. (C) Competition of CFH H402 and Y402 was assessed by adding increasing concentration of CFH Y402 (0 to 4 μM) to three fixed concentrations (0, 1 and 4 μM) of CFH H402. Note that little competition was observed between the two variants.

**Figure 27:** CFH H402 variant is equally effective at removing endogenous BrM lipoproteins. (A and B) Human BrM explants were isolated from aged (>70) donors and incubated with either 1 mM CFH Y402, 1 mM CFH H402 or 10 mM PB with matched NaCl concentration. FPLC fractionation was performed on tissue lysates and enzymatic cholesterol assays were performed on fractions to determine the lipoprotein content within the lysates. Note considerable variability exists in amount of lipoprotein present in aged donors. (C) However, analysis of relative cholesterol removal comparing the CFH H402 variant to the CFH Y402 shows equal ability to remove BrM lipoprotein from aged donors.
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1. Introduction

1.1 Age-related Macular Degeneration

Medical advances have resulted in a significant increase in life-span. However, a challenge inherent in this increase in life span is the increasing prevalence of age-related diseases. This dissertation focuses on the age-related condition, age-related macular degeneration (AMD). AMD affects about 30% of Americans over age 70 [1-3] and is the leading cause of irreversible vision loss in the elderly in industrialized nations [4]. It is a progressive, chorioretinal degenerative disease that affects the central region of the retina known as the macula (Fig. 1), which is responsible for the majority of central high acuity, photopic vision (vision under well-lit conditions). The pathognomonic lesions of early AMD are lipid and protein rich deposits known as drusen (Fig. 2). Historically, drusen were detected on fundoscopic clinical exam [5], but are now visible in a variety of imaging modalities including optical coherence tomography [6]. While the presence of a few small drusen is a normal, non-vision-impairing part of aging, the deposition of large diffuse drusen in the macula is vision impairing and indicative of early AMD. The diagnosis of early AMD places a patient at high risk of progressing to later stages of the disease. Late-stage AMD is categorized as either ‘dry’ [geographic atrophy, with photoreceptor loss and extensive RPE atrophy] or ‘wet’ [exudative, with subsequent choroidal neovascularization], however, in many patients both ‘dry’ and ‘wet’ forms are evident [7].
Figure 1: Posterior eye ocular anatomy. (A) Cross-section schematic of a human eye showing major structures. (B) Color fundus photograph from an elderly patient covering the area indicated in (A) showing a healthy macula, fovea, and optic nerve head (photograph courtesy of Eleonora Lad). (C) Histochemical cross-section of the foveal region indicated in (B), foveola (indicated by *), which contains only cone photoreceptors and no rods, is located at the center of the foveal pit. Photoreceptor layer (PR), RPE, BrM, and choroid, are indicated (fovea image courtesy of Christine Curcio) [8].
1.2 Pathobiology of Drusen

Drusen, which are visible as small yellowish deposits under the retina on fundus examination, were first identified in 1854 by the Dutch ophthalmologist, Franciscus Donders. It has long been recognized that drusen are a hallmark lesion of early and intermediate AMD as the size, number and extent of confluency of drusen determine the risk of progression to late AMD [9, 10]. Furthermore, the presence of drusen is associated with visual deficits prior to the loss of visual acuity, which include changes in color contrast sensitivity, macular recovery function, central visual field sensitivity, and spatiotemporal contrast sensitivity [11-14]. More recent studies have shown that visual acuity improves in some cases following laser photocoagulation and subsequent drusen regression [15, 16]. These observations provide support for the notion that an association exists between drusen and vision loss in patients with AMD. However, depending on
the type and extent of examination and classification, nearly 80% of patients over 60 years of age have evidence of macular drusen [17]. Thus, drusen are a sensitive but not specific lesion for AMD, since it also occurs in a variety of diseases of the posterior eye.

Drusen are classified based on their size. While characteristics and distribution of drusen can vary significantly between individuals, there is a remarkably high degree of symmetry between the two eyes of a patient [18]. There are three major classes of drusen: small, (<62µm), intermediate (62-124µm) and large drusen (>125µm). Small drusen, previously referred to as hard drusen, are tiny (usually <50µm in diameter), well-defined yellowish deposits on the fundus detected by ophthalmoscopy and are highly associated with “normal aging,” showing little risk for progression to late stage AMD. Intermediate and large drusen, previously referred to as soft drusen, are larger than 63µm. The concentration of these drusen in the macula is an established indicator of progression to late-stage AMD [17]. The specific contribution of drusen to AMD complications and progression are not well characterized. Therefore, the origin of drusen, as well as their implications in retinal health, is essential to our understanding and the development of therapeutics for AMD.

1.3 Drusen in Bruch’s Membrane

Drusen formation occurs in Bruch’s membrane (BrM), a boundary between the ocular RPE and the capillary bed of the choroid known as the choriocapillaris (Fig. 3A). BrM is not a classically defined membrane; rather it is a thin (2-4µm), acellular, five-
layered, extracellular matrix that serves as the basal lamina of the RPE and a vessel wall of the choriocapillaris (Fig. 3) [19, 20]. The five layers of BrM from the basal RPE side to the choroid are: RPE basement membrane, inner collagenous layer, elastic layer, outer collagenous layer and choroidal basement membrane (Fig. 3A). As a vessel wall of the choroid, BrM’s primary function is similar to other vessel walls and has a structure similar to vascular intima, with a subendothelial extracellular matrix and an elastic layer that is analogous to the internal elastic lamina of vessels [19, 20]. BrM differs from other vessel walls in that its abluminal surface abuts the basal lamina of the RPE, which is also a characteristic of the renal glomerulus. This morphological similarity between BrM and the glomerulus helps explain the overlap of some renal and retinal diseases, particularly AMD and Dense Deposit Disease [19]. The composition, thickness and structural properties of BrM vary with age [21-23]. In particular, the collagen type IV content and collagen cross-linking increases with age, while collagen solubility decreases and advanced glycation end-product modifications accumulate in BrM with age [19, 24-27]. These structural, age-related changes contribute to the decreased hydraulic conductivity seen in BrM with age, in fact it was theoretically extrapolated that at the age of 130 no further flow through would be possible [23].
Figure 3: Schematic of RPE and Bruch’s membrane (BrM) in normal (A) and early AMD (B) eye. (A) BrM layers: 1 Basal lamina of the RPE, 2 Inner Collagenous Layer, 3 Elastic Layer, 4 Outer Collagenous Layer and 5 Basal Lamina of the Choriocapillary endothelium. (B) Sub-RPE deposits. Note that these form between RPE basal lamina and elastic layer. (Adapted from [20])

1.4 Age-related Accumulation of Drusen-like Material

Histochemical and biochemical analysis of drusen has provided great insight into the origin and contributions of drusen to AMD. Drusen are predominantly lipid-rich with lipid containing particles occupying 37-44% of drusen by volume and each druse containing approximately 112 ng lipids compared to 42 ng protein, thus,
understanding the origin of drusen lipids will provide great insight into the pathogenesis of AMD [28]. Historically, Donders inferred the lipid content of drusen based on their small, spherical inclusions [29]. However, it was not until 1962, when Wolter and Falls used Oil Red O to stain for non-polar lipids, that the rich lipid content of drusen was confirmed [30]. Further histochemical analysis of drusen identified the presence of phospholipids, unesterified and esterified cholesterol, which accumulate dramatically with age [9, 31, 32]. There is considerable variability between individuals and the lipid composition of their drusen [9]. While some drusen contain predominantly neutral lipids, others are dominated by phospholipids [9, 31, 32].

The lipid containing particles seen in drusen resemble the non-fundoscopically detectable lipid accumulation in BrM and basal linear deposits, which are hypothesized to be the precursor to drusen [33]. BrM lipid accumulation with age and basal linear deposits appear in the same histological plane as drusen and contain similar vesicular histological hallmarks [9, 33]. Drusen are considerably modified by a variety of intrinsic and extrinsic factors making the investigation of their origin difficult based on their altered state and techniques to isolate large diffuse drusen, associated with AMD, have not been developed due to their oily and fragile structure [34]. Thus, most research on the origin of drusen has centered on understanding the origin of the lipid accumulation in BrM.
Early histological examination of BrM in 1963 and 1964 described “a series of clear roundish zones (mostly less than 100 nm in diameter) without a limiting membrane” in aged patients [35, 36]. The authors speculated that these “zones” represented morphological evidence of a fluid droplet permeation process to the epithelium. However, fatty acids are washed out during dehydration steps of conventional electron microscopy, limiting investigators analyses of the electron-permeable areas. It was not until 1990 that Pauleikhoff et al., using a neutral lipid (Oil Red O) and phospholipid (Sudan Black) histochemical staining, showed progressive accumulation of neutral lipid and phospholipid staining in BrM with age, although strong lipid variation was noted between patient samples [9]. Further analysis by thin-layer chromatography in 1993 confirmed the accumulation of lipids with age and facilitated quantification of the lipid compositions as predominately phospholipid, as well as with moderate levels of free cholesterol, fatty acids and triglycerides and small amounts of esterified cholesterol [31, 32]. The authors concluded that these lipids are consistent with a cellular origin, likely derived from the overlying RPE [32]. This is in contrast to atheroma and corneal arcus where intracellular cholesterol esters form the major fraction of total lipids [37, 38]. These studies demonstrate a novel and dramatic accumulation of lipids within BrM with age and naturally subsequent research endeavors focused on the origin of these lipids.
1.5 Origin of Lipid Accumulation: An RPE-derived Lipoprotein

Lipid Wall

In the late 1990s and early 2000s genomic analysis of AMD patients revealed an association between lipid transport pathways, including apolipoproteins, and modification of risk of the development of AMD [39]. Correlating these findings with the lipid accumulation studies in BrM, Curcio et al. investigated the role of RPE-derived apolipoproteins in age-related lipid accumulation, which led to their “Oil Spill” hypothesis of lipid accumulation in BrM [33]. Although this hypothesis does not encompass all of the complexity associated with lipid accumulation in BrM, it represents a well-developed series of experiments that greatly contribute to the understanding of lipid accumulation in AMD. Utilizing quick-freeze/deep-etch, a freeze-fracture method to preserve lipids for electron microscopy, the authors observed round space-filling vesicles 60-80nm in diameter with a core-shell structure that the authors later termed lipoprotein-like particles (Fig. 4). Subsequent analysis showed that these lipoproteins accumulate with age between the internal collagenous layer and elastic layer of BrM, forming what they termed the “lipid wall” (Fig. 4) [40, 41]. Isolation of lipoprotein-like particles by high-salt buffer and density gradient fractionation revealed two concentrations peaks, one dominated by 60-80nm vesicles containing predominantly cholesterol esters, and a larger second peak represented by a more heterogeneous population of lipids, predominantly unesterified cholesterol and phospholipids [41-43].
Furthermore, cultured RPE-J cells, an immortalized rat RPE cell line, have been shown to secrete ApoB-containing lipoprotein-like particles of 60-80 nm in size consistent with the lipoprotein-like particles seen in BrM, but in contrast to circulating high density lipoprotein (HDL) and low density (LDL) particles which range from 8-13 nm to 15-25 nm, respectively [43]. Current attempts to reproduce the results of Holz et al. HPLC lipid distribution that are consistent with a “RPE cellular origin” have been unsuccessful by Curcio and colleagues [43]. It is possible that the Holz et al. study was underpowered, since the majority of samples had some RPE contamination, as noted by the authors [31-33]. The experiments by Curcio et al. demonstrate that a portion of the lipid accumulation in BrM is lipoprotein-like particles derived from the RPE. However, the origin of the lipids in the RPE remains to be determined. There are two major cholesterol sources in an RPE cell derived from: 1) daily phagocytosis of the outermost/oldest photoreceptor outer segments (OS); and 2) the uptake of peripheral LDL particles from systemic circulation for retinal lipid supply [44-46]. Based on the high linoleate, abundant in LDL and low cholesteryl docosahexaenoate or cholesteryl stearate, abundant in OS, Curcio et al. concluded that the source of esterified cholesterol is from circulating LDL particles in blood [42, 47].
1.6 Complement Cascade

Another major contributing event in AMD pathobiology is complement dysregulation. The complement system is among the oldest evolutionary components of the immune system. It was discovered in 1896 as a heat-labile fraction of serum that led to opsonization of bacteria. Biochemical characterization showed that the complement system is composed of over 30 proteins that function to mediate removal of apoptotic cells and eliminate pathogens [48]. Three separate pathways (i.e. classical, alternative and lectin pathways) converge to convert C3 to C3b forming the C3 convertase, an enzyme capable of initiating a cascade that results in the formation of the immunostimulatory breakdown products C3a and C5a and the membrane attack complex (MAC, C5b-9), a cell membrane pore that can cause subsequent cell lysis [48].

The classical and lectin pathways are initiated by the recognition of specific protein or carbohydrate targets. The immunoglobulins IgM and IgG and C-reactive
protein activate the classical pathway. The lectin pathway is initiated by the binding of mannose binding lectin (MBL) to repeating carbohydrate moieties found on bacterial surfaces. In contrast, the alternative pathway is initiated by the spontaneous hydrolysis of a thioester bond in C3 to form C3(H₂O)[48]. Approximately 1% of total C3 per hour changes its conformation from C3 to C3(H₂O). C3(H₂O) is capable of binding to Factor B, resulting in a conformational change of factor B. This conformational change allows Factor D, a constitutively active serum protease, to cleave Factor B to Factor Bb. Factor Bb is retained within the C3(H₂O)Bb complex where it acts as a serum protease for C3 cleaving it to C3b, which can in turn also associate with factor B to generate more C3 convertases (C3bBb). This auto-activation process is known as “tickover” [48]. Blurring the trichotomy between these pathways, however, is the role of the alternative pathway in the “amplification loop.” In this system fixed C3b, generated from either the classical or lectin pathway, binds to factor B to generate more C3 convertase. This “amplification loop” has been shown to be responsible for more than 80% of the C5a and membrane attack complex formation in both the classical and lectin pathways [49, 50].

### 1.7 Complement Factor H

Due to the spontaneous nature of the alternative pathway and its importance in amplifying complement responses, continuous control is necessary. The major negative fluid-phase regulator of the alternative complement cascade is complement factor H (CFH). CFH is an abundant (0.8 – 5.3 μM, in serum) 155 kD serum protein made up of
twenty domains called short consensus repeats (SCR) [51, 52]. CFH SCR 1-4 regulate the alternative pathway C3 convertase via two mechanisms: 1) CFH blocks the binding of Factor B to C3b and 2) CFH acts as a co-factor to the serum protease Factor I which cleaves C3b to C3d and C3c [51]. CFH functions not only in the fluid phase but also in the extracellular matrix and host cell surface due to its binding domains for heparin sulfate (SCR 6-7 and 20) [53, 54].

1.8 Complement Factor H Polymorphisms and AMD

Twin studies and epidemiological studies provided some of the earliest evidence for a role of genetics in AMD [55-59]. In fact, twins studies demonstrate that the genetic factors account for 67% and 71% of the variation in disease risk for intermediate and advanced AMD, respectively [60]. Family based whole genome linkage analysis studies indicated an area near chromosome 1q was associated with increased risk of developing AMD [61-64]. Sequencing of the human genome has allowed more sophisticated methods, including haplotype mapping and single nucleotide polymorphism (SNP) analysis. Using these techniques, in 2005, four groups concomitantly revealed that a common haplotype in the CFH gene predisposes individuals to AMD [65-68]. This risk haplotype was highly associated with a non-synonomous SNP resulting in a tyrosine (Y) to histidine (H) exchange at position 402 of the CFH protein [65-67, 69]. The H402 polymorphism is located in SCR 7, a region that is known to bind to both heparan
sulfate (HS) and C-reactive protein (CRP) but is not involved in regulation of complement activation (SCR1-4) [70, 71].

Although the Y402H substitution explains 17% of the genetic risk attributed to age-related macular degeneration, due to the high linkage disequilibrium in CFH, definitive proof for the involvement of CFH H402 is lacking [72, 73]. The linkage disequilibrium in CFH results in 5 common (>2%) haplotypes (Fig. 5). The most common haplotype (H1, dark grey) shows the highest association for AMD [2.46 odds ratio (OR), confidence interval (CI) 1.95-3.11] and is 95% associated with the CFH H402 SNP [68]. Alternative hypotheses exist for the association of CFH H1 not involving CFH H402 SNP [52]. Recently, Ansari et al. have hypothesized that non-coding SNP alter the plasma CFH concentrations [52]. This hypothesis is intriguing as several non-coding SNPs in CFH showed a higher association with AMD but at the time their functionality was less obvious. Ansari et al. were able to show that non-coding SNPs appear to influence CFH plasma concentrations [52]. However, the cumulative effect on non-coding SNPs on CFH concentrations seems relatively minor as AMD patients have just 3.7% decrease (428 µg/mL versus 412 µg/mL) in plasma CFH levels [52]. Considering plasma CFH is normally distributed with a 3-fold range (218-654 µg/mL) and a standard deviation of 15% (62 µg/mL), Ansari, et al.’s statistically significant observations appear biologically insignificant [52]. Taken together, the significance of the CFH H402 association with
AMD and the alternative explanations put forth by Ansari et al., makes determining the functionality of the CFH H402 polymorphisms all the more important.

Figure 5: Association analysis of CFH haplotypes. Shown above is a set of the eight most informative SNPs in the CFH gene with all of the haplotypes with a frequency of >3%. The odds ratios (OR) for the comparison of cases and controls were calculated as shown. The major risk haplotype (H1) is shown in dark shading, and the protective haplotypes are shown in light shading. SNPs exclusively found in these risk and protective haplotypes are boxed. Note that IVS10 is a non-coding SNP but also associates exclusively with the H1 haplotype. (Adapted from [69])

### 1.9 Complement Factor H H402 Functionality

Following the association of the CFH H402 SNP with AMD the next scientific milestone was to demonstrate the functional significance of this SNP.

**CFH variants and CRP binding**

Early on it was demonstrated that the CFH H402 protein showed decreased binding to CRP compared to the CFH Y402 protein [74]. Furthermore, CRP levels were shown to be elevated in the choroid of CFH 402H patients compared to CFH 402Y [75]. However, these observations have been met with skepticism as other groups have shown that CRP and CFH do not associate at physiologic concentrations and that binding only occurs to denatured CRP [76]. However, Okemefuma et al. maintains that
during the acute phase response CRP concentrations do have an interaction with CFH [77]. In either case, the role of CFH H402 in altering the function of CRP and the relationship between that function and the development of AMD are lacking.

**CFH variants and Heparan Sulfate Proteoglycans**

In a series of publications from 2006 to 2013, using both *in vitro* assays and *ex vivo* BrM binding assays Clark et al. have detailed the decreased functionality of the CFH H402 variant in binding to heparan sulfate in BrM [53, 78, 79]. These observations were exciting because BrM is rich in heparan sulfate; thus, it was hypothesized that the CFH H402 SNP alters local BrM levels of CFH, which was shown by the authors [80]. However, these observations were made using recombinant fragments (SCR6-8) of the CFH protein and the authors cannot reproduce these results using full-length CFH [78, 79]. Furthermore, subsequent quantification of CFH binding was done using relative fluorescent intensities using fluorescent-conjugated CFH with two different fluorophores on fixed post-mortem tissue frozen sections [80]. Our lab and others have had difficulty reproducing the decreased affinity of the CFH H402 variant in binding to heparan sulfate using purified full-length CFH [81-83]. We suspect that recombinant fragments do not fully represent the three dimensional flexibility and/or post-translational modifications of the endogenous protein.

**CFH variants and oxidized motifs**
More recently, Weismann et al. and Shaw, et al. have shown the decreased ability of the CFH H402 variant to bind to oxidative motifs [84, 85]. In 2011, Weismann et al. identified an interaction between malondialdehyde (MDA), a secondary lipid peroxidation product, and CFH. The author’s show that CFH binds to MDA epitopes and “masks” their proinflammatory effects, but that the CFH H402 variant shows decreased binding to MDA motifs [85]. Shortly after, Shaw et al. demonstrated that the CFH H402 variant bound with less affinity to oxidized phospholipids than the normal Y402 form [84]. The authors hypothesized that in AMD oxidized phospholipids accumulate following chronic light exposure to the retina and that CFH influences AMD risk by modulating oxidative stress [84].

Although these in vitro binding assays are intriguing several groups including our own have been skeptical of the interactions of CFH to polystyrene plates. In fact, our lab has shown that several proteins with the capacity to interact with CFH (MDA, CRP, C3, C3b and Amyloid beta) when plated on a plastic surface show a 20-30% decreased binding comparing the H402 variant (Fig. 6). However, these relative interactions do not correlate to the fluid-phase [83]. The incongruence of these findings highlights the fact that no in vivo model exists to establish the role for CFH in AMD pathobiology. Thus it has become increasingly clear that there is an immense need for a more relevant in vivo model which replicates the age-dependent effects of AMD and CFH deficiency.
Figure 6: CFH H402 relative binding affinities on polystyrene surface. Cell culture treated plastic plates were incubated overnight with either CFH binding partners: MDA, CRP, intact C3, C3b, amyloid-beta or BSA. Plates were washed and CFH Y402 or H402 variants were added overnight. ELISA detection was performed with goat anti-CFH antibody (Quidel) and anti-goat IgG HRP. Note that CFH H402 variant causes a similar decrease in binding on a plastic surface for all binding partners (unpublished).
2. CFH deficiency as a model of AMD

2.1 Introduction

Our ability to unravel the link between CFH and the pathobiology of AMD has been hindered by the lack of a model that faithfully reconstitutes the multifactorial pathophysiology of AMD. Initially, the use of humanized mice to model the functional significance of the CFH H402 SNP was complicated by the size of the CFH gene and the lack of homology between human and mouse CFH. Previously, transgenic mice expressing a chimeric CFH, in which the human CFH sequence for SCR6-8 is flanked by mouse sequences SCR1-5 and SCR9-20, showed significant retinal pathology by 12 months of age in both CFH Y402 and CFH H402 transgenic animals [86]. Our lab used bacteria artificial chromosomes spanning the human CFH gene to insert CFH Y402 and CFH H402 associated haplotypes into Cfh-/- mice and found that the human CFH protein functions in the mouse complement pathway [87]. However, the expression of human CFH was low (~10% in CFH Y402 and ~5% in CFH H402 mice compared to WT mice) and no significant ocular phenotype is distinguishable in the CFH H402 expressing mice [87]. Thus, in order to test the function of CFH in vivo we elected to study the role of the AMD-like phenotype in mice deficient in the native mouse CFH protein and follow up these observations using isolated human CFH and human tissue ex vivo.
The use of aged Cfh-/- mice was expected to model the age-related progression of AMD pathogenesis [88]. However, the absence of CFH in these mice leads to the rapid consumption of C3 and the development of severe MPGN type II kidney disease often resulting in death between 12-24 months of age in Cfh-/- mice [89]. Animals that survive to 2 years have a mild retinal pathology including photoreceptor loss and reduced electroretinogram (ERG) b-wave amplitudes [88]. In Cfh-/- mice, C3 is cleaved as fast as it is generated, thus, neither intact C3 nor FB can be detected in the plasma or locally in BrM. The mild retinal phenotype that is observed can be explained by the absence of intact C3, which has a known role in regulating synaptic pruning both in development and in disease states [89, 90]. This idea is supported by data showing that the double transgenic Cfh-/-, C3-/- retinal phenotype is similar to the phenotype observed in C3-/- or Cfh-/- mice [91]. Due to the deficiency of intact C3 in Cfh-/- mice, in this study we investigated the AMD-like ocular phenotype of aged Cfh+/- mice placed on an HFC diet, as these mice show evidence of complement dysregulation but maintain an intact C3 reservoir [89].

2.2 Results

*Fluid phase complement dysregulation in aged Cfh+/- mice versus complement deficient Cfh-/- mice.*

In order to systematically test the role of CFH in AMD pathogenesis and
progression *in vivo* we developed a model system based on age, CFH deficiency, complement dysregulation and cholesterol/oxidative stress using wild type C57BL/6J (B6), Cfh+/- and Cfh-/- mice aged to 2 years that were then switched from a normal rodent chow diet (ND) to a high fat, cholesterol-enriched (HFC) diet for 8 weeks. As reported previously [89], Cfh+/- mice have approximately half the circulating plasma levels of CFH compared to B6 mice on either diet, while there is no detectable CFH in the plasma of Cfh-/- mice (Fig. 7A). CFH levels showed the similar genotype-dependent distribution in RPE/choroid tissue lysates (Fig. 7B).

As previously reported, we found that decreased levels of CFH in aged Cfh+/- mice fed either diet leads to a decrease in intact C3 in the plasma, with no measureable C3b (Fig. 7C) [89, 92]. In contrast, there is no intact C3 in plasma of Cfh-/- mice due to immediate conversion of C3 to C3b (Fig. 7C). Normally, when the alternative pathway is activated by C3 cleavage, Factor B (FB) binds to C3b and is subsequently cleaved by Factor D to form the alternative pathway convertase, C3bBb. Strikingly, in Cfh-/- mice, FB is almost entirely consumed, whereas Cfh+/- and B6 mice have ample intact FB (Fig. 7D). In order to further verify the lack of intact C3 in the plasma of Cfh-/- animals as well as reduced C3 in Cfh+/- plasma, we used a sensitive and quantitative hemolytic assay for the measurement of intact, fully functional mouse C3 [87]. As expected, Cfh+/- mice had approximately half of the intact C3 compared to wild type B6 mice, further validating that the fluid-phase tick-over of C3 is accelerated with CFH deficiency (Fig. 7E). In
contrast, no hemolysis was detected in Cfhl−/− plasma, indicating that all of the C3 in the Cfhl−/− mouse was already cleaved (Fig. 7E). This effect was not rescued by the addition of CFH to the assay and could only be rescued with the addition of C3 (Fig. 7F). These data clearly demonstrate that Cfhl+/− mice have increased fluid-phase consumption/conversion of C3 due to the decreased CFH levels, compared to B6 controls. Furthermore, the data show that the total lack of CFH in the Cfhl−/− mice accelerates the consumption/conversion of C3 and FB to the point that there are no intact downstream components of the alternative pathway detectable in the plasma.

**Figure 7:** Fluid phase complement dysregulation in old Cfhl+/− mice versus age-matched Cfhl−/− mice, which are deficient in key activators of the complement cascade. (A-B) Densitometric analysis of CFH immunoblots from plasma (A) and lysates of RPE/choroid (B) across genotype and diet. Note, Cfhl+/− mice have
approximately half as much CFH as wild type (B6) mice. (C-D) Densitometric analysis of C3/C3b (C) and FB (D) immunoblots from plasma of B6, Cfh+/− and Cfh−/− mice fed a normal (ND) or high fat, cholesterol-enriched (HFC) diet. The dose effect of CFH on circulating C3 in plasma shows fluid phase complement dysregulation in Cfh+/− mice, compared to B6 mice and the absence of circulating C3 and FB in Cfh−/− mice. (E) Red blood cell (RBC) hemolysis assay in each genotype, Cfh+/− mice had approximately half of the intact C3 compared to wild type B6 mice, confirming fluid phase complement dysregulation. In contrast, as expected, Cfh−/− mice were unable to lyse antibody primed sheep RBCs due to the lack of reserve intact C3. (F) In order to establish the C3 deficiency as the primary mechanism for the inability of Cfh−/− plasma to lyse RBCs 2 μg of C3 and/or CFH were added to the Cfh−/− plasma samples. Only addition of C3 restored the hemolytic activity of the Cfh−/− plasma. Data is presented as mean ± SE. N=5-8 per group. Albumin (Alb) served as a loading control in A, C and D. GAPDH served as loading control in B.

*Decreasing CFH increases sub-RPE deposit formation.*

Extracellular lesions that form between the RPE and BrM characterize early AMD [93-95]. We used quantitative electron microscopy of RPE/BrM to analyze the height of similar sub-RPE deposits that accumulated in aged mice in response to Cfh genotype and diet. Sub-RPE deposit measurements were made from the elastic lamina of BrM (Fig. 8A, arrowhead) to the top of deposits. Strikingly large (>4 μm) basal laminar deposits (BLamD) [95] were often seen in the Cfh+/−-HFC and Cfh−/−-HFC mice, extending from the elastic lamina to the basal infoldings of the RPE (Fig. 8A). Deposits of this magnitude were absent in control B6-HFC mice. Vesicular structures with an electron dense shell of 80-160 nm across were frequently observed (Fig. 8A, arrows) within a primarily amorphous deposit. The height of BLamD changed abruptly and
varied from less than 0.2 µm to as much as 5.7 µm for a given section. Therefore, rather than estimating the amount of sub-RPE deposits per eye by examining a few transmission electron microscopy (TEM) images, we developed a method to systematically and objectively sample and measure the height/thickness of sub-RPE deposits by TEM [87]. Using this method, 98 +/- 18 (mean +/- SD) images were taken per mouse eye from sections obtained from the center of the posterior eyecup near the optic nerve sampling from ora to ora.

Quantitative analysis of mean deposit height by genotype and diet demonstrated that sub-RPE deposits increased only in the mice fed a HFC diet that had reduced levels (Cfh+/--HFC) or no CFH (Cfh-/--HFC) (p<0.05) (Fig. 8B). The distribution of sub-RPE deposit thickness was graphed by cumulative frequency and spatial distribution plots (Fig. 8C and D). The cumulative frequency curve that is furthest to the left (closest to the y-axis) in the plot represents the genotype with the least deposits (B6, black trace, Fig. 8C and D, left panels). The cumulative frequency plots revealed an inverse relationship between CFH levels and deposit load in the HFC fed mice (Fig. 8C-D). In B6 and Cfh+/- mice fed a normal diet very few deposits are seen, although there is some deposit accumulation in the Cfh-/-- mice on normal diet as previously described [87]. Most strikingly, however, in mice fed a HFC diet substantially more deposit was observed in Cfh+/--HFC and Cfh-/--HFC mice while no increase in deposits was detected in the B6--HFC mice (Fig. 8). Furthermore, maximum deposit height is inversely related to
CFH dose following HFC diet (Cfh/-→Cfh+/->B6) (Fig. 8C, left panels, bars). Spatial distribution of sub-RPE deposits revealed the accumulation of sub-RPE deposit in the central retina is also inversely related to CFH levels following HFC diet (Fig. 8C and D, right panels).

Figure 8: Decreased CFH increases sub-RPE basal deposit load. (A) Transmission electron micrograph images of basal deposits along Bruch’s membrane (BrM). Large (>4μm height) deposits were often seen in the Cfh+/-→HFC and Cfh-/-→HFC BrM. Vesicular structures with an electron dense shell of 80-160nm across were also frequently observed (A, Cfh+/-→HFC, arrows) within a primarily amorphous deposit. Arrowhead, elastic lamina of BrM. Scale bars = 1 μm. (B) ANOVA quantitative analysis was performed on the mean basal deposit height (N=84-124 images per mouse) of each animal where the graph represents the mean ± SE across genotype and diet. Statistically significant increases in basal deposits were observed in the Cfh+/-→HFC and Cfh-/-→HFC mice. Asterisks indicate post-hoc Tukey test for a p<0.05 following a statistically significant genotype by diet interaction by ANOVA. N=6-8 mice per group. (C and D) Distribution of basal deposits for the three mouse genotypes fed a ND (C) or switched to a HFC diet (D) are represented by cumulative frequency curves showing the frequency of sub-RPE deposit heights (left panels) and mean spatial distribution of sub-RPE deposit heights (right panels). For the cumulative frequency plots the genotype with the least number of deposits (B6, black trace) is represented by the curve that is furthest left (closest to the y axis).
Vertical bars at the top of the cumulative frequency traces indicate the maximum deposit size for each genotype and diet combination. The cumulative frequency curves reveal a modest accumulation of basal deposits in the Cfh⁻/⁻ ND, compared to the other genotypes on ND, but strikingly significant basal deposit accumulation occurs in both the Cfh⁻/⁻ and Cfh⁺/⁻ HFC fed animals and not in B6–HFC animals. Spatial distribution of sub-RPE deposits shows a central > peripheral decrease in sub-RPE deposit height following HFC diet (D, right panels).

**Attenuated scotopic visual function in Cfh⁺/⁻ HFC mice.**

Visual function of the retinas of aged Cfh mice on and off a HFC diet was measured by scotopic electroretinogram (ERG), which primarily measures the rod photoreceptor function [96]. Strikingly, visual function decline was only observed in Cfh⁺/⁻ mice compared to the Cfh⁻/⁻ and wild type B6 mice after HFC diet (Fig. 9). Similar trends shown for b-waves in Fig. 9 were also observed in the ERG a-waves. In addition, there were no measureable statistical changes in a-/b-wave ratios indicating that the attenuation of the b-wave responses originated in the photoreceptor [97, 98]. Quantitative analysis of b-wave rod- and cone-driven responses was achieved by determining amplitude of Bmax1 (rod-dominant response) and Bmax2 (cone-dominant response) (data shown in [98]). Analysis of variance showed that the 40% reduction in the rod-dominant response in Cfh⁺/⁻ following HFC diet was statistically significant (p<0.05), while the 22% reduction in cone-dominant response did not reach statistical significance. No statistically significant changes were detected among other genotype or diet interactions. These data were supported by a moderate (8.8%) but statistically...
significant (p<0.05) reduction in photoreceptor outer nuclear layer (ONL) area in Cfh+/− HFC mice (data shown in [98]). The data show that Cfh+/− mice have a significant visual function decline following HFC diet, whereas, wild type and Cfh−/− mice do not.

Figure 9: Significant vision loss in response to the HFC diet is only detected in Cfh+/− mice. Scotopic electroretinogram (ERG) flash responses in wild type B6 (A), Cfh+/− (B) and Cfh−/− mice (C) fed a ND or HFC diet. Stimulus response curves of b-wave amplitudes. Data is expressed as mean and ± SE of the stimulus response curve overlaid with B = (Bmax1*I/I+I1)/(Bmax2*I/I+I2) comparing ND (black) to HFC (green) with B6−ND overlaid (gray in Cfh+/− and Cfh−/− graphs) for genotype comparisons. Cfh−/−ND mice were slightly worse at baseline compared to B6−ND and Cfh+/−ND mice; however this failed to reach statistical significance by ANOVA. (A and C) B6 and Cfh−/− mice showed no statistically significant depression of ERG amplitude with HFC diet. (B) Cfh+/−HFC mice showed a marked decrease in b-wave amplitude (middle graph). * indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype by diet interaction by ANOVA for the mean Bmax1. N=6-10 mice per group.

RPE damage in aged Cfh+/−HFC mice.

In order to assess damage to the RPE cells in aged Cfh+/− and Cfh−/− mice resulting from exposure to the HFC diet, multinucleate RPE cells were quantified by immunostaining RPE flat mounts with the tight junction protein, zonula occludens-1
Quantitative analysis showed that there is a small, but statistically significant, increase in multinucleate RPE cells (≥ 3 nuclei) in aged Cfh+/− mice compared to control, age-matched, B6 mice maintained on a ND. However, exposure to a HFC diet resulted in a large, statistically significant, increase in enlarged multinucleate RPE cells in the Cfh+/− mice (21.4 SE +/- 2.8 vs. 11.2 SE +/- 1.4, p<0.05) (Fig. 10). In contrast, no detectable morphological differences in RPE were seen between wild type and Cfh−/− mice in response to HFC diet (Fig. 10). Damage to the RPE was further supported by the observation of RPE thinning in plastic sections of retina from Cfh+/−HFC mice (Data shown in [98]). Together these data demonstrate RPE dysmorphogenesis in the Cfh+/−−HFC mice, which is not detected in Cfh−/−HFC mice.

Figure 10: RPE damage in response to the HFC diet is only detected in Cfh+/− mice. (Left) Representative confocal fluorescence images of flat mounts of the central RPE from >90 week old mice Cfh+/− and Cfh−/− mice fed a ND or HFC diet that were stained with Hoechst 33342 (blue, nuclei) and anti-ZO-1 (green) and imaged RPE apical side up with the neural retina removed. In Cfh+/−HFC mice there are many more enlarged,
multinucleate cells. [Right] Quantification of multinucleate (nuclei ≥ 3) RPE cells per field view demonstrating that Cfh+/-~HFC RPE flat mounts have the largest number of multinucleate cells per field view of all the groups. Data are presented as mean ± SE. * indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype by diet interaction by ANOVA. # indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype interaction by ANOVA. N=6-8 per group.

**APOE4, Cfh+/--HFC mice have a similar ocular phenotype to Cfh+/-~HFC mice**

Our lab has previously shown that aged apolipoprotein E4 targeted replacement (APOE4) mice [100] when placed on an HFC diet for 8 weeks develop sub-RPE deposit formation, as well as, RPE dysmorphogenesis and electroretinogram deficits in an amyloid-beta dependent manner [99, 101]. Thus, we hypothesized that adding the APOE4 background to the Cfh+/ mice would exacerbate their ocular phenotype. APOE4, Cfh+/ mice were generated and aged to 90 weeks of age and placed on HFC diet for 8 weeks, along with APOE4, Cfh+/- and APOE4, Cfh-/- controls. APOE4, Cfh+/- mice have approximately half the circulating plasma levels of CFH compared to APOE4 mice on either diet, while there is no detectable CFH in the plasma of APOE4, Cfh-/- mice (Fig. 11A). As previously reported, we found that decreased levels of CFH in aged Cfh+/- mice fed either diet leads to a decrease in intact C3 in the plasma, with no measureable C3b (Fig. 11B) [89, 92]. In contrast, there is no intact C3 in plasma of APOE4, Cfh-/- mice due to immediate conversion of C3 to C3b (Fig. 11B).
**Figure 11:** Fluid phase complement dysregulation in old APOE4, Cfh+/- mice. (A) Densitometric analysis of CFH immunoblots from plasma across genotype and diet. Note, APOE4, Cfh+/- mice have approximately half as much CFH in the plasma as APOE4 mice. (B) Densitometric analysis of C3/C3b immunoblots from plasma of APOE4, APOE4, Cfh+/- and APOE4, Cfh-/- mice fed a normal (ND) or high fat, cholesterol-enriched (HFC) diet. The dose effect of CFH on circulating C3 in plasma shows fluid phase complement dysregulation in APOE4, Cfh+/- mice, compared to APOE4 mice and the absence of circulating intact C3 in APOE4, Cfh-/- mice.

**APOE4 background decreases the increased sub-RPE deposit formation seen in Cfh+/-~HFC mice**

Initially we sought to investigate the relative changes to sub-RPE deposit formation seen in APOE4, Cfh+/-~HFC mice compared to Cfh+/-~HFC mice. Within APOE4 transgenic mice the same trend was observed in sub-RPE deposits as shown in CFH deficient mice with mouse APOE (Fig. 12A, B) [99]. However, we consistently observed smaller sub-RPE deposits in APOE4, Cfh+/-~HFC mice transgenic mice than what was previously
observed in Cfh+/-~HFC mice (Fig. 12B, C). These data show that combining CFH deficiency with the APOE4 background does not result in larger deposits than CFH deficiency alone and suggests a role of APOE in regulating sub-RPE deposit formation.

**Figure 12:** Sub-RPE deposit formation in APOE4,Cfh+/-~HFC mice is decreased compared to Cfh+/-~HFC mice. (A) Transmission electron micrograph (25,000x, scale = 0.5 µm) of normal retinal pigment epithelium (RPE), Bruch’s membrane (BrM), and choriocapillaris (CC) in an aged APOE4 ND mouse. (B-C) Representative images of sub-RPE deposits (arrows) observed in an aged APOE4,Cfh+/-~HFC mouse (B) and Cfh+/-~HFC mouse (C). Significant, basal deposits were also observed in the APOE4,Cfh+/-~HFC mouse, and preliminary data shows a similar trend observed in non-APOE4 transgenic mice is also seen but to a lesser degree in APOE4 mice (data not shown). Scale bar = 0.5 µm.

**APOE4 background does not increase multinucleate RPE seen in Cfh+/-~HFC mice**

Multinucleate RPE cells were quantified by immunostaining RPE flat mounts with the tight junction protein, ZO-1, as shown in Fig. 10A and 13A [99]. Quantitative analysis showed that there is a statistically significant, increase in multinucleate RPE
cells (≥ 3 nuclei) in APOE4, Cfh/+ mice following HFC mice, whereas, no statistically significant changes were seen in other genotypes following HFC diet (Fig. 13B). In contrast, no detectable morphological differences in RPE were seen between APOE4 and APOE4, Cfh/-/ mice in response to HFC diet (Fig. 13). Although, APOE4, Cfh+/--HFC mice showed increased numbers of multinucleate RPE it was not higher than detected in Cfh+/--HFC mice (Fig. 10).

**Figure 13:** APOE4, Cfh+/--HFC mice show RPE damage in response to the HFC. (A) Representative confocal fluorescence images of flat mounts of the central RPE from >90 week old APOE4, APOE4, Cfh+/- and APOE4, Cfh/-/ mice fed a ND or HFC diet that were stained with Hoechst 33342 (blue, nuclei) and anti-ZO-1 (green) and imaged RPE apical side up with the neural retina removed. In APOE4, Cfh+/---HFC mice there are many more enlarged, multinucleate cells. (B) Quantification of multinucleate (nuclei ≥ 3) RPE cells per field view demonstrating that APOE4, Cfh+/---HFC RPE flat mounts have the largest number of multinucleate cells per field view of all the groups, however, the effect does not appear exacerbated compared to Cfh+/---HFC mice. Data are presented as mean ± SE. * indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype
by diet interaction by ANOVA. # indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype interaction by ANOVA. N=6-8 per group.

**APOE4 background exacerbates photoreceptor loss following HFC diet**

Outer nuclear layer (ONL) loss in Cfh+/−HFC mice was quite moderate. The role of complement dysregulation on APOE4 retinal morphology was assessed by morphometric analysis of plastic retinal sections. Representative retinal sections (Fig. 14A) and ONL thickness histograms (Fig. 14B) are shown to highlight the retinal thinning observed in APOE4 and APOE4, Cfh+/−HFC mice compared to APOE4, Cfh−/−HFC mice. Quantitative analysis of ONL and inner nuclear layer (INL) area showed no statistically significant changes in ONL or INL area in APOE4−HFC or APOE4, Cfh−/−HFC mice (Fig. 14C, D). However, it should be noted that in APOE4−HFC mice an 11% decrease in ONL area and a 10% decrease in INL area were observed but did not reach statistical significance (Fig. 14C, D). This effect appeared to be exacerbated in APOE4, Cfh+/−HFC mice where a statistically significant 15% decrease (p<0.05) in ONL area and 17% decrease (p<0.05) in INL area was observed in APOE4, Cfh+/−HFC mice (Fig. 14C, D). ONL/INL ratios were unaffected in all genotypes (Fig. 14E). Retinal thinning was absent in APOE4, Cfh−/−HFC mice, further, highlighting the dependence of retinal thinning on the complement system. Notably, this is greater in magnitude than the 8% decrease in ONL area (p<0.05) and no statistically significant thinning of INL in Cfh+/−HFC mice.
Figure 14: Significant Retinal thinning in APOE4, Cfh+/-~HFC mice. (A) Representative light microscopic images of toluidine blue-stained sections from eyes of aged APOE4, APOE4, Cfh+/- and APOE4, Cfh-/- mice fed a HFC diet. OS, outer segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. (B) Photoreceptor degeneration was quantified by using the mean and ± SE of total ONL area. APOE4, Cfh+/-~HFC mice have decreased ONL thickness. (C) Significant INL thinning was also observed in APOE4, Cfh+/-~HFC mice. (D) INL and ONL thickness was measured at 200 µm intervals from the optic nerve to the ora serrata. Data are expressed as mean and ± SE of ONL thickness versus distance from optic nerve comparing mice fed ND (black) to HFC (green) for each genotype. * indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype by diet interaction by ANOVA. # indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype interaction by ANOVA. N=6-8 per group
2.3 Discussion

The Cfh+/--HFC mice we have characterized here display a robust AMD-like ocular phenotype, with substantial sub-RPE deposit formation, multinucleate RPE and atrophy, photoreceptor cell loss and visual function decline. It should be noted that nearly 10% of sub-RPE deposits were greater than 4 \( \mu \)m in height, which represents the largest deposits detected in any AMD mouse model within our lab [87, 101]. Comparisons to other studies are difficult due to the lack of systematic quantification and the use of younger mice [87, 88, 91, 101-109]. Data indicate that RPE damage and visual function loss in the Cfh+/--HFC model are initiated by sub-RPE deposit formation, primarily based upon the findings that Cfh-/-HFC mice develop substantial sub-RPE deposits without subsequent damage to the RPE and photoreceptors and that age-matched wild type B6 mice, which have an intact complement system, barely develop RPE deposits or damage to their RPE and photoreceptors. Thus, our data show that sub-RPE deposit formation occurs with the combination of diet and CFH deficiency prior to RPE/photoreceptor dysfunction. Furthermore, it is important to note that the cornerstones of the Cfh+/--HFC model are predominant factors established within the human disease: advanced age, genetic alteration of CFH, complement dysregulation and cholesterol/lipid metabolism perturbation [65, 110, 111]. Analysis of the ocular phenotype of these mice revealed two mechanisms which are the focus of the remainder of my thesis project: 1) the role of complement activation in RPE damage; and 2) the role
of CFH in the regulation of sub-RPE deposit formation, which will be interrogated in the proceeding chapters.

*APOE4*–HFC mouse model of AMD is established within our lab and we hypothesized that adding the *APOE4* background to the *Cfh+/-* would exacerbate the AMD-like phenotype seen in these mice. Within this study, the *APOE4*–HFC mice showed ERG declines and ONL loss. Furthermore, ERG decline and ONL loss appeared exacerbated on *APOE4, Cfh+/-*–HFC mice compared to mice not on the *APOE4* background. The effect of C3 depletion in *APOE4, Cfh+/-*–HFC mice suggests that much of the ocular phenotype in the *APOE4*–HFC mice, including ERG decline and ONL loss, is complement dependent. However, overall, similar trends were seen in *APOE4, Cfh+/-*–HFC and *APOE4, Cfh-/-*–HFC mice compared to mice not on the *APOE4* background. In fact, sub-RPE deposit formation appeared attenuated on the *APOE4* background.

Interestingly, the *APOE4* genotype is protective in genetic analysis of AMD patients [112]. ApoE plays major roles in the maturation of HDL particles for reverse cholesterol transport [113, 114]. Genetic and biochemical studies suggest that the reverse cholesterol transport system including ApoE, ABCA1, Cyp46A and HDL play a role in AMD development [112, 114-117]. However, the role of the reverse cholesterol transport system in AMD has not been determined. Our data suggest a role of ApoE in sub-RPE deposit formation. Potentially, ApoE has a protective role in RPE by modulating the maturation of basally deposited lipoproteins.
2.4 Methods

Mice

Mice were maintained in accordance with the Institutional Animal Care and Use Committee at Duke University. Cfh+/+ (B6), Cfh-/-, Cfh+-/- mice were generated as described [89]. We have confirmed that none of the mice carry the rd8 mutation [118]. Aged male B6, Cfh+-/- and Cfh-/- mice (n = 84; 91-110 wks) were maintained on a normal rodent chow diet [normal diet (ND), Isopurina 5001; Prolab], and a subset of cage- and litter-mate mice were switched to a HFC diet (n = 53; TD 88051; Harlan Teklad) for 8 wks. All mice were housed conventionally on a middle rack under ambient light conditions to control for light exposure. Mice were randomly assigned to treatment groups with an even distribution by age.

Western blots analysis for quantification of FH, C3/C3b and FB in plasma and tissue lysates

Western blot analysis was performed as previously described [87]. Briefly, plasma and RPE/choroid samples were diluted and run, non-reduced, on 10% Bis-Tris Criterion XT gels in MOPS buffer; transferred to nitrocellulose, then probed with goat anti-FH (Quidel), goat anti-FB (Kent Laboratories) or rabbit anti-C3d (Dako). Loading controls for plasma and RPE/choroid were rabbit anti-albumin (Abcam) and mouse anti-GADPH (Chemicon), respectively.
**Hemolytic assay for the functional measurement of complement activity in mouse plasma**

Hemolytic assays were performed as previously described [87]. Sheep red blood cells were coated with rabbit anti-sheep erythrocyte antiserum (Hemolysin). Six doubling dilutions of mouse plasma were added to a fixed amount of C3-depleted human serum and incubated for 1 hour at 37°C with the antibody primed sheep erythrocytes. Ice cold PBS with EDTA was added to stop the reaction and tubes were spun at 3000 rpm and optical density was measured at 412 nm. The reciprocal of the dilution for which 50% hemolysis was achieved gave the hemolytic units for that sample. 200 ng/μL of C3 and/or CFH was added to the reaction tubes for the experiment shown in Fig. S1.

**In vivo visual function analysis by electroretinography**

Electroretinography was performed as previously described [87, 99]. Briefly, mice were dark-adapted overnight, pupils dilated with 0.5% Tropicamide and 1.25% Phenylephrine, and anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). Scotopic ERGs were recorded using an Espion E2 system (Diagnosys LLC), at increasing flash intensities. The data points from the b-wave stimulus-response curves were fitted according to a previously published equation using the least-square fitting
procedure [97] (OriginPro 9.0; OriginLab). Statistical significance was determined using the mean values for Bmax1 and Bmax2.

Quantification of sub-RPE deposits by electron microscopy

Quantification of sub-RPE deposits was performed as previously described [87]. Briefly, thin sections of 60 - 80 nm were cut for each part, collected onto 400 mesh thin-bar copper grids (T400-Cu, Electron Microscopy Sciences), and stained with Sato’s lead. Images containing the basal RPE and BrM were taken on the parts of the section adjacent to the grid’s bar. Using this method, 98 +/- 18 (mean +/- SD) images were taken per mouse, sampling most the retina. For each image, the height of the sub-RPE deposits was measured using ImageJ software (version 1.46, NIH) and the cumulative frequency and spatial distribution of the deposit height was plotted using OriginPro 9.0 software.

Analysis of RPE damage based on flat mount quantification of multinucleate cells

RPE flat mount preparation was performed as previously described [99]. Briefly, flat mounts of RPE cells were stained with a rabbit antibody against ZO-1 (40–2200, 1:100; Invitrogen) and Hoechst 33342, and confocal images were captured on a Nikon Eclipse C1 microscope. Morphometric analysis was performed by a masked grader from each central quadrant. The number of multinucleate (nuclei ≥ 3) cells per central field view was counted.
Quantification of ONL thickness and assessment of RPE atrophy on toluidine blue plastic sections

ONL thickness was analyzed as previously described [87]. Briefly, posterior eyes were osmicated, dehydrated with ethanol, infiltrated and embedded in a mixture of Epoxy and Spurrs resin. Semi-thin sections were cut superior to inferior, mounted, and stained with toluidine blue. 40X images were merged with Photoshop and ONL was measured versus distances from the optic nerve. Data is presented as ONL height versus distance from optic nerve. INL thickness was also measured versus distance from the optic nerve and ONL/INL area was used to control for angle of the plastic section cut, which was also statistically significant in Cflh+- following HFC diet. For RPE height measurements, 100X sections were required, thus, field views were taken from the optic nerve to the ora serrata and RPE cell height was averaged between the first and last possible RPE height measurement for each image and this observation was presented as graph of the mean +/- standard error (SE) of RPE height for each field view from optic nerve. RPE heights measurements were not an outcome measure of the original experiment and are thus not subject to post-experimental statistical analysis, but rather are presented as a quantitative observation.
3. Complement Activation leads to RPE damage in \textit{Cfh}+/\textasciitilde \textit{HFC} model

\textit{3.1 Introduction}

The size and number of drusen is diagnostic for early AMD and has a clinically predictive value for the progression to late stage AMD where RPE atrophy occurs and visual acuity is significantly affected [17]. Although sub-RPE deposit formation has been well established in AMD, the relationship between the deposition of sub-RPE debris and the development of the RPE and photoreceptor pathology in AMD has not been established. Sub-RPE deposits can be oxidized and have been shown to activate complement through the binding of C-reactive protein binding to oxLDL [119, 120], or by oxidation-specific neo-epitopes rendering the modified lipoproteins immunogenic [121, 122]. As a consequence, membrane attack complexes (MAC, C5b-9) can be found in sub-RPE deposits. Interestingly, however, MAC is not found on RPE cells themselves. In fact, RPE have high levels of membrane complement regulatory proteins and only after high levels of oxidants or anti-RPE antibody insults does MAC formation occur on RPE cells \textit{in vitro} [123, 124]. Thus, although it is clear that complement dysregulation is a critical predisposing step in AMD development, it is unclear how complement activation and sub-RPE deposits contribute to cellular dysfunction [125, 126]. Our data show that aged \textit{Cfh}+/\textasciitilde \textit{HFC} and \textit{Cfh}+/\textasciitilde \textit{HFC} mice both accumulate significant sub-RPE deposits in response to HFC diet but only the \textit{Cfh}+/\textasciitilde \textit{HFC} mice develop RPE and photoreceptor dysfunction in response to this extracellular debris. This suggested a role
of complement activation in RPE damage following sub-RPE deposit formation. In order
to test the role of complement activation in RPE damage following sub-RPE deposit
formation we analyzed local RPE/choroid complement activation, including the
chemoattractant C5a, and the potential for consequent immune cell recruitment in the
$\text{Cfh}^{+/--}\text{HFC}$ and $\text{Cfh}^{-/-}\text{HFC}$ mice. Following these observations we treated $\text{Cfh}^{+/--}\text{HFC}$
mice with an anti-C5a antibody to determine the effect C5a on immune cell recruitment
and RPE damage.

### 3.2 Results

**Local ocular deficiency of key complement components in $\text{Cfh}^{-/-}$ mice.**

Since sub-RPE deposits accumulated in both aged $\text{Cfh}^{+/}$- and $\text{Cfh}^{-/-}$ mice fed the
HFC diet, we sought to unravel why the $\text{Cfh}^{+/}$- mice develop RPE dysmorphogenesis
and visual function loss in response to HFC diet whereas the $\text{Cfh}^{-/-}$ animals do not.

Based on the absence of intact C3 in the $\text{Cfh}^{-/-}$ plasma, we suspected a lack of
complement activation in the BrM of $\text{Cfh}^{-/-}\text{HFC}$ mice, which would explain why there
was no pathological response to the increased sub-RPE deposit formation in these mice.
Western blot quantification of C3, C3b/iC3b (Fig. 15A) and FB (Fig. 15B) in the
RPE/choroid revealed almost no intact C3, an absence of FB and very little C3b/iC3b in
$\text{Cfh}^{-/-}$ mice. C3 was also undetectable in BrM by immunohistochemistry in $\text{Cfh}^{-/-}\text{HFC}$
mice, whereas, substantial C3 was localized to BrM of the $\text{Cfh}^{+/--}\text{HFC}$ mice (Fig. 15C). In
contrast to the Cfh-/- mice, Cfh+/---HFC mice have decreased C3 levels following HFC diet (p<0.05) in the RPE/choroid (Fig. 15A), showing increased local C3 consumption/activation, as plasma C3 levels are unchanged (Fig. 7C). C3 consumption/activation is not seen following HFC diet in control wild type B6 mice due to their abundance of CFH or in Cfh-/- animals due to their lack of C3. These results confirm that Cfh-/---HFC mice lack key activators of the complement cascade in the RPE and BrM whereas that local complement activation is occurring in Cfh+/---HFC mice.

**Figure 15:** Complement activation in Cfh+/---HFC mice versus local deficiency of key complement components in Cfh-/- mice. (A-B) Densitometric analysis of C3 (A) and FB (B) immunoblots in the RPE/choroid across genotype and diet. Cfh-/- mice have almost no detectable levels of intact C3, only minimal detectable C3b/iC3b and no detectable levels of FB in the RPE/choroid lysates, whereas, Cfh+/- mice have
significant intact C3 and show a statistically significant decrease in intact C3 following HFC diet (p<0.05), which is not seen in wild type B6 or Cfh-/- mice. Data is presented as mean ± SE. N=6-8 per group. (C) Anti-C3 (green) immunohistochemistry with Hoechst 33342 (blue, nuclei) staining of posterior eyecups. 100x images at the RPE/BrM interface show no detectable C3 can be localized to BrM, where sub-RPE deposit formation occurs in Cfh-/-−HFC mice, whereas in Cfh+/-−HFC mice significant amounts of C3 can be detected in BrM. N=3 mice per group. Scale bars = 5 μm.

Monocytosis and increased extravascular monocytes in the RPE/choroid of Cfh+/-−HFC mice.

A major role of the complement system is the activation of the innate and adaptive immune systems by the complement cleavage products, C3a and to a greater extent, C5a [70]. As expected, increased C5a levels are detected in the plasma of Cfh+/-−HFC mice compared to Cfh-/-−HFC mice, further demonstrating the impairment of complement activation in Cfh-/- mice (Fig. 16A). C5a has an established role in immune cell recruitment and has also been implicated in the development of atherosclerotic lesions [127-131]. We were particularly interested in monocytes due to the role of monocytes within AMD patients and the potential role of this population in the phagocytosis sub-RPE debris [7, 132-134]. Significant populations of myeloid cells (CD11b+) were observed in the RPE/choroid near BrM and the RPE by immunohistochemistry (IHC) of Cfh+/-−HFC mice (Fig. 16B). Thus, we hypothesized that a functional consequence of dysregulated complement activation in the Cfh+/-−HFC
mice compared to the Cflh/-/-HFC mice is enhanced mononuclear phagocyte (MNP) recruitment, which we tested by flow cytometry. Mononuclear phagocytes are non-polymorphic neutrophils of myeloid lineage and included, bone marrow-derived monocytes, macrophages, dendritic cells and microglia [135]. In the peripheral blood an established gating strategy was used to determine the percentage of classical and non-classical monocytes [98, 136, 137]. We were unable to detect changes between either monocyte subpopulations in Cflh/- mice with diet (Fig. 17). In contrast, in Cflh+/- mice there was a 5.3-fold increase in classical monocytes (2.4% versus 12.8%) and a 2.5-fold increase (3.9% versus 9.8%) in non-classical monocytes following HFC diet, both of which reached statistical significance by chi-squared analysis (p<0.05). In addition, in wild type mice there was a 1.5-fold increase (5.1% versus 7.9%) and 2.6-fold increase (2.1% versus 5.5%) in classical and non-classical monocytes (both of which are CD11b+), respectively, which was also significant by chi-squared analysis (p<0.05) (Fig. 17). Overall the Cflh+/-HFC mice had the largest population of classical (12.8%) and non-classical (9.8%) monocytes of the three Cflh genotypes and this was statistically significantly higher than in Cflh/-/-HFC animals for non-classical monocytes (9.8% versus 4.1%, p<0.05) and B6-HFC for both classical (12.8% versus 7.9%, p<0.05) and non-classical monocytes (9.8% versus 5.5%, p<0.05) (Fig. 17). The role of classical and non-classical monocytes in AMD has not been established, however, these populations have diverse functions warranting sub-population analysis [135].
Figure 16: C5a levels and CD11b+ myeloid in Cfh+/--HFC mice. (A) C5a ELISA was performed on mouse plasma from Cfh+/+ and Cfh+/-- mice fed a ND or switched to a HFC diet. Cfh+/--HFC mice have significantly increased C5a levels compared to Cfh+/--HFC mice. Data is presented as mean ± SE. N=7-12 per group. (B) Anti-CD11b (green) with Hoechst 33342 (blue, nuclei) staining of posterior eye cups of Cfh+/--HFC mice shows myeloid cells in and around the RPE/choroid and RPE/BrM.

Figure 17: Monocytosis in Cfh+/--HFC mice versus local deficiency of key complement components in Cfh+/-- mice. Classical and non-classical monocyte populations were identified by Ly6C and CD43 gating [98]. A statistically significant 5.3-fold increase (p<0.05) in classical monocytes was detected in Cfh+/--HFC mice in response to diet, in contrast Cfh+/-- have a baseline increase in classical monocytes but
no change was observed in Cfh/-/-HFC mice, while B6 mice show a 1.5 fold increase (p<0.05). Non-classical monocytes showed a distinct, statistically significant, 2.5-fold increase (p<0.05) in Cfh+/-HFC mice which was not observed in Cfh/-/-HFC mice, but was also observed to in B6 mice (p<0.05). Note that Cfh+/-HFC mice represent the largest population of classical (12.8%) and non-classical (9.8%) monocytes in all genotypes.

In order to further test our hypothesis that Cfh+/-HFC mice develop a complement response to sub-RPE deposits, which is not seen in Cfh/-/-HFC mice, we tested for recruitment of MNPs in the RPE/choroid. Due to the high vascularity of the choroid, fluid perfusion is not a sufficient means to flush intravascular cells from contaminating flow cytometry. Therefore we injected anti-CD45 fluorescent conjugated antibodies (CD45-IV) intravascularly 5 minutes prior to euthanasia to distinguish cells located in the intravascular space from those in the extravascular space [138]. With this method we were able to identify the presence of extravascular MNPs recruited to the RPE/choroid within our aged Cfh+/-HFC model and Cfh/-/-HFC controls [139]. MNP cells were increased in Cfh+/- mice following HFC diet (p<0.05) but were unchanged in Cfh/-/- mice (Fig. 18A, upper panel). Using Ly6C and CD64 to differentiate MNPs, three clear subpopulations could be identified within the RPE/choroid, a Ly6C<sup>lo</sup>, a Ly6C<sup>hi</sup> and a Ly6C<sup>int</sup> CD64<sup>+</sup> population (Fig. 18A, lower panel). The CD64<sup>+</sup> MNP subpopulation in the Cfh+/-HFC model represents the largest myeloid cell population, increasing 2.8-fold over ND controls (10.9% versus 3.9%, p<0.05) whereas it is barely detectable in Cfh/-/-HFC mice (Fig. 17A, bottom panel and 17B). Further analysis showed that the CD64+
MNP population has elevated expression of F4/80 and MHC class II and it could not be found in peripheral blood or the choroidal intravascular space [139]. Together these findings help to establish the absence of a complement mediated effector response to sub-RPE deposits in the Cfh-/-HFC mice.

**Figure 18:** In order to assess RPE/choroid monocyte populations, intravascular flow cytometry analysis was performed [98]. (A, top panel) MNP were increased in Cfh+/- mice following HFC diet but were unchanged in Cfh-/- mice. (A, bottom panel) Three clear MNP populations were evident in the extravascular space a Ly6C^hi^, a Ly6C^lo^ and a CD64^+^ population. (B) Note that the CD64^+^ subpopulation in Cfh+/- HFC animals represents the largest myeloid cell population (CD64^+, Ly6C^hi^, Ly6C^lo^ and polymorphic neutrophils (PMN)) across all genotypes and diets within the study. The statistical significance of the MNP population changes was determined by using a Chi-squared test for p<0.05. * indicates statistical significant diet interaction. Data shown represents a pooled analysis of 6 mice per group.

*Mouse Anti-C5a antibody in vitro characterization and in vivo dose response*

In order to establish that the anti-C5a antibody was able to deplete C5a levels, chinese ovarian hamster (CHO) cells expressing C5aR were incubated with 0.4 nM of mC5a agonists and increasing concentrations of mouse anti-C5a antibody (4C9, Rinat).
To determine the IC50 of the 4C9 antibody FLIPR analysis was done to determine the influx of calcium following agonist-receptor interaction. The IC50 of 4C9 antibody was calculated to be 0.21 nM (Fig. 19A).

In order to test the *in vivo* dose response of the 4C9 in the eye we made use of the complement-dependent ERG effects seen in the sodium iodate (NaIO4) model of AMD (unpublished, Pfizer). Pre-treatment with increasing concentrations of 4C9 (0 mg/kg, 3 mg/kg, 10 mg/kg, 20 mg/kg, 30 mg/kg or 60 mg/kg) or isotype control (60 mg/kg), showed a dose effect of 4C9 protection of electroretinogram responses following NaIO4 treatment (Fig. 19B). This analysis found a sub-therapeutic dose to be 10 mg/kg or less and a therapeutic dose to be 30 mg/kg or more.

**Figure 19**: 4C9 anti-C5a antibody characterization and *in vivo* dose response. (A) C5aR cells cultured in the presence of Calcium 3 dye given 0.4 nM mC5a agonist with increasing concentration of antibody and Calcium efflux was measured over a period of 2 minutes by fluorescence imaging plate reader (FLIPR). An IC50 of 0.12 nM was determined for 4C9 against 0.4 nM mC5a. (B) To determine the *in vivo* dose response of anti-C5a (4C9) in the context of posterior eye inflammation 25 mg/kg of sodium iodate was injected into the intravitreal space with a single injection of increasing concentrations of intraperitoneal anti-C5a (3, 10, 20, 30 and 60 mg/kg) or
60 mg/kg of isotype control (Iso 60 mg/kg). Electroretinogram b-wave amplitudes are presented to increasing flash intensity of light (B). The therapeutic dose was 30 mg/kg and sub-therapeutic dose was 10 mg/kg.

**Anti-C5a treatment blocks monocytosis and CD64+ MNP recruitment**

Our previous work established a classical and non-classical monocytosis in the peripheral blood coinciding with increases in a CD64+ MNP population in the RPE/choroid. Thus, we hypothesized that CD64+ monocyte recruitment was due to increased C5a levels, which we tested by anti-C5a treatment and flow cytometry. Aged Cfh+/-~HFC mice were given either 10 mg/kg (sub-therapeutic dose) or 30 mg/kg (therapeutic dose) of anti-C5a (4C9) or PBS vehicle weekly for 8 weeks coinciding with the start of HFC diet. Following 8 weeks of diet with or without anti-C5a therapy, flow cytometry was performed on the peripheral blood and RPE/choroid. In the peripheral blood an established gating strategy was used to determine the percentage of classical and non-classical monocytes [136, 137]. Classical and non-classical monocyte populations (2.5% versus 15.8% and 2.9% versus 5.2%) were increased significantly (p<0.05) following HFC diet as previously described and unchanged with 10 mg/kg of anti-C5a (15.8% versus 11.3% and 5.2% versus 5.8%), while 30 mg/kg of anti-C5a led to significantly decreased levels of classical (15.8% versus 4.5%) (p<0.05) and non-classical (5.2% versus 0.6%) (p<0.05) monocytes (Fig. 20A,B). In order to further test our hypothesis we sought to test if Cfh+/-~HFC mice develop a C5a-dependent complement-
mediated CD64+ MNP recruitment to the RPE/choroid. The percent of intravascular CD64+ MNP cells were increased in Cflt+/- mice following HFC diet (1.8% versus 10.9%) (p<0.05) and unchanged with 10 mg/kg anti-C5a (10.9% versus 8.3%) (Fig. 20C, D). 30 mg/kg of anti-C5a treatment led to a statistically significant decrease in CD64+ MNP (10.9 versus 2.9) (p<0.05) (Fig. 20C, D). Together these findings establish that the complement break down product C5a leads to peripheral blood monocytosis and CD64+ MNP recruitment following HFC diet in aged Cflt+/- mice.

**Figure 20:** Anti-C5a blocks the monocytosis and increased RPE/choroid extravascular CD64+ MNP in Cfh+/- HFC model. (A) Classical and non-classical monocyte populations were determined by Ly6C and CD43 gating as shown previously described (Toomey et al). A statistically significant 6-fold increase (p<0.05) in classical monocytes and 1.8 fold increase (p<0.05) in non-classical monocytes was detected in Cfh+/- HFC mice in response to diet. 30mg/kg of anti-C5a therapy was able to prevent this classical and non-classical monocytosis, whereas sub-therapeutic dosing at 10mg/kg was not. (B and C) A 2.8 fold increase (p<0.05) in RPE/choroid CD64+ MNP was seen in Cfh+/- mice on HFC diet. 30mg/kg of anti-C5a therapy was able to prevent this classical and non-classical monocytosis, whereas sub-therapeutic dosing at 10mg/kg was not.
therapy was able to prevent this increase in CD64+ MNPs, whereas sub-therapeutic dosing at 10mg/kg was not.

Anti-C5a therapy protects RPE damage following sub-RPE deposit formation

Extracellular lesions that form between the RPE and BrM characterize early AMD and these can be focal (drusen) or diffuse (basal deposits) [93-95]. Based on our previous study we show that sub-RPE deposits form in a CFH dependent manner. We used electron microscopy of RPE/BrM to analyze the thickness of sub-RPE deposits that accumulated in aged mice in response to Cfh genotype and diet following anti-C5a therapy. As expected, anti-C5a therapy did not affect sub-RPE deposit formation previously seen in Cfh+/- HFC mice (Fig. 21A). In order to assess damage to the RPE cells in aged Cfh+/- and Cfh-/- mice resulting from exposure to the HFC diet, multinucleate RPE cells were quantitated by immunostaining RPE flat mounts with the tight junction protein, zonula occludens-1 (ZO-1) (Fig. 21B) [99, 139]. Exposure to a HFC diet resulted in a large, statistically significant, increase in enlarged multinucleated RPE cells in the Cfh+/- mice. Strikingly, anti-C5a therapy protected RPE cells from damage following HFC diet (Fig. 21B and C). Together these data demonstrate that anti-C5a therapy protects RPE dysmorphogenesis in the Cfh+/- HFC mice in spite of the presence of significant sub-RPE deposit formation.
**Figure 21:** Anti-C5a protects RPE cells from damage in spite of significant sub-RPE deposit formation. (A) Transmission electron micrograph images of basal deposits along Bruch’s membrane (BrM). Large (>2μm) deposits were often seen in the Cflt+/−HFC and Cflt+/−HFC mice treated with 30mg/kg of anti-C5a, while only minimal sub-RPE deposit could be detected in aged-matched Cflt+/− fed a normal diet. N=3 mice per group. (B) Confocal fluorescence images of flat mounts of the central RPE from >90 week old mice Cflt+/− mice fed a ND or HFC diet treated with 0 or 30 mg/kg of anti-C5a that were stained with Hoechst 33342 (blue, nuclei) and anti-ZO-1 (green) and imaged RPE apical side up with the neural retina removed. In Cflt+/−HFC mice there are many more enlarged, multinucleate cells following HFC diet, whereas RPE cells were protected from damage in Cflt+/−HFC mice treated with 30mg/kg of anti-C5a. (C) Quantification of multinucleated (nuclei ≥ 3) RPE cells per field view demonstrating that Cflt+/−HFC mice treated with 30mg/kg of anti-C5a are protected. Data are presented as mean ± SE. * indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype by diet interaction by ANOVA. # indicates post-hoc Tukey for a p<0.05 following a statistically significant genotype interaction by ANOVA. N=6-8 per group.

**Anti-C5a therapy protects scotopic visual function**

Visual function of the retinas of aged Cflt+/−HFC mice treated with 0 or 30 mg/kg of anti-C5a was measured by scotopic electroretinogram (ERG). Scotopic visual function
decline was seen in Cfh+/− mice following HFC diet (p<0.05). A moderate but statistically significant (p<0.05) improvement was seen in Cfh+/--HFC mice treated 30 mg/kg of anti-C5a (Fig. 22). The data show that Cfh+/− have a significant visual function decline following HFC diet, which appears to be improved with anti-C5a therapy, however, this did not reach statistical significance.

Figure 22: Anti-C5a ameliorates visual function attenuation in Cfh+/--HFC mice. Scotopic electroretinogram (ERG) flash responses in wild type B6, Cfh+/− and Cfh+/-- mice fed a ND or HFC diet. Stimulus response curves of b-wave amplitudes. Data are expressed as mean and ± SE of the stimulus response curve overlaid with B = (Bmax1*I/I+I1)/(Bmax2*I/I+I2) comparing ND (black), HFC (green) and HFC 30mg/kg of anti-C5a (pink). Cfh+/--HFC mice were significantly worse following HFC diet, which was partially ameliorated with anti-C5a therapy. N=6-12 mice per group.

3.3 Discussion

This difference between the two observed ocular phenotypes observed in Cfh+/--HFC and Cfh+--HFC mice can be explained by the lack of a reservoir of intact C3 in Cfh+--HFC mice during HFC diet challenge. We hypothesize that sub-RPE deposits form at an accelerated rate in Cfh+--HFC mice compared to normal age-matched
controls (B6–HFC) due to the lack of CFH, however, in the absence of C3 and FB no subsequent complement activation occurs; thus failing to initiate recruitment of MNPs, which may be the root cause of RPE dysfunction and subsequent photoreceptor degeneration. This hypothesis is supported by the absence of complement breakdown products, RPE/choroid monocyte recruitment, RPE damage, ONL thinning and scotopic ERG attenuation, in spite of the significant sub-RPE deposit formation following HFC diet in Cfh⁻/⁻ mice.

Contrasting the abundance and absence of complement breakdown products in the Cfh⁺/⁺–HFC and Cfh⁻/⁻–HFC models, respectively, led us to investigate immune cell recruitment within the Cfh⁺/⁺–HFC model due to the established role of C5a in immune cell recruitment [129]. In addition, immune cell recruitment has been hypothesized to be a contributing factor to human AMD pathogenesis [29, 126]. Evidence for monocyte involvement stems from analysis of genetic polymorphisms and immunohistochemical analysis of AMD post-mortem tissue; however, the contribution of this immune cell recruitment to the disease phenotype had not been defined [140-146]. Using the Cfh⁺/⁺–HFC model we established the presence of both a classical and non-classical monocytosis in the blood following HFC diet. These findings are consistent with previous reports of monocytosis following HFC diet challenge in rodents [147]. We were also able to distinguish a significant, exclusive increase in a CD11b+, CD64+, F4/80+, I-
A/E+ MNP subpopulation in Cfh+/--HFC choroid, which we suspect to be a monocyte-derived macrophage population.

In subsequent analysis we investigated the role of C5a monocyte recruitment and how these events affect the RPE dysfunction seen within the Cfh+/--HFC model. We show that blockade of C5a blocks monocyte recruitment and prevent RPE dysfunction in the Cfh+/--HFC mice. Elner et al. has published a series of co-culture experiments with human monocyte derived-PBMCs and RPE cells, where they show that via both direct contact and production of cytokines monocytes can cause apoptosis and ROS production in RPE [148-151]. It is possible that the root cause of RPE dysfunction is a direct pathological effect of monocytes on RPE cells. In order to further support a causal relationship between monocytes and RPE damage in the Cfh+/--HFC mice we are currently doing serial immunohistochemical localization of monocytes in Cfh+/--ND, Cfh+/--HFC and Cfh+/--HFC anti-C5a mice to establish the proximity of recruited monocytes to the RPE/BrM area.

3.4 Materials and Methods

Mice

Mice were maintained in accordance with the Institutional Animal Care and Use Committee at Duke University. Cfh-/-, Cfh+/-- mice were generated as described [89]. We
have confirmed that none of the mice carry the rd8 mutation [118]. Aged male Cfh+/− and Cfh−/− mice (n = 84; 91-110 wks) were maintained on a normal rodent chow diet [normal diet (ND), Isopurina 5001; Prolab], and a subset of cage- and litter-mate mice were switched to a HFC diet (n = 53; TD 88051; Harlan Teklad) for 8 wks. All mice were housed conventionally on a middle rack under ambient light conditions to control for light exposure. Mice were randomly assigned to treatment groups with an even distribution by age.

**Western blots analysis for quantification of FH, C3/C3b and FB in tissue lysates**

Western blot analysis was performed as previously described [87]. Briefly, RPE/choroid samples were diluted and run, non-reduced, on 10% Bis-Tris Criterion XT gels in MOPS buffer; transferred to nitrocellulose, then probed with goat anti-FB (Kent Laboratories) or rabbit anti-C3d (Dako). Loading controls for RPE/choroid was mouse anti-GADPH (Chemicon).

**Immunohistochemical localization for C3 and CD11b**

Immunohistochemistry for C3 and CD11b were done as previously described [87]. Briefly, free-floating sections were blocked and incubated overnight with anti-mouse C3d (1:500, R&D Systems) or anti-mouse CD11b (1:500, Pierce), then incubated
for 2 hours in Alexa fluorophore-conjugated secondary antibody (1:500, Invitrogen), and counterstained with Hoechst 33342 (1:500, Invitrogen). Confocal images were acquired using a Zeiss 710 inverted confocal microscope.

**Anti-C5a ELISA**

Briefly, 1 μg/100μL of anti-C5a (Pfizer, 4C9) was incubated at 4°C overnight in 96 well plates. After washing unbound antibody and blocking with 1% BSA, a 1:50 dilution of plasma was incubated overnight at 4°C. Following the wash steps the plate was sequentially incubated with biotinylated rat anti-mouse C5a (BD Biosciences), streptavidin-peroxidase (Sigma) and TMB substrate reagent (BD Biosciences) and the OD 450nm read. mC5a (R&D systems) was used as a standard curve.

**Peripheral blood and extravascular RPE/choroid monocyte analysis with flow cytometry**

Extravascular staining was performed with the following modifications to the previously published method [138]. Five minutes prior to euthanasia mice were injected retro-orbitally with 3μg/50ul of APC/Cy7 anti-mouse CD45 (BioLegend) (CD45-IV) in the right eye. Mice were then euthanized and perfused post-mortem with 30mL of PBS to wash unbound antibody from vasculature [138]. Peripheral blood was analyzed as a positive (>90%) control of vascular localization (Fig. S3B) [138]. Peripheral blood samples
were subjected to RBC lysis and staining for cell viability (eBiosciences #65-0863), CD45 (BioLegend, #110735), CD11b (BD Biosciences, #562950), CD115 (eBiosciences #61-1152), CD43 (BD Pharmingen #562866), Ly6C (BioLegend #128022) and Ly6G (BioLegend #127621) to determine the percent of classical and non-classical monocytes per CD45+ cells based on established methods (Fig. S3A) [136]. The left eye was enucleated and the RPE/choroid/sclera was isolated by microdissection. RPE/choroid was mechanically removed from the sub-adjacent sclera. Six eyes were pooled to obtain cell numbers sufficient for analysis. Samples then underwent DNase I and collagenase treatment with mechanical stimulation to free immune cells. Subsequently samples were filtered and stained for cell viability, CD11b, CD45, Ly6C, Ly6G, CCR2 (R&D Systems, #FAB5538A), CD11c (BD Biosciences, #563048), CD64 (BioLegend, #100539), F4/80 (BioLegend, #123109) and I-A/E (BioLegend, #107629). Using gating strategies established in other organs we found that using Ly6C and CD64 we could identify three clear subpopulations: Ly6C\textsubscript{lo}, Ly6C\textsubscript{hi} and a Ly6C\textsubscript{int}, CD64+ population (Fig. S3C) [136, 137, 152, 153]. Chi-squared statistical test for p<0.05 was used to determine the statistical significance of the cell population frequencies normalized to total extravascular CD45+ cells for a pooled sample.

**Characterization of 4C9 anti-C5a antibody**

C5aR cells were plated in a 96-well black, clear bottom plate so that confluence was reached the day of the experiment. All media was removed and replaced with
Calcium 3 dye (Molecular Devices) containing 0.1%BSA, 20mM Hepes and 2.5mM Probenecid in HBSS. The cells were then incubated for 1hr at 37C, and then equilibrated at room temperature before reading the plate. Meanwhile in a V-bottomed plate, agonist/antibody mixtures were made up in the same buffer (without the dye). For this experiment, recombinant mC5a had a final concentration of 0.4nM in all wells, with the antibody 4C9 titrated down from 50nM by a factor of 5 with the lowest concentration at 16pM. FLIPR analysis was done using a Flexstation II 384 (Molecular Devices). Briefly, agonist/antibody mixtures were added to each well and then fluorescent signal was measured over a period of 2 minutes with an interval of 1.6 seconds between each measurement, thereby creating an excitation curve. These curves were then analyzed for IC50 using the transform function in OriginPro 9.

*Anti-C5a treatment following intravitreal sodium iodate (NaIO3)*

8-10 week old C57BL/6J mice were pre-treated (Day 0) with either 0, 3, 10, 20, 30 or 60 mg/kg of anti-C5a or 60 mg/kg of isotype control in the peritoneal space. 24 hours later (Day 1), the mice were given an intravitreal injection of 25 mg/kg of NaIO3 or PBS control. 48 hours later (Day 3) mice were dark adapted over night and on day 4 scotopic electroretinograms were performed as previously described at 0.0001, 0.001, 0.1 and 2.5 cds of light intensity.

*Quantification of sub-RPE deposits by electron microscopy*
Sub-RPE deposits analysis was performed as previously described above [154]. Briefly, thin sections of 60 - 80 nm were cut for each part, collected onto 400 mesh thin-bar copper grids (T400-Cu, Electron Microscopy Sciences), and stained with Sato’s lead. Images containing the basal RPE and BrM were taken on the parts of the section adjacent to the grid’s bar, representative images were shown.

**Analysis of RPE damage based on flat mount quantification of multinucleate cells**

RPE flat mount preparation was performed as previously described [99]. Briefly, RPE flat mounts cells were stained with a rabbit antibody against ZO-1 (40–2200, 1:100; Invitrogen) and Hoechst 33342, and confocal images were captured on a Nikon Eclipse C1 microscope. Morphometric analysis was performed by a masked grader from each central quadrant. The number of multinucleated (nuclei ≥ 3) cells per central field view was counted.

**In vivo visual function analysis by electroretinography.**

Electroretinography was performed as previously described [139]. Briefly, mice were dark-adapted overnight, pupils dilated with 0.5% Tropicamide and 1.25% Phenylephrine, and anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). Scotopic ERGs were recorded using an Espion E2 system (Diagnosys LLC), at increasing flash intensities. The data points from the b-wave stimulus-response curves
were fitted according to a previously published equation using the least-square fitting procedure [97] (OriginPro 9.0; OriginLab). Statistical significance was determined using the mean values for Bmax1 and Bmax2.
4. CFH regulates sub-RPE lipoprotein accumulation

4.1 Introduction

A major consequence of RPE-choroid pathology in AMD is the deposition and sequestration of cellular and acellular debris sub-RPE between the RPE and Bruch’s membrane (BrM) that leads to drusen formation. BrM is a pentalaminar extracellular matrix between the RPE and choroid comprised of distinct sub lamina containing collagen and elastin-based fibrous connective tissue elements and a variety of proteoglycans, including heparan sulfate-rich proteoglycans [155, 156]. Lipoprotein particles accumulate in BrM with aging, before the development of sub-RPE deposits and drusen, as neutral lipids that are characterized by an abundance of esterified cholesterol that arise from RPE cells as components of apolipoprotein-B-containing (ApoB) lipoproteins [33, 157]. There is also a systemic contribution of ApoB-100-containing lipoproteins, including LDLs, which appear to be particularly important for the retina [45]. The accumulation of lipoproteins leads to formation of a lipid “wall” in the inner collagenous layer of BrM that is proposed to be the precursor of the lipoprotein-derived debris in AMD that forms the sub-RPE deposits clinically detected as drusen [158, 159]. Cholesterol (esterified and unesterified) and its transporter, ApoE, are also major constituents of these lipid-rich, sub-RPE deposits in AMD [125]. Lipoprotein deposits that accumulate in AMD can be oxidized [160] and have been shown to activate complement through the binding of C-reactive protein to oxidized
LDL [119, 120], or by oxidation-specific neo-epitopes rendering the modified LDL immunogenic [121, 122]. Our results demonstrate that sub-RPE deposit formation occurs in a CFH-dependent manner that is independent of increased complement activity, immune cell recruitment, RPE dysfunction and visual function decline. These findings provide a proof of concept that lipoproteins can be retained in mouse, if the right retention matrix is present. Because mouse does not form basal linear deposit (a layer of rich in lipoproteins), we tested these ideas further in human eye samples. We hypothesized that CFH regulates lipoprotein deposit formation, accumulation of which in BrM is proposed to be the precursor of the lipoprotein-derived debris that forms the sub-RPE deposits [158, 159]. Specifically, we hypothesized that CFH plays a direct role in competing with lipoproteins for binding to heparan sulfate proteoglycans (HSPGs) since lipoproteins interact with heparan sulfate [161], which is the main binding partner for CFH in human BrM [78, 162]. Using a variety of binding assays we show that CFH regulates lipoprotein accumulation by competition with lipoproteins for heparin sulfate binding in BrM. Furthermore, we show that CFH H402 variant alters binding to BrM heparin sulfate; however, this does not appear to dramatically affect lipoprotein regulation.

4.2 Results

*CFH competes with lipoproteins for binding to heparin*
Based on our *in vivo* observations, we performed a series of experiments using heparin binding columns and both young porcine and aged human BrM tissue to test the hypothesis that decreasing CFH increases lipoprotein binding to BrM HSPGs. Initially, heparin-sepharose columns were pre-incubated with increasing concentrations of CFH or equimolar albumin and then human lipoproteins were added to the column to test the effect of CFH on the lipoprotein-heparin interaction. Our results clearly show that while CFH competes with lipoproteins for binding to heparin (*Fig. 23A*), albumin does not (*Fig. 23B*).

**Figure 23:** CFH competes with lipoproteins for binding to heparin-sepharose beads. (*A-B*) Heparin sepharose columns were pre-incubated with doubling concentrations of CFH or albumin, after washing off excess protein, 2µl of a lipoprotein preparation was added to all the columns. (*A-B*) SDS-PAGE gels showing Coomassie blue
staining of separated proteins following incubation with increasing concentrations of CFH and elution with 1M NaCl. The identity of the bands was determined by MALDI-TOF mass spectrometry. Western blot was used to identify ApoE eluted from heparin column following increasing concentrations of CFH or albumin. The density of each band was measured to determine the relative concentration changes of each apolipoprotein with increasing CFH (A, bottom panel) or albumin (B, bottom panel).

**CFH levels regulate lipoprotein binding in BrM and remove endogenous lipoproteins in human BrM**

To further test our hypothesis in an *ex vivo* setting, we determined the range of concentrations of CFH that could bind to a 6 mm biopsy punch of BrM/choroid isolated from a young pig eye (**Fig. 24A**). We show that the EC50 of this interaction is 1 μM, establishing the specificity and affinity of the interaction (**Fig. 24A**). We then added a fixed amount of lipoproteins with increasing concentrations of CFH to these porcine explants and determined that by increasing the concentration of CFH we could dramatically reduce the binding of lipoprotein to the tissue (**Fig. 20B**).
Figure 24: CFH levels regulate lipoprotein binding in porcine BrM. Six mm porcine RPE/BrM tissue explants were incubated with doubling concentrations of CFH (62 nM-4 μM) in the presence of a 200 μM excess of albumin. (A) The amount of CFH bound to the tissue explant was determined by Western blot of the tissue lysate to determine EC50 binding. (B) Similar porcine tissue explants were incubated 2 μl of lipoprotein and increasing concentrations of CFH (0, 60, 120 nM) and lipoprotein binding to the explants was assessed by Western blot analysis for ApoB-100 and ApoE. Data are expressed as mean ±SE. * indicates p<0.05 for ApoE and ApoB-100 by ANOVA compared to 0 nM CFH. Data presented are a representative of experiment of three independent experiments (N=12-15). Actin served as a loading control in A-B.

CFH competes with endogenous human BrM lipoproteins present in aged donors

The use of heparin binding columns and young porcine BrM tissue are advantageous due to the absence of endogenous lipoprotein deposition, allowing control over lipoprotein deposition. In a final experiment, we validated the interaction using endogenous human BrM lipoproteins from aged human donors (Fig. 25 A,B). To test the interaction of CFH and endogenous human BrM lipoproteins, RPE/BrM/choroid punches obtained from aged (>75 years of age) human donor eyes were incubated overnight with or without 1μM CFH (Fig. 25A and B). Immunohistochemical analysis of
human BrM explants showed that while some endogenous CFH exists in untreated RPE/BrM preparations (Fig. 25A, top panel), following overnight incubation in 1 μM human CFH, CFH accumulates significantly above endogenous levels in BrM (Fig. 25A, bottom panel). FPLC fractionation of human BrM lysates shows that 1 μM CFH removes endogenous VLDL-like sized particles from human BrM (Fig. 25B). Together these results provide a mechanistic link that explains the increased sub-RPE deposit formation and accumulation detected in vivo in animals with decreased CFH.

**Figure 25:** CFH levels regulate lipoprotein binding and remove endogenous lipoproteins human in BrM. (A) Since aged human BrM contains endogenous sub-RPE lipoproteins we tested the ability of CFH to remove these accumulated lipoproteins ex vivo. Anti-CFH (green) immunohistochemistry of aged human BrM donor tissue before (upper panel) and after (lower panel) overnight incubation with 1 μM exogenous CFH, shows accumulation of CFH in BrM. Scale bar = 5 μm. (B) FPLC fractionation of human BrM lysates shows endogenous lipoproteins present in aged BrM tissue are removed with the addition of CFH (1 μm CFH, green trace). * indicates p<0.05 for total cholesterol in each fraction comparing 0 μM CFH to 1 μM CFH. Three independent experiments each with an N=3 were performed to confirm these results of human BrM FPLC experiments.

*CFH H402 variant shows equal accumulation in BrM but binds to different epitopes*
Due to the association of CFH Y402 with AMD risk we sought to investigate the role of CFH H402 polymorphism in BrM binding and lipoprotein removal. Using aged human BrM explants incubated with either 1 μM full-length isolated CFH Y402 or H402, we show that accumulation of CFH is unaffected by the CFH H402 variant (1.25 μg/punch vs 1.26 μg/punch, SD ± 0.08 μg/punch, β<0.05) (Fig. 26A). Furthermore, EC50 curves of CFH Y402 and CFH H402 on porcine BrM are indistinguishable (EC50, CFH Y402 130 nM and CFH H402: 160 nM) (Fig. 26B). However, mixtures of 0.5 μM CFH Y402 and 0.5 CFH H402 μM increase binding above plateaus achieved at 1 μM of either CFH Y402 or H402 in isolation (Fig. 26B). These data suggested that CFH H402 binds to alternative sites in BrM. To test competition between CFH H402 variants, increasing concentration of CFH Y402 (0 to 4 μM) was added to three fixed concentrations (0, 1 and 4 μM) of CFH H402. As expected, only a small competitive (22% decrease in the CFH H402 maximum binding with 4 μM CFH Y402) is seen between CFH Y402 and CFH H402 for BrM binding sites (Fig. 26C). Together these data show that although AMD risk-associated CFH H402 does not alter accumulation in BrM, the proteins have the ability to interact with different epitopes.
Figure 26: CFH H402 variant shows equal accumulation in BrM but binds to different epitopes. (A) Human BrM explants from aged (73-79 years old) donors were incubated with 1 μM CFH Y402 or CFH H402. Bound CFH was quantified by western blot using CFH standard curves with of goat anti-CFH established to recognized CFH variants equally [87]. Equal binding was noted and type II error was calculated to establish statistical significance of the null hypothesis (β<0.05) (B) Porcine BrM explants were incubated with increasing concentrations of full-length CFH Y402 or CFH H402 to establish EC50 values for CFH Y402 (130 nM) and H402 (160 nM). Maximum binding was achieved at 0.5 mM with CFH Y402 and CFH H402. Addition of 0.5 mM CFH Y402 and 0.5 mM CFH H402 showed increased binding compared to 1 mM of CFH variants in isolation. (C) Competition of CFH H402 and Y402 was assessed by adding increasing concentration of CFH Y402 (0 to 4 μM) to three fixed concentrations (0, 1 and 4 μM) of CFH H402. Note that little competition was observed between the two variants.

CFH H402 variant is equally effective at removing endogenous BrM lipoproteins

Due to the variation in CFH binding seen with CFH H402, we sought to determine if this differential binding affects the removal of endogenous lipoproteins from human BrM tissue. Aged (>70 years of age) human post-mortem BrM tissue had varying levels of endogenous lipoprotein: some with high levels (Fig. 27A) and other with lower levels of endogenous lipoprotein (Fig. 27B). However, the CFH H402 variants did not show any decreased ability to remove endogenous lipoprotein (Fig. 27...
A, B). In six aged donors, removal of BrM lipoproteins was significant with both CFH Y402 and CFH H402. These data suggest that the altered binding of CFH H402 variant does not affect the ability of CFH to regulate BrM lipoprotein accumulation.

Figure 27: CFH H402 variant is equally effective at removing endogenous BrM lipoproteins. (A and B) Human BrM explants were isolated from aged (>70) donors and incubated with either 1 mM CFH Y402, 1 mM CFH H402 or 10 mM PB with matched NaCl concentration. FPLC fractionation was performed on tissue lysates and enzymatic cholesterol assays were performed on fractions to determine the lipoprotein content within the lysates. Note considerable variability exists in amount of lipoprotein present in aged donors. (C) However, analysis of relative cholesterol removal comparing the CFH H402 variant to the CFH Y402 shows equal ability to remove BrM lipoprotein from aged donors.

4.3 Discussion

Characterization of the AMD-like phenotype in the Cfh+-/-HFC mouse model also revealed a novel role for CFH in regulating the pathogenesis of sub-RPE deposit formation. A recent study uncovered a function for CFH in blocking the damaging effects of the lipid peroxidation product, malondialdehyde [85]. Both Weismann et al. [85] and Shaw et al. [163] describe the ability of CFH to bind to chemically modified
LDL, either malondialdehyde-acetaldehyde LDL (MAA-LDL) or CuSO₄ modified oxidized LDL (oxLDL), with much higher affinity than unmodified LDL. This binding prevents the detrimental inflammatory effects that the oxidized epitopes can potentiate. Our work focuses on the interaction of CFH and endogenous BrM lipoproteins and demonstrates that the amount of deposit - rather than the pathological effects of the deposit - appear to be regulated by CFH. In a series of experiments using aged BrM tissue and RPE cultures, Curcio et al. identified 60-80 nm RPE-derived lipoproteins which accumulate in BrM with age forming a “lipid wall” in BrM which is hypothesized to be the precursor to basal linear deposits and eventually visible on fundoscopic exam as drusen [33]. Since the main binding partner for CFH in human macular BrM is heparan sulfate [78, 162] and lipoproteins interact with heparan sulfate [161], we hypothesized that CFH competes with lipoproteins for binding to HSPGs. This hypothesis is further supported by the high concentrations of HSPGs found in BrM as well as studies implicating HSPGs in AMD [53, 78, 80, 162, 164]. Here, we show that CFH plays a direct role in modulating the interaction between BrM and endogenous lipoprotein particles that accumulate with age in human BrM. These lipoproteins resemble, in size, the RPE-derived lipoprotein-like particles described by Curcio et al., which are hypothesized to play a major role in the formation of the lipid-wall and drusen-biogenesis [33, 43, 165]. Future studies will be aimed at investigating the role of CFH variants and HSPGs in regulating BrM sub-RPE deposit formation [166, 167].
The CFH H402 variant is hypothesized to be responsible for the genetic attributable risk of AMD associated with CFH H1 haplotype. However, the functional significance of the CFH H402 substitution remains unclear (Section 1.9 Complement Factor H H402 Functionality). Here we show that CFH H402 does not alter the binding capacity CFH in aged human BrM. Previous studies reporting significant differences in CFH H402 BrM binding used recombinant SCR6-8 protein fragments for CFH Y402 and H402 that were labeled with different fluorophores and their relative fluorescent intensities were determined by IHC of BrM [53, 79, 80]. Several explanations exist for the discrepancy in results, however, the use of recombinant protein fragments and quantification of relative fluorescent intensity comparing proteins labeled with different fluorophores introduce significant variables not present in our analysis. Langford-Smith et al. predicted that HSPG sulfation patterns create a three dimensional diversity within the BrM extracellular matrix which act as “zip codes” for specific HSPG-CFH interactions [167]. The three dimensional nature of this diversity further highlights the requirement for studying full length human CFH, as both SCR 6-8 and SCR 19-20 contain HSPG binding domains. Furthermore, this group also suggested that these “zip codes” change with age [80]. We show in aged donors both competitive and non-competitive binding on human BrM. We suspect this is due to the diversity of HSPG sulfation in BrM and the multiple HSPG binding sites on CFH. Interestingly, the H402 variant shows equal ability to remove lipoproteins from BrM. This suggests that the
H402 variant does not alter lipoprotein deposition and subsequent sub-RPE deposit formation seen in AMD. Other explanations also could explain the CFH H1 association and AMD development including: non-coding SNP altering levels of CFH [52], association with rare highly penetrant SNPs [168] and CFHR1/3 [52], CFH H402 alteration of CRP binding during acute phase response [74, 75, 77, 169] and/or the decreased ability of CFH H402 to mask oxidative stress epitopes on sub-RPE deposits [85, 170]. Over the past seven years our lab has studied the function of CFH in vivo and ex vivo, and the unifying finding within our lab has been the importance of CFH dosage in AMD-like progression seen in our animal models [87, 139]. Based on these observations, small differences in CFH levels seen in AMD patients and associated with CFH H1 haplotype have the potential to result in a 2-3 fold increase in risk of AMD progression.

4.4 Materials and Methods

**CFH and lipoprotein binding to heparin-sepharose beads**

Heparin-sepharose columns (5 µl washed beads, GE Healthcare) were pre-incubated with increasing concentrations of CFH (prepared as previously described) [83] or albumin (0, 400, 800, and 1600 nM) for 20 minutes at room temperature, after washing off excess protein, 2 µl of a lipoprotein preparation isolated from human donor blood using ultracentrifugation of human plasma adjusted to a sp. gravity of 1.21 was added to
all the columns for an additional 20 minutes at room temperature. Following washing, the bound proteins were eluted with 1M NaCl and these fractions were run on 10% Bis Tris XT Criterion gels. One gel was stained by Coomassie and used to quantify (by densitometry and ImageJ) and identify (using a quantitative quadrupole time of flight mass spectrometer [Waters Synpt G2 with a LC-MS/MS workflow], CFH, albumin, ApoB-100 and ApoA-1. The other gel was transferred to nitrocellulose and Western blot analysis was used to measure ApoE (goat anti-human ApoE, Calbiochem).

**Porcine RPE/BrM lipoprotein/CFH binding assays**

For porcine BrM experiments, human serum lipoproteins were isolated as described above. BrM explants were isolated as previously described [83]. Porcine eyes were chosen because they have little to no BrM lipid accumulation and show low background for anti-human ApoB-100 and ApoE antibodies on a Western blot. EC50 values were determined by incubation of porcine BrM explants overnight in 10 mM PB with increasing concentrations of CFH (60 nM-4 μM). 200 μM albumin was added to each sample to block non-specific binding. Following five, 10 minutes washes in 10 mM PB, proteins were extracted and Western blot analysis was performed using 1:5000 goat anti-FH (Quidel) and an HRP conjugated secondary as previously described using 25ng of total protein lysate. The actin loading control had 5μg lysate/well and these samples were diluted 1:200 to observe the CFH in the linear range [171]. Data presented are a representative of two independent experiments (N=4-8). For competition assays a fixed
amount of human lipoproteins (2 µl) with increasing concentrations of CFH (60 nM and 120 nM) was added overnight at 4°C to 6mm porcine explants in a well of a 96-well plate. After five, 10 minutes washes in 10 mM PB, proteins were extracted and Western blot analysis was performed using 1:30,000 goat anti-ApoB-100 (EMB Millipore), 1:5000 goat anti-ApoE (CalBiochem) and 1:3000 mouse anti-Actin (Santa Cruz) primary antibodies and an HRP conjugated secondary as previously described [87]. Data is expressed are a representative experiment of three independent experiments (N= 12-15).

Aged Human RPE/BrM endogenous lipoprotein/CFH competition assays

Human donor eyes were obtained from the North Carolina Eye Bank. Due to the previously described age-related accumulation of lipoproteins in human BrM, eyes from donors >75 years of age were used for this study [9, 31, 32]. RPE/BrM punches were isolated as previously described [83] then were randomly assigned to incubation overnight at 4°C in 200 µl 10 mM PB with or without 1 µM CFH of CFH Y402 or H402 variant. Following incubation, the tissue was washed in 10 mM PB five times. For IHC tissue was fixed with 4% paraformaldehyde for 20 minutes and then washed with PBS for overnight storage. Tissue was embedded in 10% low melting temperature agarose (Sigma-Aldridge) and 70 µM sections vibratome sections were cut. IHC staining was performed as previously described [87]. For lipoprotein analysis, 80 µl of RIPA buffer with protease inhibitors and mechanical homogenization was used to isolate
lipoproteins. Homogenates were pooled, and 50 µL was run on an FPLC system (Pharmacia LKB) with a Superose 6HR 10/30 column (GE Healthcare) and 40, 500 µL fractions were collected. Total cholesterol was determined for each fraction by enzymatic cholesterol assay (Amplex Red Cholesterol Kit, Invitrogen). Data is expressed as a pooled N=3. Three independent experiments were performed.

**Human RPE/BrM CFH variant binding**

Human RPE/BrM punches from donors over 75 years old were isolated as previously described [83] and then were randomly assigned to incubation overnight at 4°C in 200 µl 10 mM PB with or without 1 µM CFH of CFH Y402 or H402 variant. Following incubation, the tissue was washed in 10 mM PB five times. 80 µl of RIPA buffer with protease inhibitors and mechanical homogenization was used to determine CFH binding. Western blot analysis was performed with 1:1000 dilution of 3 µL of homogenate. Western blot analysis was performed using 1:5000 goat anti-FH (Quidel) and an HRP conjugated secondary.

**Porcine RPE/BrM CFH variant binding**

To assess CFH variant binding on porcine BrM, porcine BrM were prepared as described above. EC50 values were determined by incubation of porcine BrM explants overnight in 10 mM PB with increasing concentrations of CFH (9 nM-1 µM). A mixture of 0.5 µM
CFH Y402 and 0.5 μM CFH H402 was also incubated overnight in porcine BrM and compared to 1 μM of CFH Y402 and 1 μM CFH H402 incubated separately. 1 μM albumin was added to each sample to block non-specific binding. Following five, 10 minutes washes in 10 mM PB, proteins were extracted and Western blot analysis was performed using 1:5000 goat anti-FH (Quidel), which has equal affinity for CFH H402 variant [87], and an HRP conjugated secondary as previously described using 25 ng of total protein lysate. Data presented are a representative of two independent experiments (N=3).
5. Conclusions and Implications

In conclusion we have elucidated a previously unidentified non-canonical function of the CFH protein in regulating sub-RPE lipoprotein accumulation. This role of the CFH protein more readily explains the large genetic and biochemical associations seen between CFH and AMD. Furthermore, we provide evidence that complement dysregulation is involved in AMD pathogenesis and address the efficacy of targeting specific complement breakdown products to prevent immune cell recruitment and RPE damage. These results have significant implications in treatment of AMD as several randomized controlled clinical studies have been performed targeting the complement system, including systemic anti-C5 therapy which failed to show significant improvement in patients with geographic atrophy [8]. Our findings have provided insights into why therapies targeting the complement system have had only limited success: 1) our results suggest that some of the genetic factors associated with AMD within the complement system are due to non-canonical functions of CFH no associated with complement dysregulation and 2) we show that complement dysregulation does play a role in AMD pathogenesis, however, targeting complement breakdown products, including C5a, in the earlier stages of the disease appears to be more efficacious.
References


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132. !!! INVALID CITATION !!!


Biography

Christopher B. Toomey was born on January 28th, 1987 in San Diego, CA, he grew up in San Diego and graduated from Mt. Everest Academy in 2005. Subsequently, he attended University of California, San Diego where he majored in Neurosciences and Physiology, receiving a Bachelor of Science, Magna Cum Laude, and was inducted into the Muir College Caledonian Society. During his undergraduate tenure Christopher did research in the laboratory of Dr. Kenneth M. Pollard at the Scripps Research Institute in San Diego, CA, from 2005 to 2010. He enrolled in the Medical Scientist Training Program (MSTP) at the Duke University School of Medicine in 2010 and, after two years of medical training, began his graduate training in the Department of Cell Biology, joining the laboratory of Catherine Bowes Rickman in 2013. Following receipt of M.D. and Ph.D. degrees, Christopher plans to attend residency as the next step in becoming a physician-scientist in ophthalmology.