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VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

The Membrane Attack Complex in Aging Human Choriocapillaris

Relationship to Macular Degeneration and Choroidal Thinning

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Age-related macular degeneration (AMD) is a common disease that can result in severe visual impairment. Abnormal regulation of the complement system has been implicated in its pathogenesis, and *CFH* polymorphisms contribute substantially to risk. How these polymorphisms exert their effects is poorly understood. We performed enzyme-linked immunosorbent assay (ELISA) analysis on young, aged, and AMD choroids to determine the abundance of the membrane attack complex (MAC) and performed immunofluorescence studies on eyes from 117 donors to evaluate the MAC in aging, early AMD, and advanced AMD. Morphometric studies were performed on eyes with high- or low-risk *CFH* genotypes. ELISA confirmed that MAC increases significantly with aging and with AMD. MAC was localized to Bruch's membrane and the choriocapillaris and was detectable at low levels as early as 5 years of age. Hard drusen were labeled with anti-MAC antibody, but large or confluent drusen and basal deposits were generally unlabeled. Labeling of retinal pigment epithelium was observed in some cases of advanced AMD, but not in early disease. Eyes homozygous for the high-risk *CFH* genotype had thinner choroids than low-risk homozygotes ($P < 0.05$). These findings suggest that increased complement activation in AMD and in high-risk genotypes can lead to loss of endothelial cells in early AMD. Treatments to protect the choriocapillaris in early AMD are needed. *(Am J Pathol 2014, 184: 3142—3153; http://dx.doi.org/10.1016/j.ajpath.2014.07.017)*

Age-related macular degeneration (AMD) is a complex disease that frequently results in loss of visual acuity. AMD, the most common cause of irreversible blindness in the elderly, is projected to affect 3 million Americans by 2020.[1,2] Clinically, the early stages of AMD are characterized by structural abnormalities in the posterior pole that include an abnormal appearance of the retinal pigment epithelium (RPE) and the presence of drusen, which are extracellular deposits that form between the RPE and its blood supply, the choriocapillaris.[3] Although for most patients early AMD does not progress to severe end-stage disease, a subset of patients will develop extensive degeneration of photoreceptor cells and RPE cells in the macula, caused by either pathological invasion of new blood vessels from the choroid into the sub-RPE or subretinal spaces [ie, choroidal neovascularization (CNV)] or by idiopathic loss of the macular RPE and photoreceptor cells (ie, geographic atrophy).

Although our understanding of the pathophysiology of AMD is incomplete, recent genome-wide association studies

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Table 1 Characteristics of Donor Eyes Used in Enzyme-Linked Immunosorbent Assay Study

Donor	Donor age (years)	MAC concentration (ng/mL)	Cause of death	D–P time (hours)	Allele	
					Y402H	V62I
Young (<50 years of age)						
1	23	2.75	Methane asphyxia	6.75	HY	VV
2	48	18.68	Cancer	6.5	HY	VV
3	45	15.67	Respiratory failure	6.5	HY	VV
4	21	30.71	Hodgkin lymphoma	6	HY	VV
5	34	26.78	Intercranial hemorrhage	6.5	HY	VV
6	46	4.46	Cardiopulmonary arrest	<8	YY	IV
7	46	45.34	Pneumonia	2.75	YY	II
8	43	34.19	Lung cancer, renal failure	5.25	HY	IV
9	46	19.97	Renal disease	6.5	HY	VV
10	45	1.64	Pneumonia	6.75	YY	IV
Aged control, without AMD						
11	77	38.77	Pulmonary embolism	5.5	YY	VV
12	71	28.72	Intracerebral hemorrhage	7	HY	IV
13	82	83.68	Heart failure	5.5	YY	II
14	83	68.07	Respiratory failure	5.75	YY	IV
15	96	30.16	Respiratory failure	7	HY	IV
16	85	47.80	Metastatic bladder cancer	4.75	HY	VV
17	79	52.29	Congestive heart failure	5.25	HY	IV
18	95	43.95	Stroke	7	YY	VV
19	82	49.05	NA	6	YY	II
20	78	43.72	Lung cancer	6	YY	IV
Aged AMD						
21	89	185.44	NA	4.5	HH	VV
22	88	221.13	Myocardial infarction	6	HH	VV
23	99	26.96	Stroke	5.75	YY	IV
24	78	113.91	Respiratory failure	5	HH	VV
25*	77	89.88	NA	6	HH	VV
26*	85	114.65	Acute coronary syndrome	7	HY	VV
27	82	40.67	Stroke	5.5	HY	IV
28	93	165.16	Urosepsis	5.5	HY	VV
29	89	70.34	Congestive heart failure	7.25	HY	VV
30	91	77.24	Perforated bowel	5.5	HY	IV

*Earlier punches from these donor eyes were analyzed in a previous study.[10]

AMD, age-related macular degeneration; D–P time, maximum time between death and preservation of eye in liquid nitrogen; NA, not available; sC5B-9, soluble terminal complement complex.

and candidate gene approaches have led to the identification of various polymorphisms that affect the risk of AMD at all stages. These include single-nucleotide polymorphisms in a number of genes encoding members and regulators of the complement pathway, including *C3*, *CFI*, *C2* and/or *CFB*, and *CFH* (recently reviewed by Khandhadia et al[4]). One polymorphism in the *CFH* gene (rs1061170) increases risk of AMD by approximately twofold to sevenfold, depending on the population studied.[5–8] This variant results in the substitution of histidine for tyrosine at amino acid residue 402. The effect of this polymorphism in the human eye is not well understood, although adults harboring the Y402H polymorphism show increased choroidal C-reactive protein[9] and increased membrane attack complex (MAC).[10]

Formation of the MAC is the final event in the terminal portion of the complement cascade and results from the binding of C5b to plasma complement proteins C6, C7, C8, and multiple molecules of C9. MAC forms transmembrane channels that lead to cell lysis and death. The MAC has been found in drusen of older eyes with AMD.[11] However, the relative abundance and distribution of MAC in aging, early AMD, and advanced AMD have not been comprehensively studied. Inhibition of MAC components such as C6 can inhibit CNV,[12] and other complement pathway inhibitors are in active clinical trials for the treatment of AMD.[13] Because it is the ultimate downstream effector of the complement pathway, understanding the role of the MAC in the pathophysiology of AMD is important for the development of new therapies.

We evaluated the MAC in a large series of donor eyes. MAC was present in Bruch's membrane and choriocapillaris in very young eyes, but the concentration increased with age; we observed the highest levels in eyes with AMD. We further evaluated the MAC in a series of eyes from young and old donors, and from donors with early and advanced AMD. Although in early AMD the MAC is associated exclusively

Table 2 Characteristics of Donor Eyes Used for Immunohistochemistry

Donor ID	Age (years)	AMD Dx	Cause of death	D—P time (hours)
1	0	None	Cardiac arrest	7:00
2	1	None	Cardiac arrest	7:00
3	5	None	Myelogenous leukemia	8:06
4	21	None	Hemorrhagic pancreatitis/leukemia	5:23
5	21	None	Hodgkin lymphoma	6:03
6	28	None	Metastatic carcinoma	4:12
7	41	None	Ovarian cancer	4:50
8	42	None	Anaplastic T-cell lymphoma/sepsis	4:12
9	48	None	Septic shock	5:53
10	50	None	Adenocarcinoma/respiratory failure	7:35
11	53	None	Cardiac arrest	6:30
12	57	None	Cardiogenic shock/heart disease	5:50
13	59	None	Lymphoma	4:59
14	60	None	Sepsis	7:45
15	62	None	Cardiac arrest	7:18
16	63	None	Myocardial infarction	5:06
17	65	None	Renal failure	4:56
18	66	None	Unknown	6:32
19	67	None	Lung cancer	4:22
20	67	None	Sepsis	5:50
21	68	AMD: OS GA	NA	6:48
22	68	None	Acute leukemia	6:57
23	69	AMD: OU dry	Respiratory failure	7:22
24	70	AMD: OU wet	NA	3:54
25	70	None	Acute respiratory failure	5:57
26	70	AMD: OD CNV	Cardiac arrest	7:50
27	71	None	Sepsis	7:02
28	72	None	Perforated bowel	5:03
29	73	None	Throat cancer	5:45
30	74	None	Ovarian cancer	7:25
31	75	None	NA	5:15
32	76	AMD: OU dry	Subdural hematoma	4:44
33	76	AMD: OU dry	Basal ganglion bleed	6:01
34	76	None	NA	7:38
35	76	AMD: OU dry	NA	8:03
36	77	None	Brain tumor	5:07
37	77	AMD: OD wet, OS dry	NA	5:58
38	77	AMD: OU dry	Sepsis	6:35
39	77	None	Aortic valve stenosis/congestive heart failure	6:41
40	78	AMD: OD dry	Respiratory failure 2 COPD	4:07
41	78	AMD	Respiratory failure	4:56
42	78	AMD: OU dry	Subarachnoid hemorrhage	5:34
43	78	None	Sepsis	5:47
44	79	None	Metastatic lung cancer	4:50
45	79	None	NA	5:32
46	79	None	Bladder cancer	5:52
47	79	None	NA	6:26
48	79	AMD: OU dry	NA	6:47
49	79	AMD: OD dry, OS wet	Lymphoma	7:55
50	79	AMD: OU dry	NA	NA
51	80	None	NA	4:51
52	80	AMD: OD dry	COPD	4:53
53	80	None	NA	5:34
54	80	None	Hemorrhagic shock	8:00
55	81	None	Cancer	4:44
56	81	None	NA	6:02
57	81	None	Renal failure	6:30
58	82	None	Respiratory failure	5:12
59	82	AMD: OU GA	Respiratory failure	6:04

(*table continues*)

Table 2 (*continued*)

Donor ID	Age (years)	AMD Dx	Cause of death	D—P time (hours)
60	82	None	Perforated bowel	6:52
61	82	None	Multisystem cancer	8:20
62	83	AMD: OU GA	NA	5:24
63	83	AMD: OU dry	Motor vehicle collision	5:55
64	83	None	NA	6:32
65	83	AMD: OU wet	Myocardial infarction	8:40
66	84	AMD: OU dry	Respiratory failure	4:16
67	84	None	Hypoxia due to congestive heart failure	5:43
68	85	None	NA	6:56
69	86	AMD: OU dry	NA	3:13
70	86	AMD: OS dry	Small bowel obstruction/renal insufficiency	3:35
71	86	None	Heart disease	6:05
72	86	AMD: OU dry	Myocardial infarction	6:54
73	86	AMD: OU wet	Colon cancer	7:20
74	87	AMD: OD GA, OS wet	Heart and respiratory failure	4:37
75	87	None	NA	5:26
76	87	None	Stroke	5:30
77	87	AMD: OU dry	Motor vehicle accident with head injury	5:42
78	87	AMD: OU wet	Myocardial infarction	5:45
79	87	AMD: OU GA	Prostate cancer	8:50
80	88	None	NA	2:35
81	88	AMD: OU dry	GI bleed	4:12
82	88	None	Respiratory failure	5:35
83	88	None	Complications after a fall	5:37
84	88	None	NA	5:56
85	88	AMD: OD GA, OS wet	Heart failure	6:43
86	88	None	Respiratory failure/sepsis	8:10
87	88	None	Cardiogenic shock	9:15
88	88	AMD: OU dry	Cerebrovascular accident	NA
89	89	None	NA	4:20
90	89	None	Stroke	5:08
91	89	AMD: OU dry	Congestive heart failure	7:10
92	90	None	Cardiac arrest	4:27
93	90	AMD: OD wet, OS dry	Cerebrovascular accident	5:30
94	90	None	Cerebrovascular accident	6:46
95	90	None	NA	8:50
96	91	None	Cerebrovascular accident	5:06
97	91	AMD: OD wet, OS early dry	NA	5:10
98	91	None	NA	5:32
99	91	AMD: OU dry	Aspiration pneumonia	7:48
100	91	AMD: OU wet	Intracerebral hemorrhage	NA
101	92	AMD: OU dry	Acute myocardial infarction	4:25
102	92	AMD: OU dry	Lung cancer	5:25
103	92	AMD: OU dry	Cardiac arrest	7:49
104	93	None	Respiratory failure	5:50
105	93	AMD: OU dry	Renal insufficiency	6:30
106	94	None	Congestive heart failure	5:34
107	94	None	Respiratory failure	5:34
108	94	AMD: OU dry	Cardiac arrest	5:46
109	94	AMD: OU dry	Cardiac arrest	7:40
110	95	AMD: OD GA	Stroke/atrial fibrillation	6:20
111	95	AMD: OU dry	Heart and renal failure	6:35
112	96	AMD: OU dry	NA	5:00
113	98	None	NA	5:41
114	98	AMD: OU dry	COPD	6:14
115	98	AMD: OU dry	Multisystem organ failure/renal failure	6:17
116	99	AMD: OD GA	NA	1:42
117	100	AMD: OD dry	Found unresponsive	5:55

CNV, choroidal neovascularization; COPD, chronic obstructive pulmonary disease; D—P, maximum time between death and preservation in liquid nitrogen or fixative; Dx, diagnosis; GA, geographic atrophy; GI, gastrointestinal tract; NA, not available; OD, right eye; OS, left eye; OU, both eyes.

with the choriocapillaris, in advanced AMD the RPE may be exposed as well. Morphometric experiments suggest that high-risk *CFH* genotypes may contribute to thinning or atrophy of the choroid. Overall, these studies suggest that choroidal endothelial cells are targets of the MAC and that approaches to prevent their injury from complement-mediated lysis may be useful in the treatment of AMD.

Materials and Methods

Human Donor Eyes

Whole globes from human donors were obtained from the Iowa Lions Eye Bank (Iowa City, IA). Full consent for research was obtained from the donor's next of kin in all cases, and all experiments were performed in accordance with the Declaration of Helsinki.

Eyes were processed within 9.5 hours of death (range, 1 hour 42 minutes to 9 hours 15 minutes). For biochemical studies, a 6-mm juxtamacular, inferotemporal punch was acquired. Neural retina and RPE–choroid layers were collected separately and snap-frozen in liquid nitrogen, before long-term storage at −80°C. Macular punches and/or superotemporal wedges were collected from each eye and preserved in 4% paraformaldehyde in phosphate-buffered saline within 8 hours of death. After 2 hours of fixation, eyes were washed in phosphate-buffered saline and then were cryoprotected in sucrose and embedded in sucrose–optimal cutting temperature medium, as described by Barthel and Raymond.[14]

Quantification of Soluble C5b-9/MAC

Samples were chosen for MAC quantification from a collection of frozen juxtamacular punches of RPE–choroid, centered approximately 7 mm temporal to the fovea. Ten RPE–choroid samples were selected from each of three groups: young (mean age, 39.6 years; range, 21 to 48 years); aged, with a clinical and/or histological diagnosis of dry AMD (mean age, 87.1 years; range, 77 to 99 years); and age-matched control, without AMD (mean age, 82.8 years; range, 71 to 96 years) (Table 1). Of the 30 samples studied, 2 samples in the AMD group were new punches from donor eyes reported previously.[10] Samples were homogenized for 90 seconds using a Kontes disposable pestle (Thermo Fisher Scientific, Waltham, MA) and motorized tissue grinder (Sigma-Aldrich, St. Louis, MO) in 30 μL of phosphate-buffered saline with 1% Triton X-100 and protease inhibitors (cOmplete kit; Roche Diagnostics, Indianapolis, IN). Total protein concentration was determined using the Lowry method (Bio-Rad Laboratories, Hercules, CA); 30 μg of each sample was loaded in duplicate wells into a MAC enzyme-linked immunosorbent assay (ELISA) plate (MicroVue SC5b-9 Plus enzyme immunoassay kit; Quidel, San Diego, CA), and MAC levels were determined according to the manufacturer's instructions.

Immunohistochemistry

Sections were collected on a cryostat and dual-labeled with antibodies directed against a neoepitope present in activated complement C9 that is exposed during formation of the MAC (anti–C5b-9 complex; Dako, Carpinteria, CA) and the vascular marker *Ulex europaeus* agglutinin I (UEA-I; Vector Laboratories, Burlingame, CA), which labels fucosylated glycoconjugates on viable human endothelial cells. MAC and endothelial cells were detected using Alexa Fluor 488–conjugated goat anti-mouse IgG (Life Technologies, Carlsbad, CA) and avidin–Texas Red (Vector Laboratories), respectively. Immunohistochemistry and lectin histochemistry were performed as described previously.[9,15] Eyes from 117 donors were evaluated (Table 2).

Genotyping

Genotyping for the Y402H allele (rs1061170) and the V62I allele (rs800292) of the *CFH* gene was performed on DNA from subsets of donor eyes using a microfluidics station (Fluidigm, South San Francisco, CA) with TaqMan (Life Technologies) reagents. DNA was isolated either from whole blood, collected during enucleation, or from extraocular muscle, using a DNeasy kit (Qiagen, Valencia, CA). Genotyping assays were performed as described previously.[4,10]

Morphometry of Choroidal Thickness

Eyes from 100 donors genotyped at the Y402H risk allele of *CFH* were used for quantitative analyses. The distribution was 43 donors homozygous for the low-risk allele (YY), 25 donors homozygous for the high-risk allele, and 32 donors

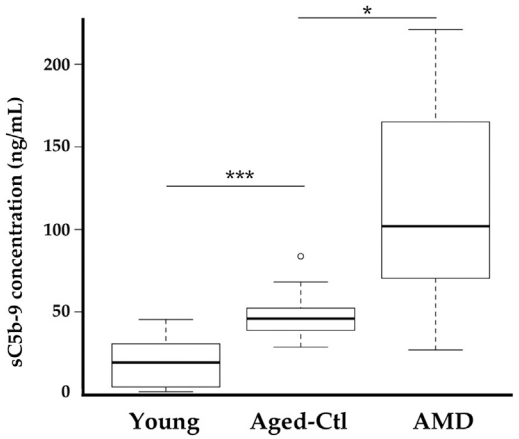

Figure 1 Enzyme-linked immunosorbent assay analysis of soluble C5b-9 MAC in young eyes, aged eyes without AMD (control), and eyes with atrophic AMD. Increased MAC levels were related to increased age (***$P < 0.001$) and to diagnosis of AMD (*$P < 0.05$). Data are expressed as box-and-whisker plots, indicating median, interquartile range, and minimum and maximum values; any outliers are indicated by individual symbols. $n = 10$ per group. AMD, age-related macular degeneration; Ctl, control; MAC, membrane attack complex.

heterozygous (HY). Cryostat sections were collected and assigned a random identifier by a masked observer. Choroidal thickness (measured from the outer edge of Bruch's membrane to the inner surface of the sclera[16]) was quantified in sections from all donors using ImageJ software version 1.60_65 (NIH, Bethesda, MD). At least five measurements were taken from each section, and a single mean value was determined for each eye. Data were analyzed using linear regression in the R version 3.0.3 statistical computing environment.

Results

ELISA Analysis

The concentration of MAC was quantified in a series of punches of RPE–choroid from donor eyes. Standards included in the kit, ranging from 15 to 172 ng/mL, showed excellent correlation between concentration and absorbance ($r^2 > 0.99$). For RPE–choroid samples, duplicate wells showed very good reproducibility ($r^2 > 0.989$).

Samples from young donors had relatively low levels of MAC, and these levels were significantly lower than those in the aged control non-AMD group ($P < 0.001$) (Figure 1). Samples from those with AMD had variable but significantly higher levels of MAC than either age-matched control eyes ($P < 0.05$) or young eyes ($P < 0.01$). In both older age groups, MAC levels were significantly higher in eyes with one or more risk alleles ($P < 0.05$, Student's *t*-test), as we have reported previously.[10]

MAC Immunofluorescence

We evaluated a series of eyes from 117 human donors (Table 2). Consistent with previous report,[17] MAC in aging maculae was predominantly localized to the outer aspect of Bruch's membrane and in extracellular domains surrounding the choriocapillaris (Figure 2). The eye of one

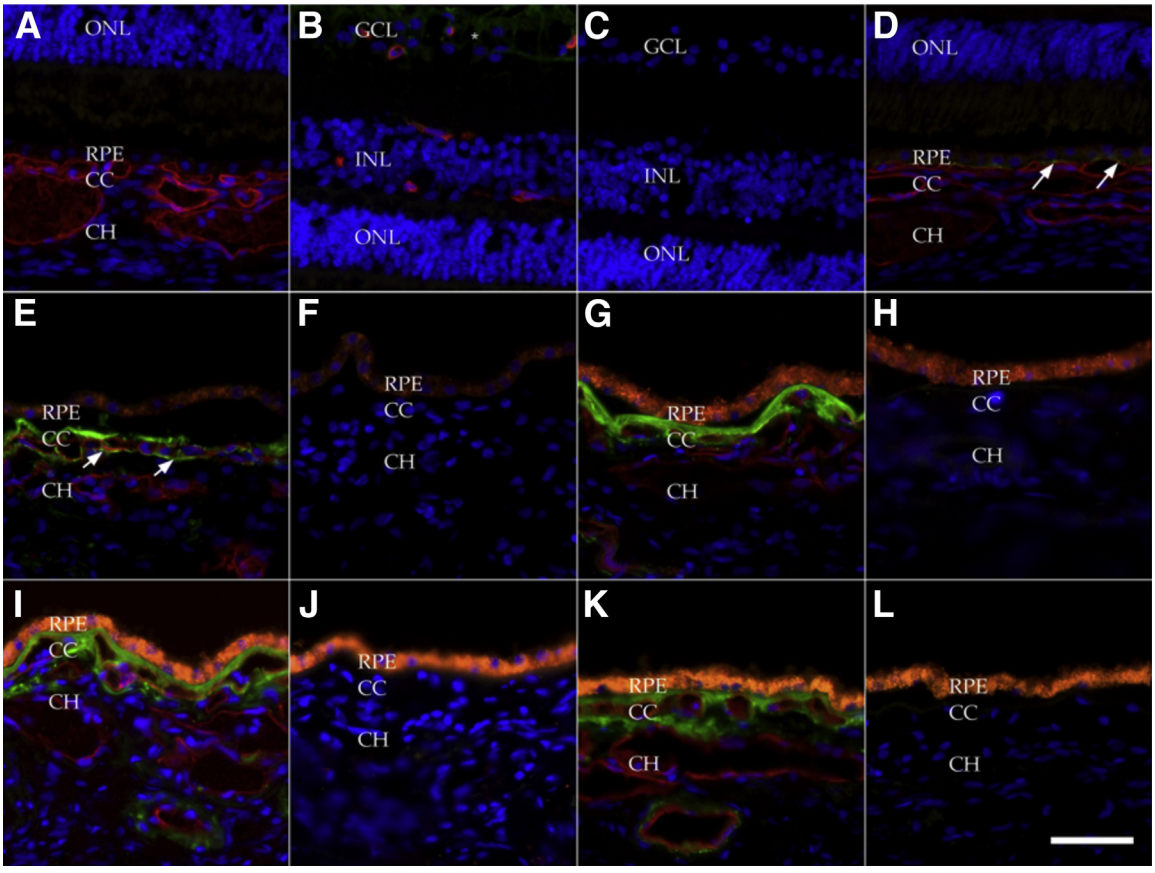

Figure 2 Localization of the membrane attack complex (MAC) in young (<50 years) and aged donor eyes without AMD: newborn (**A**–**C**), 16 months (**D**), 5 years (**E** and **F**), 32 years (**G** and **H**), 41 years (**I** and **J**), and 62 years (**K** and **L**). Sections were dual-labeled with anti-C5b-9 MAC antibody (green) and UEA-I lectin (red) (**A**, **B**, **D**, **E**, **G**, **I**, and **K**); the primary antibody and lectin were omitted for negative control (**C**, **F**, **H**, **J**, and **L**). Orange fluorescence of the RPE (**G**–**L**) indicates age-related lipofuscin accumulation. **A**–**C:** Eyes from a newborn infant showed no MAC labeling in Bruch's membrane or choroid (**A**). Minor immunoreactivity was observed in the ganglion cell layer (**B**, **asterisks**; **C**, negative control) of this newborn, but in none of the older specimens. **D:** At 16 months of age, very minor labeling was observed as puncta (**arrows**) between the RPE and choriocapillaris. **E:** In a 5-year-old donor heterozygous for the Y402H variant, considerable MAC labeling (**arrows**) in Bruch's membrane extended around the choriocapillaris. **G**–**L:** More robust labeling was observed in older eyes. Note the lack of choriocapillaris labeling and the constitutive RPE autofluorescence in the negative controls (**H**, **J**, and **L**). All images were processed identically. Scale bar = 50 μm. CC, choriocapillaris; CH, outer choroid; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.

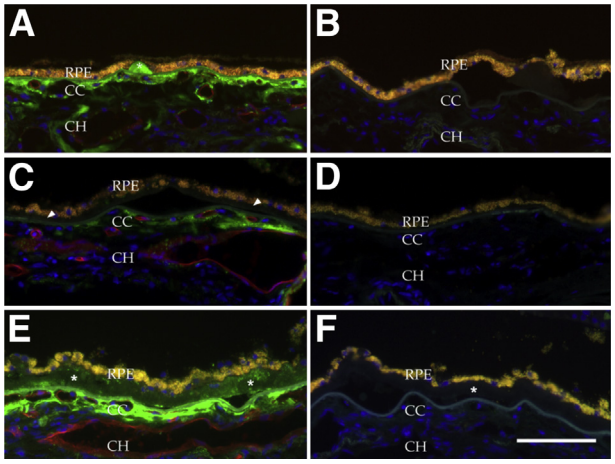

Figure 3 Localization of the MAC in eyes with early AMD. **A:** MAC was localized to solitary, hard drusen (**asterisk**). **C:** By contrast, basal deposits characteristic of early AMD[19] were generally unreactive with anti-MAC antibody (**arrowheads**). **E:** Modest punctate immunoreactivity was observed where drusen were confluent (**asterisks**). Note immunoreactive extracellular material extending into the outer choroid. **B**, **D**, and **F:** Secondary antibody controls from adjacent sections. Scale bar = 100 μm.

very young donor, a newborn, showed lack of labeling (Figure 2, A–C); this eye was also notable (compared with older donors) for lack of RPE lipofuscin. Interestingly, in this individual, robust MAC was detected in the inner retina, consistent with the proposed role of complement in developmental axon pruning.[18] Bruch's membrane and choriocapillaris from a 16-month–old donor (Figure 2D) showed tiny puncta of labeling, but were generally negative for MAC. Labeling in the eye of a 5-year-old donor who died of myelogenous leukemia showed modest MAC labeling, in a pattern that was predominantly on the inner aspect of Bruch's membrane and did not wrap around the choriocapillaris endothelium (Figure 2, E and F). By 21 years of age, lipofuscin in the RPE was frequently already remarkable (data not shown). Donors in the third and fourth decade of life had detectable MAC both in Bruch's membrane and around the outer aspect of the choriocapillaris (Figure 2, G and H). This distribution was also observed in the fifth decade (Figure 2, I and J), as well as in older donors without AMD (Figure 2, K and L).

In eyes with early AMD, consistent with previous report,[11,17,20] small, hard drusen were almost invariably labeled with anti-MAC antibody (Figure 3, A and B), whereas basal deposits and large confluent drusen were generally negative (Figure 3, C and D). Some large macular drusen showed punctate immunoreactivity (Figure 3, E and F). In contrast to younger eyes and aged control eyes, extension of the MAC-reactive domain often extended into the outer choroid (Figure 3A). Extramacular drusen were invariably positive for MAC, consistent with previous report.[11,21]

Eyes with advanced AMD were also evaluated. In eyes with geographic atrophy (n = 15 eyes from 11 donors), MAC was present outside of areas of RPE and photoreceptor loss in a pattern similar to that seen in early AMD (ie, choriocapillaris) (Figure 4, A–C), although reactivity on outer vessel walls was more notable in eyes with geographic atrophy (Figure 4A). In areas of extensive atrophy (Figure 4, D and F) the intensity of immunoreactivity at the choroiocapillaris–Bruch's membrane interface was lower than elsewhere, although a moderate level of anti-MAC labeling was found to persist when RPE, photoreceptor, and choriocapillaris loss was complete (Figure 4F). In one case, near the interface of the healthy RPE and geographic atrophy, MAC deposition was observed on the RPE (Figure 4E).

In addition, eyes from 11 donors with CNV were evaluated. Choroidal neovascular membranes, with or without subretinal fibrosis, were identified in eyes with RPE degeneration (Figure 5, A–D) or between the outer layers of Bruch's membrane and an intact layer of RPE (type I or occult CNV) (Figure 5, E–H). Eyes with CNV showed persistent labeling of MAC at the level of the choriocapillaris even after degeneration of the endothelium was complete (Figure 5A). Eyes with CNV frequently show a detached layer of basal laminar deposits.[22,23] Interestingly, in one donor with neovascularization, the MAC was

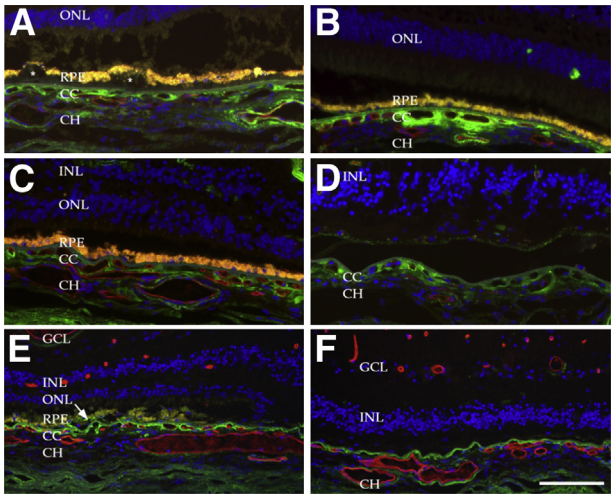

Figure 4 Localization of the MAC in the choriocapillaris of eyes with geographic atrophy. The choroid is thinner in eyes with advanced dry AMD than in control eyes.[16] Shown are areas outside (**A–C**), within (**D** and **F**), and at the junction (**E**) of the central atrophy. **A:** Drusen deposits (**asterisks**), extensive MAC in the choriocapillaris layer, and vascular atrophy with loss of endothelium (ghost vessels) in an 83-year-old donor eye. **B:** Choriocapillaris atrophy, with nonreactive basal deposits, in an 82-year-old donor eye. **C:** An area of atrophy in an 89-year-old donor eye, with some thinning and attenuation of the ONL and shortening of the inner–outer segment. **D:** An area of central atrophy, with loss of the RPE, in an 87-year-old donor eye. Note the modest amount of RPE lipofuscin in a gliotic scar. MAC immunoreactivity was still present in the largely atrophied choriocapillaris. **E** and **F:** The atrophic interface (**E**) and the central atrophic zone (**F**) of an eye from a 68-year-old donor homozygous for the high-risk Y402H allele. Note localization of MAC to RPE cells near the interface (**E**, **arrow**) and persistence of MAC in the degenerated choroid in the area of central atrophy. Scale bar = 100 μm. CC, choriocapillaris; CH, outer choroid; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.

present on or near the RPE (Figure 5, G and H), as well as in the outer aspect of the basal laminar deposits.[24] This labeling of RPE with anti-MAC antibody was not observed in early AMD.

Genotype and Choroidal Thickness

We hypothesized that the Y402H variant in *CFH* affects morphological features of the macula. Choroidal thickness measurements were collected in the macula in a masked fashion. The *CFH* high-risk H allele was associated with thinner choroids (Figure 6). Compared with eyes from donors homozygous for the low-risk Y allele, eyes from donors homozygous for the Y402H single-nucleotide polymorphism were 23.6% thinner ($P < 0.05$ for HH versus YY). Linear regression indicated a significant association between choroidal thickness and the number of copies of the H allele ($P = 0.019$).

Discussion

The complement system comprises an interacting set of evolutionarily ancient proteins, with members represented in animal phyla as far removed from humans and other

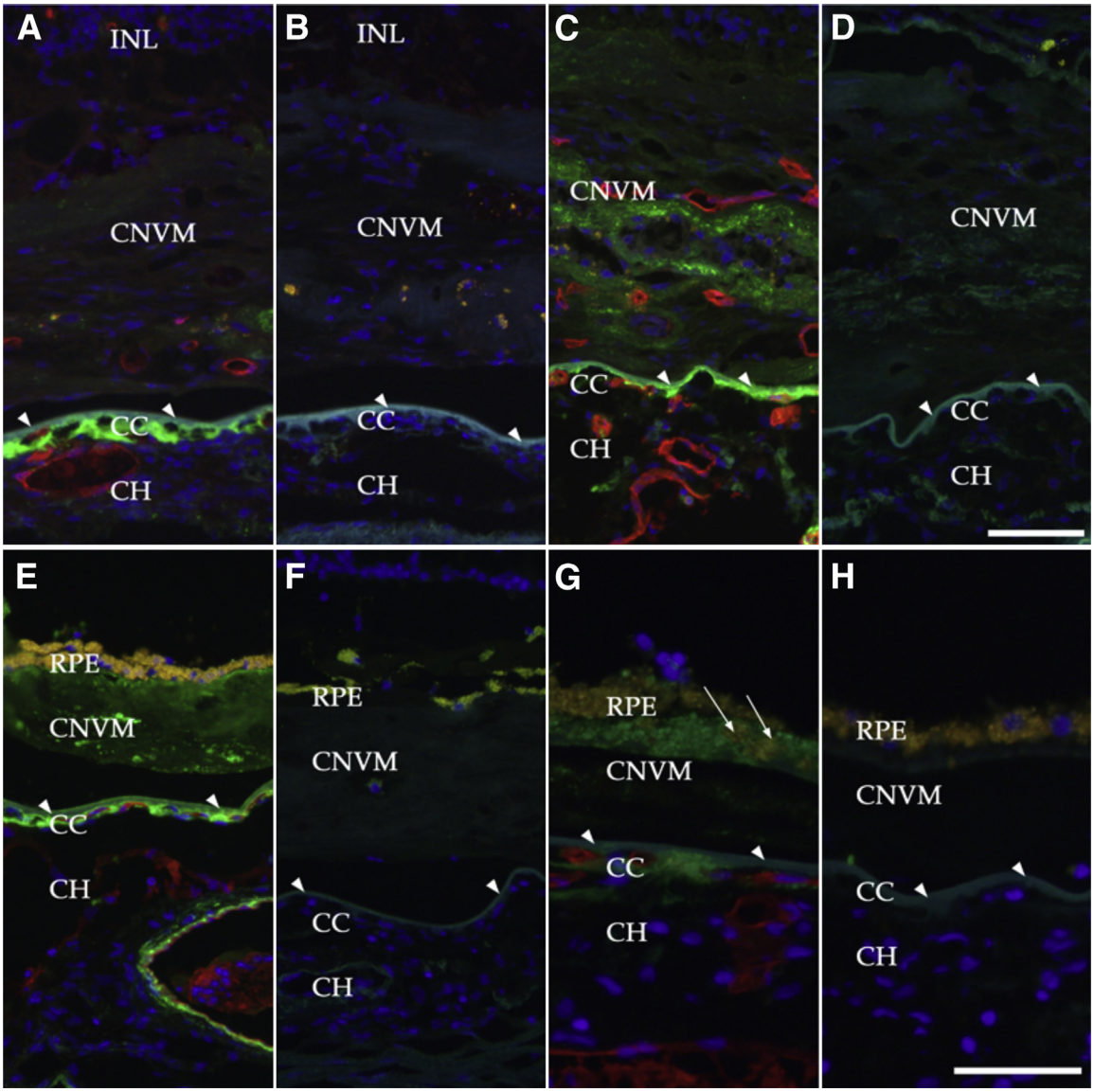

Figure 5 Localization of MAC in eyes with choroidal neovascularization under dual labeling with anti-C9 neoepitope antibody (green) and the vascular-labeling lectin UEA-I (red). Sections were incubated with primary and secondary antibody (**A**, **C**, **E**, and **G**); adjacent unlabeled sections served as negative controls (**B**, **D**, **F**, and **H**). Bruch's membrane is indicated by **arrowheads**. **A** and **C:** MAC persisted in domains surrounding the choriocapillaris even after extensive atrophy. MAC was also present in some choroidal neovascular membranes in association with dedifferentiated RPE cells (**C**, **arrows**). **E:** Punctate labeling could also be observed in subretinal fibrosis and beneath the RPE that resided on the surface of these fibrotic membranes. **G:** In an eye with type I or occult CNVM, MAC labeling of the RPE was robust (**arrows**). Scale bars: 50 μm (**G** and **H**); 75 μm (**A**–**F**). CC, choriocapillaris; CH, outer choroid; CNV, choroidal neovascularization; CNVM, choroidal neovascular membrane; INL, inner nuclear layer; RPE, retinal pigment epithelium.

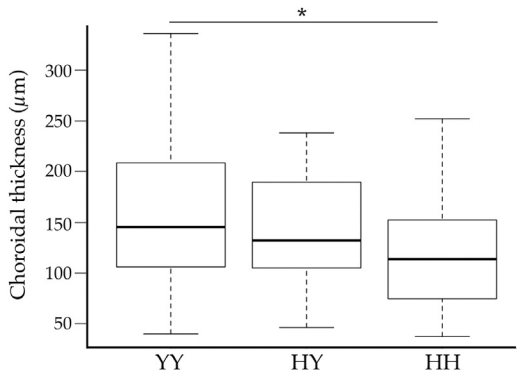

Figure 6 Choroidal thickness in donor eyes of the three Y402H *CFH* genotypes. Measurements of choroidal thickness were performed as described previously,[16] and genotypes were determined for the Y402H allele (rs1061170). Eyes from donors homozygous for the high-risk H allele had thinner choroids than eyes from donors heterozygous or homozygous for the low-risk Y allele. Eyes homozygous for the H allele had significantly thinner choroids than those homozygous for the Y allele ($P < 0.05$), and there was a significant association ($P = 0.019$) between choroidal thickness and the number of copies of the H allele. Data are expressed as box-and-whisker plots, indicating median, interquartile range, and minimum and maximum values. $n = 43$ (YY); $n = 32$ (HY); $n = 25$ (HH). *$P < 0.05$.

vertebrates (Chordata) as the Cnidaria and Echinodermata.[25] Metazoan species lacking complement genes (eg, *Drosophila*) are thought to have undergone selective loss of genes that were present in a shared ancestor.[26] Although antimicrobial protection is the principal role for complement, roles for this system in tissue repair and regeneration have also been described.[27] Although the complement system is a critical component of innate immunity, abnormal activation can lead to bystander injury of resident cells.[28]

The striking abundance of the MAC of complement in the human macular choriocapillaris has been reproducibly observed.[8,17] Seth et al[17] previously evaluated MAC semiquantitatively in aging eyes and found an increase in MAC abundance in aging. Hageman et al[8] reported that MAC increases with aging and that its localization is predominantly in the macular region. Our immunofluorescence and ELISA results are in accord with these findings, although in fixed frozen sections using immunofluorescence we detected MAC at a much younger age and consistently in older eyes. Although the youngest donor eye in our cohort showed little or no labeling, we observed MAC as early as 2 years of age, with modest labeling by age 5 and striking accumulation by age 21. Thus, choriocapillaris endothelial cells are potentially exposed to some level of MAC for decades before any development of drusen or vision loss.

Given the role of the MAC in cell lysis and opsonization, its high concentration in the delicate tissues of the macula seems surprising, especially in eyes much too young to develop AMD and preceding the appearance of RPE lipofuscin. One possible explanation for complement activation in Bruch's membrane and the choriocapillaris is that some beneficial role for this state of MAC formation offsets its risks for bystander injury, especially at younger ages. The photoreceptor–RPE–choriocapillaris interface is physiologically unusual. Photoreceptor cells, or at least rod cells, completely renew their outer segments approximately every 10 days, and thus 1/10 of the entire outer surface of the retina is phagocytosed and processed every day. Each RPE cell is responsible for the maintenance and phagocytosis of 12 to 40 photoreceptor outer segments.[29] Assuming that the outer segment is 25 μm long and 1 μm wide, the RPE must remove and lyse a volume of approximately 2 μm^3 per photoreceptor cell per day; at some eccentricities, this amounts to approximately as one red blood cell per RPE cell per day. That the RPE is able to perform this function, in addition to pumping fluid out of the retina, participating in retinal adhesion, and directing the visual cycle, suggests a highly efficient process.[30,31] It is conceivable that the complement activation in Bruch's membrane is an adaptive response to help opsonize and remove incompletely digested cellular debris. In this context, is notable that, in an *in vitro* model of RPE deposition of material into a porous substrate, the MAC was found to opsonize the debris.[32] A similar event *in vivo* may facilitate clearance of Bruch's membrane. The age-related accumulation coincides with other structural and molecular changes in Bruch's membrane, including accumulation of lipids and advanced glycation end products.[19,33–36]

Moreover, the photoreceptor cells of the retina have long chain and very long chain fatty acid molecules with a restricted distribution.[37] Docosahexaenoic acid, for example, can be metabolized into neuroprotective[38,39] or pathogenic[40,41] derivatives. Carboxyethylpyrrole-modified macromolecules are targets of autoantibodies in AMD, and lead to activation of the complement system in mouse.[42] In light of the enrichment of docosahexaenoic acid in the retina, and its metabolism by the RPE, this pathway may explain the abundant complement activation in the aging macular choriocapillaris.

Unlike the major allele, *CFH* molecules harboring a histidine at residue 402 have an altered affinity for C-reactive protein (itself increased in the choroid of donors homozygous for the risk allele[9]), altered behavior in the presence of zinc,[43] and impaired binding to glycosaminoglycans in Bruch's membrane.[44,45] In addition, the 402H form of *CFH* has a reduced affinity for Bruch's membrane malondialdehyde.[46] Moreover, eyes homozygous for the Y402H polymorphism have increased deposition of MAC.[10] In light of the observed MAC assembly on RPE cells in advanced AMD in the present study, it is also of interest that cultured RPE cells harboring protective haplotypes are more resistant to MAC injury, compared with RPE cells with high-risk haplotypes.[47]

We also addressed the question of whether the increased MAC found in eyes with high-risk genotypes is associated with morphological changes in the macula. Masked studies of choroidal thickness showed evidence for choroidal atrophy associated with homozygosity for the Y402H polymorphism.

The finding that eyes with high-risk genotypes show loss of endothelial cells supports the notion that changes occur in the choroidal vasculature early in AMD pathogenesis. In previous gene expression studies with AMD and age-matched RPE−choroids using unbiased gene set enrichment criteria, we found that, as a group, endothelial cell−associated transcripts decline in eyes with early non-neovascular AMD,[48] a finding that is consistent with proteomic,[49] histopathologic,[15,50−52] novel imaging,[53,54] and blood flow[55,56] studies.

The combined elements of localization of MAC to the choriocapillaris, increased choroidal MAC in high-risk genotypes,[10] and evidence for the loss of vasculature in eyes with high-risk genotypes suggest a mechanism by which *CFH* risk alleles may contribute to AMD. By increasing the overall level of complement activation, *CFH* polymorphisms that result in impaired function or localization of the protein may allow for an increased level of MAC formation that is additive to the high complement activation of normal aging, which in turn leads to injury of the choriocapillaris endothelium in early AMD. Morphometric,[15,57] biochemical,[49] and gene expression[48] studies support early loss of vascular endothelial cells in the choroid in early AMD.

The finding of robust MAC in small hard drusen, but less abundant or undetectable MAC in confluent drusen or basal deposits, suggests that complement may have the opportunity to stress the RPE overlying a druse early in its development, but that other, non-MAC−mediated responses are more important during drusen growth. Moreover, if MAC injury to the RPE up-regulates expression of drusen-associated gene products, as has been shown *in vitro*,[58] this response is more likely in small hard drusen than in confluent drusen. We recently reported that MAC is present in the unusual laminated drusen-like deposits in an eye with Malattia Leventinese and suggested that these deposits have features of both hard drusen and basal deposits.[20]

Overall, the histological findings from our studies and those of others suggest that the choriocapillaris endothelium experiences more substantive challenge by the MAC than does the RPE. MAC was not observed on the RPE surface in either early AMD or age-matched control eyes, but exposure of RPE to MAC was observed in some cases of advanced AMD. An example of robust deposition of MAC on the RPE in an eye with neovascular AMD is shown in Figure 5G. A number of investigators have shown a requirement for complement activation, as well as a protective effect of complement deficiency or inhibition, in the development of experimental CNV in mice. Various mouse studies using cobra venom factor,[12] targeted deletion of specific complement genes,[59−61] and virally delivered[62,63] or pharmaceutical[64,65] complement inhibitors have all shown impaired formation of choroidal neovascular membranes, compared with controls. Moreover, cultured RPE cells respond to sublytic complement attack by increased synthesis of proinflammatory and proangiogenic molecules that may exacerbate the progression of AMD.[58,66]

In summary, aging and the high-risk H allele *CFH* genotypes are associated with increased MAC deposition at the level of the choriocapillaris. Eyes with AMD exhibit increased C-reactive protein and decreased complement factor H, consistent with a choroidal AMD microenvironment that favors complement activation.[67] Choriocapillaris degeneration, whether due to MAC or some other source of injury, is notable in eyes with early AMD,[15] and morphometric studies support a role for the *CFH* genotype in choroidal atrophy. Treatments that protect the choriocapillaris in early AMD, and the RPE in advanced AMD, would be beneficial in addressing this blinding disease.

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