⌘ *Author's Choice*

Assessing Susceptibility to Age-related Macular Degeneration with Proteomic and Genomic Biomarkers*

Jiayin Gu,[a,b,c] Gayle J. T. Pauer,[a,b] Xiuzhen Yue,[a,b] Umadevi Narendra,[a,b] Gwen M. Sturgill,[d] James Bena,[b,e] Xiaorong Gu,[a,b] Neal S. Peachey,[a,b,d,f] Robert G. Salomon,[c,g] Stephanie A. Hagstrom,[a,b,f,h] John W. Crabb,[a,b,c,f,i] and the Clinical Genomic and Proteomic AMD Study Group

Age-related macular degeneration (AMD) is a progressive disease and major cause of severe visual loss. Toward the discovery of tools for early identification of AMD susceptibility, we evaluated the combined predictive capability of proteomic and genomic AMD biomarkers. We quantified plasma carboxyethylpyrrole (CEP) oxidative protein modifications and CEP autoantibodies by ELISA in 916 AMD and 488 control donors. CEP adducts are uniquely generated from oxidation of docosahexaenoate-containing lipids that are abundant in the retina. Mean CEP adduct and autoantibody levels were found to be elevated in AMD plasma by ~60 and ~30%, respectively. The odds ratio for both CEP markers elevated was 3-fold greater or more in AMD than in control patients. Genotyping was performed for AMD risk polymorphisms associated with age-related maculopathy susceptibility 2 (*ARMS2*), high temperature requirement factor A1 (*HTRA1*), complement factor H, and complement C3, and the risk of AMD was predicted based on genotype alone or in combination with the CEP markers. The AMD risk predicted for those exhibiting elevated CEP markers and risk genotypes was 2–3-fold greater than the risk based on genotype alone. AMD donors carrying the *ARMS2* and *HTRA1* risk alleles were the most likely to exhibit elevated CEP markers. The results compellingly demonstrate higher mean CEP marker levels in AMD plasma over a broad age range. Receiver operating characteristic curves suggest that CEP markers alone can discriminate between AMD and control plasma donors with ~76% accuracy and in combination with genomic markers provide up to ~80% discrimination accuracy. Plasma CEP marker levels were altered slightly by several demographic and health factors that warrant further study. We conclude that CEP plasma biomarkers, particularly in combination with genomic markers, offer a potential early warning system for assessing susceptibility to this blinding, multifactorial disease. ***Molecular & Cellular Proteomics 8:1338–1349, 2009.***

Age-related macular degeneration (AMD)[1] is the most common cause of legal blindness in the elderly in developed countries (1). It is a complex, progressive disease involving multiple genetic and environmental factors that can result in severe visual loss. Early risk factors include the macular deposition of debris (drusen) on Bruch membrane, the extracellular matrix separating the choriocapillaris from the retinal pigment epithelium (RPE). Later stages of "dry" AMD involve the degeneration of photoreceptor and RPE cells resulting in geographic atrophy. In "wet" AMD, abnormal blood vessels grow from the choriocapillaris through Bruch membrane (choroidal neovascularization (CNV)). CNV occurs in 10–15% of AMD cases yet accounts for over 80% of debilitating visual loss in AMD. Anti-vascular endothelial growth factor treatments can effectively inhibit the progression of CNV (1), and antioxidant vitamins and zinc can slow dry AMD progression for select individuals (2). However, there are no universally effective therapies for the prevention of dry AMD or the progression from dry to wet AMD nor are there therapies to repair retinal damage in advanced AMD. The prevalence of advanced AMD in the United States is projected to increase by 50% to ~3 million by the year 2020 largely because of the rapidly growing elderly population (3). Accordingly early identification of AMD susceptibility and implementation of preventive measures are important therapeutic strategies (1).

From the [a]Cole Eye Institute, [b]Lerner Research Institute, and [e]Department of Quantitative Health Sciences, Cleveland Clinic Foundation, Cleveland, Ohio 44195, [c]Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106, [d]Louis Stokes Veterans Affairs Medical Center, Cleveland, Ohio 44106, and [f]Department of Ophthalmology, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, Ohio 44195

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[1] The abbreviations used are: AMD, age-related macular degeneration; CEP, carboxyethylpyrrole; CFH, complement factor H; C3, complement C3; CI, confidence interval; CNV, choroidal neovascularization; DHA, docosahexaenoic acid; HTRA1, high temperature requirement factor A1; ARMS2, age-related maculopathy susceptibility 2 (also known as LOC387715); OR, odds ratio; ROC, receiver operating characteristic; RPE, retinal pigment epithelium.

The molecular mechanisms causing AMD remain unknown, although inflammatory processes have been implicated by the identification of AMD susceptibility genes encoding complement factors (4–10) and the presence of complement proteins in drusen (11–13). Oxidative stress has long been associated with AMD pathology as shown by the finding that smoking significantly increases the risk of AMD (14) and that antioxidant vitamins can selectively slow AMD progression (2). A direct molecular link between oxidative damage and AMD was established by the finding that carboxyethylpyrrole (CEP), an oxidative protein modification generated from docosahexaenoate (DHA)-containing phospholipids, was elevated in Bruch membrane and drusen from AMD patients (11). Subsequently CEP adducts as well as CEP autoantibodies were found to be elevated in plasma from AMD donors (15), and CEP adducts were found to stimulate neovascularization *in vivo*, suggesting a role in the induction of CNV (16). From such observations, oxidative protein modifications were hypothesized to serve as catalysts of AMD pathology (11, 15, 17). In support of this hypothesis, mice immunized with CEP-adducted mouse albumin develop a dry AMD-like phenotype that includes sub-RPE deposits resembling drusen and RPE lesions mimicking geographic atrophy (18).

Although identified AMD susceptibility genes account for over half of AMD cases (19), many individuals carrying AMD risk genotypes may never develop the disease. Likewise only a fraction of those diagnosed with early AMD progress to advanced stage disease with severe visual loss (2). Toward the discovery of better methods to predict susceptibility to advanced AMD, we quantified CEP adducts and autoantibodies in over 1400 plasma donors and also genotyped many of these donors for AMD risk polymorphisms in complement factor H (*CFH*) (4–7), complement *C3* (9, 10), age-related maculopathy susceptibility 2 (*ARMS2*; also known as *LOC387715*) (19–22), and high temperature requirement factor A1 (*HTRA1*) (23, 24). The results demonstrate that combined CEP proteomic and genomic biomarker measurements are more effective in assessing AMD risk than either method alone.

EXPERIMENTAL PROCEDURES

Case-Control Study Design—Clinically documented AMD and control blood donors were recruited prospectively between 2003 and 2008 from the Cole Eye Institute, Cleveland Clinic Foundation, and the Eye Clinic, Louis Stokes Cleveland Veterans Affairs Medical Center with the approval of each Institutional Review Board and according to Declaration of Helsinki principles. All patients received a comprehensive eye examination by a clinician in the Clinical Study Group and provided written informed consent. All human identifiers were removed from blood specimens and were encoded by the Clinical Study Group to protect donor confidentiality. AMD disease progression was categorized based on fundus examination, and patients were included in the study from Age-Related Eye Disease Study AMD categories 2, 3, and 4 (2). Briefly AMD category 2 patients exhibited early stage disease with multiple small drusen, single or nonextensive intermediate drusen (63–124 μm), RPE pigmentary abnormalities, or any combination of these in one or both eyes and visual acuity of 20/30 or better in both eyes. AMD category 3 patients exhibited midstage disease with at least one eye having visual acuity of 20/30 or better and one large drusen (125 μm), extensive intermediate drusen, or geographic atrophy that did not involve the macula or any combination of these. Category 3 patients lacked advanced AMD in either eye. AMD category 4 patients exhibited advanced AMD with substantial CNV or geographic atrophy involving the macula in one or both eyes. Control donors lacked macular drusen and exhibited no clinical evidence of any retinal disorder. Plasma and DNA pellets were prepared from blood specimens and stored frozen until analysis as described below.

Human Plasma Preparation—Nonfasting blood specimens were collected in BD Vacutainer® K_2EDTA tubes, and plasma was prepared within 6 h and aliquoted to vials containing the antioxidant butylated hydroxytoluene (1 mg/ml plasma) and a protease inhibitor mixture (Sigma product number P 8340; 10 μl/ml plasma) (15). The plasma was flushed with argon, quench frozen in liquid nitrogen immediately, and stored at −80 °C. Storage time at −80 °C prior to analysis ranged from 1 to 10 months and averaged 4 months over the 5-year study period. All samples were frozen and thawed only once.

ELISA—CEP adducts were detected with IgG purified rabbit anti-CEP polyclonal antibody and quantified as described previously using a competitive ELISA using CEP-modified BSA (CEP-BSA) as coating agent and known amounts of CEP-modified human serum albumin as reference protein (ELISA method B in Ref. 15). CEP autoantibody titers were measured by direct ELISA using CEP-BSA as coating antigen as described previously (ELISA method C in Ref. 15). CEP-BSA and CEP-modified human serum albumin were synthesized and characterized as described previously (15, 25).

Western Analysis—Western analysis of plasma proteins from AMD and normal donors was performed as described previously (11). CEP immunoreactivity was detected by chemiluminescence and quantified by densitometry using a Bio-Rad GS-710 instrument.

Genotyping—DNA was isolated from blood using standard procedures. AMD risk polymorphisms were genotyped in *HTRA1* (rs11200638 in the promoter region), *C3* (rs2230199 encoding an R102G interchange), and *CFH* (rs1061170 encoding a Y402H interchange) by restriction analysis with EagI, HhaI, and Hsp92II, respectively. Those in *ARMS2* (*LOC387715* rs10490924 encoding an A69S interchange) were determined by direct DNA sequence analysis using an Applied Biosystems model 3130 XL instrument.

Statistical Analysis—Continuous measures were summarized using means, standard deviations, medians, and interquartile ranges, whereas categorical factors were described using frequencies and percentages. Differences in plasma CEP adduct concentration and CEP autoantibody titer between control and AMD patients were evaluated using two-sample *t* tests in Minitab Release 15 (Minitab Inc.). To evaluate a relationship between CEP adducts and autoantibody titer with AMD susceptibility, a logistic regression model was fit with both variables as predictors of AMD using Proc Logistic in SAS 9.1 (SAS Institute Inc., Cary, NC). C-statistics measured the ability of the model to discriminate between AMD and controls, whereas odds ratios (ORs) showed the change in risk of AMD based on the predictors. ORs, c-statistics, and *p* values were determined based on log-transformed CEP marker concentrations. Validation of c-statistics was performed using 2000 bootstrap (random) resamplings to calculate empirical 95% confidence intervals (CI) and by performing 10-fold cross-validation. Sensitivity and specificity were calculated to maximize the sum of the two values using receiver operating characteristic (ROC) curves constructed with SAS 9.1 from the output of logistic regression analysis fit with either CEP adduct concentrations plus autoantibody titers, homozygous risk genotype, or the combination of the CEP markers and the risk genotype. C-statistics and *p* values comparing ROC curves were determined with SAS 9.1. For association analyses of combined

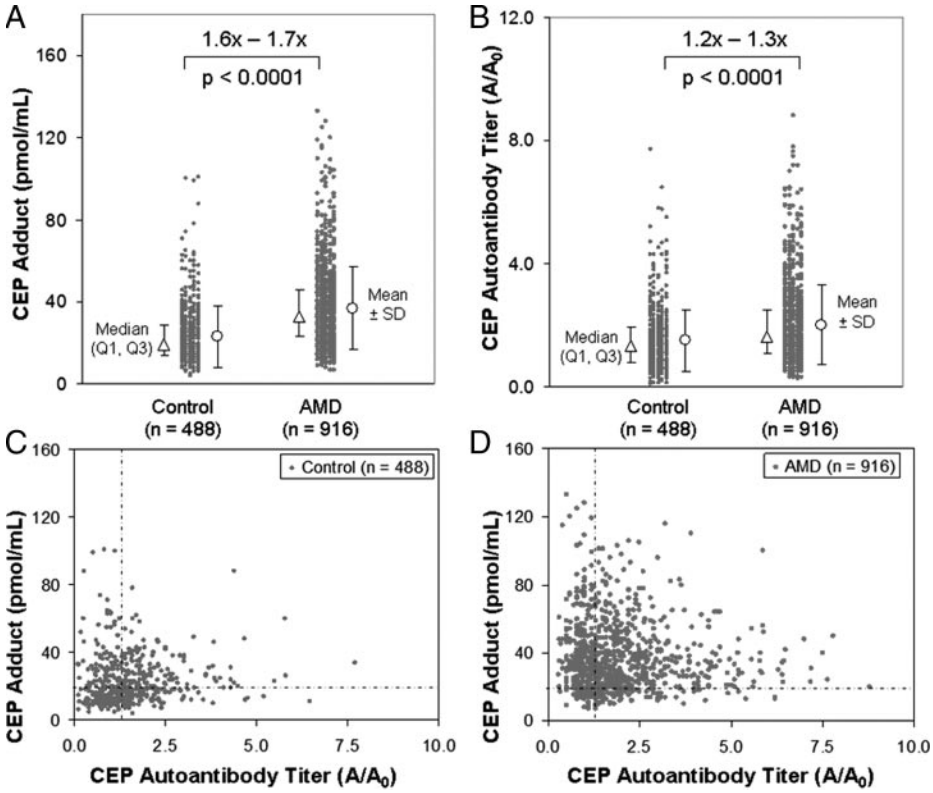

FIG. 1. **CEP adducts and autoantibodies are elevated in AMD plasma.** CEP adduct concentrations (*A*) and autoantibody titers (*B*) quantified by ELISA from control ($n = 488$) and AMD ($n = 916$) plasma donors are shown with median (△) results ± first and third quartiles (*Q1*, *Q3*) and mean (○) results ± S.D. indicated. *p* values (two-sided *t* test) were determined from log-transformed concentrations. These data are presented in Table I by category of AMD progression. Correlation between CEP adduct levels and autoantibody titers is shown for the control (*C*) and AMD (*D*) cohorts with *horizontal* and *vertical dashed lines* indicating median control values. Significantly more donors with both CEP markers elevated are apparent in AMD patients than in the controls (*upper right quadrants* in *C* and *D*).

TABLE I

CEP markers in AMD and control plasma

CEP adduct concentration and autoantibody titer were determined by ELISA. Odds ratios reflect the AMD risk for donors exhibiting elevated levels of both CEP markers relative to median control levels (≥19 pmol/ml CEP adducts and ≥1.3 CEP autoantibody titer). *p* values were determined using the Fisher exact test. AMD categories are based on the Age-Related Eye Disease Study classification system (2). Odds ratios, 95% CI, and *p* values are based on log-transformed CEP marker concentrations. Ab, antibody.

	n	CEP adduct		CEP auto-Ab titer (A/A_0)		Both CEP adduct and auto-Ab elevated		
		Mean ± S.D.	Median (Q1, Q3)	Mean ± S.D.	Median (Q1, Q3)	Odds ratio	95% CI	*p* value
		pmol/ml						
Control	488	23 ± 15	19 (14, 29)	1.5 ± 1.0	1.3 (0.8, 1.9)	1.00	Reference	
All AMD	916	37 ± 20	33 (23, 46)	2.0 ± 1.3	1.6 (1.1, 2.5)	3.17	2.51, 4.02	<0.001
AMD category 2	177	35 ± 19	31 (22, 46)	2.2 ± 1.3	1.8 (1.3, 2.6)	4.24	2.95, 6.10	<0.001
AMD category 3	130	32 ± 17	28 (20, 39)	2.2 ± 1.4	1.7 (1.2, 2.8)	3.25	2.18, 4.84	<0.001
AMD category 4	609	39 ± 21	34 (24, 48)	1.9 ± 1.2	1.5 (1.0, 2.3)	2.91	2.26, 3.75	<0.001

effects of plasma CEP adducts and autoantibody titer with AMD risk genotypes, ORs with 95% CI and Fisher exact *p* values were calculated with SAS 9.1 software. Pearson's correlation analysis in Minitab Release 15 was used to compare CEP marker concentrations with plasma donor age.

RESULTS

Elevated CEP Adducts and Autoantibodies in AMD Plasma—Plasma samples from a total of 488 control subjects and 916 AMD subjects, including 177 with early stage dry AMD, 130 with midstage dry AMD, and 609 with advanced stage AMD, were analyzed by ELISA. The results (Fig. 1, *A* and *B*, and Table I) demonstrate higher mean levels of CEP adducts (~1.6×) and autoantibody titers (~1.3×) in AMD patients relative to control plasma. Comparison of log-transformed values confirmed the results and yielded *p* values <0.0001. Data from duplicate adduct and triplicate autoantibody measurements exhibited average intra-assay variability of ~4% for adducts and ~8% for autoantibodies. Interassay, day-to-day variability, as measured by IC_{50}, averaged ~25%. Western analysis of AMD and control plasma ($n = 10$ each) demonstrated that CEP adducts are associated with proteins and also supported higher levels of the adducts in AMD plasma (Fig. 2). Plasma from all categories of AMD progression, including early stage AMD, exhibited elevated mean levels of CEP adducts and autoantibodies with no significant difference between disease categories (Table I).

Correlation of CEP adduct and autoantibody levels (Fig. 1, *C* and *D*) revealed both markers to be elevated above median

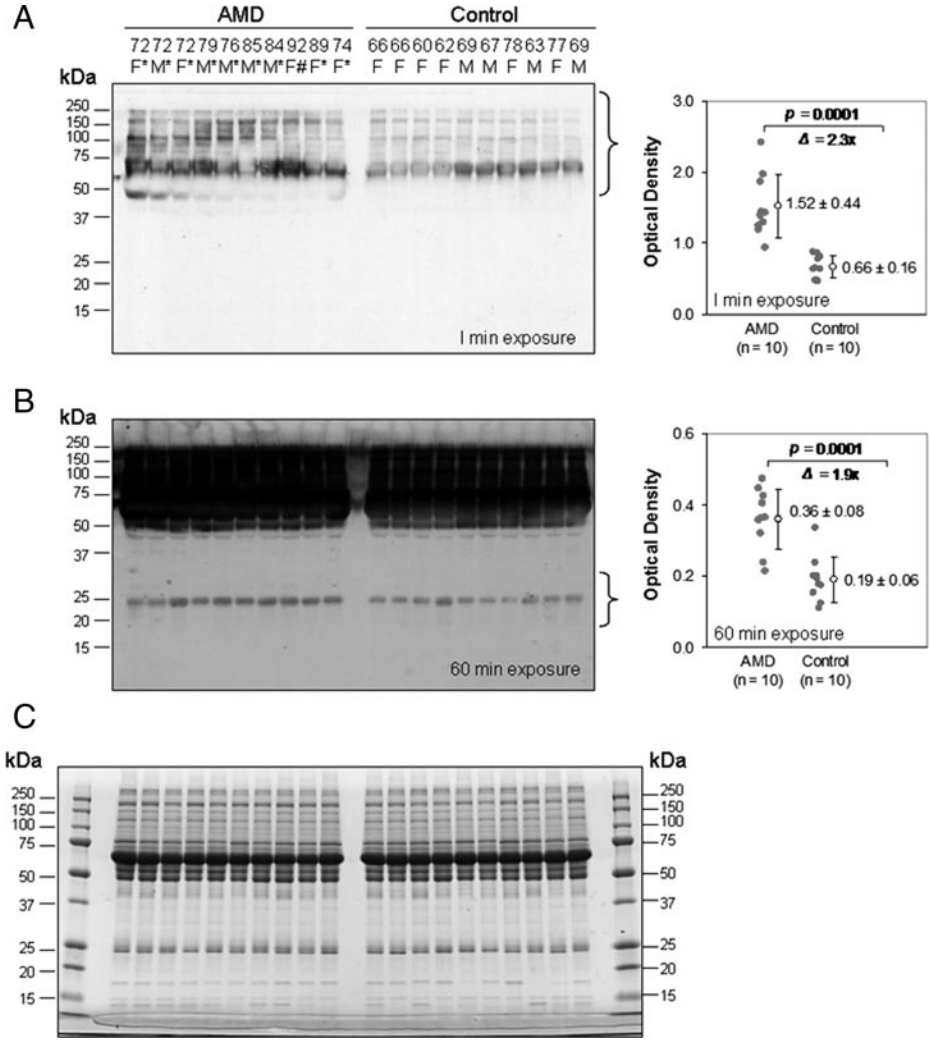

FIG. 2. **Western analysis of AMD and control plasma for CEP adducts.** Human AMD and control donor plasma samples were subjected to SDS-PAGE (~15 μg/lane), electroblotted to PVDF, and probed with mouse monoclonal anti-CEP antibody. CEP adducts (immunoreactivity) are shown to be associated with high molecular mass components (>~40 kDa) after 1-min autoradiography (*A*) and also with components of ~25 kDa after 60-min autoradiography (*B*). Additional components become apparent with longer autoradiography time. Densitometric quantification of CEP immunoreactivity in the indicated bands supports more CEP-adducted proteins in AMD than normal donor plasma (*p* values are from the two-sided *t* test). *Error bars* reflect standard deviation. The Coomassie Blue-stained gel (*C*) shows that approximately equal amounts of protein were applied per lane for the Western analysis. The age and sex of each donor are listed, and for AMD samples, the asterisk (*) and # symbols denote donors with CNV or geographic atrophy, respectively. *F*, female; *M*, male.

TABLE II
c-statistics for CEP markers

The c-statistic and 95% CI were determined by a logistic regression model fitted with log-transformed CEP adduct concentration and autoantibody titer as independent variables and were validated by bootstrap resampling and 10-fold cross-validation.

	n	Total		Bootstrap validation		Cross-validation	
		Estimate	95% CI	Estimate	95% CI	Estimate	95% CI
Control	488						
All AMD	916	0.76	0.73, 0.79	0.76	0.73, 0.79	0.76	0.73, 0.78
AMD category 2	177	0.76	0.72, 0.80	0.76	0.72, 0.80	0.75	0.71, 0.79
AMD category 3	130	0.73	0.68, 0.77	0.73	0.69, 0.78	0.72	0.68, 0.77
AMD category 4	609	0.77	0.74, 0.80	0.77	0.74, 0.80	0.77	0.74, 0.80

control levels in 56% of AMD patients (515 of 916) compared with only 29% of control donors (141 of 488). Logistic regression modeling of all control and AMD data yielded a c-statistic equal to 0.76 for AMD patients, supporting a 76% likelihood that a randomly selected person with both CEP variables elevated will be an AMD case rather than a control (Table II). Little difference in c-statistics (0.73–0.77) was observed from logistic regression modeling of the data from early, mid-, or late stage AMD patients (Table II), and bootstrap resampling and 10-fold cross-validation supported all c-statistics and 95% CIs. The predicted risk of AMD was measured by OR for donors with both CEP markers elevated relative to the median of the control plasma. The ORs for all (3.17), for midstage (3.25), and for advanced AMD patients (2.91) were found to be similar, whereas that for early stage AMD patients was slightly higher (4.24) but with a larger 95% CI (Table I).

AMD Risk Based on CEP Markers and Genotype—Genotyping for AMD risk polymorphisms associated with *ARMS2*,

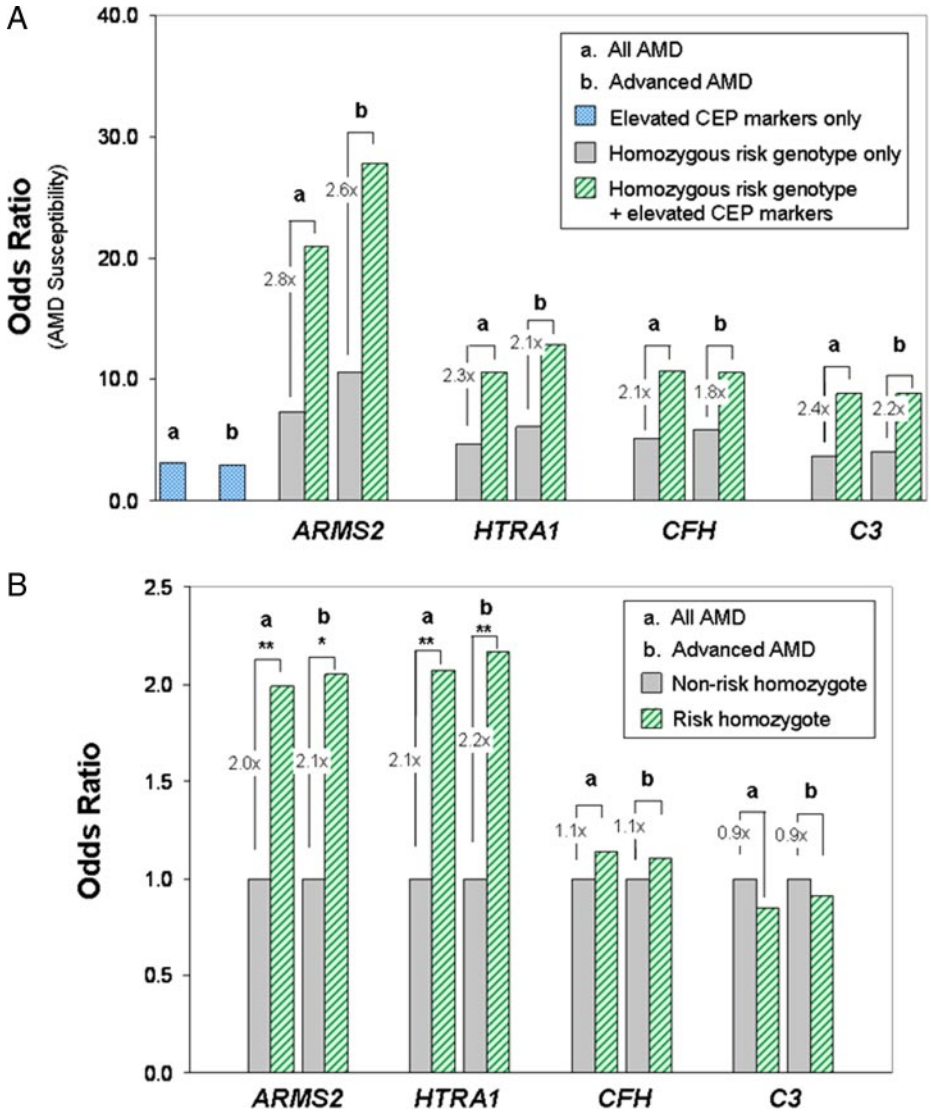

FIG. 3. **AMD risk predicted by CEP markers and genotype.** *A*, odds ratios for AMD risk based on elevated CEP markers only, genotype only (specific for the homozygous risk alleles *ARMS2*, *HTRA1*, *CFH* and *C3*), and the joint effect of both are shown for all AMD (*a*) and advanced AMD (*b*) patients. *B*, odds ratios for both CEP markers to be elevated in AMD risk and non-risk homozygous genotypes are shown for all AMD or advanced AMD patients. Differences in CEP marker concentrations between homozygous risk and non-risk donors were statistically significant (**, $p < 0.01$; *, $p < 0.05$; Fisher exact test) for *ARMS2* and *HTRA1* but not for *CFH* and *C3*. Sample size, gene frequencies, OR with 95% CI, and *p* values are presented in Tables III, IV, and VI. OR, 95% CI, and *p* values were determined with log-transformed CEP marker concentrations.

HTRA1, *CFH*, and *C3* was performed on control ($n = 233–404$) and AMD ($n = 708–788$) patients, and the predicted risk of AMD was estimated by OR for the heterozygous and homozygous risk genotypes. Fig. 3*A* shows that the ORs based on homozygous genotype alone were greater than those based on CEP markers alone for advanced AMD as well as for all AMD donors. Combining CEP measurements and genotype resulted in higher ORs for AMD than from genotype alone (Fig. 3*A*). For example, for individuals homozygous for the *ARMS2* risk genotype, elevated CEP levels increased the OR for AMD from 7.32 to 20.88 (~2.8-fold) for all AMD cases and from 10.54 to 27.74 (~2.6-fold) for advanced AMD cases. Similarly the OR for AMD based on genotype alone increased ~2–3-fold for the *HTRA1*, *CFH*, and *C3* risk alleles when combined with elevated CEP levels. Genotype frequencies and ORs with 95% CI for homozygous risk, non-risk, and heterozygous genotypes are presented in Table III (all AMD) and Table IV (advanced AMD). Notably combining CEP measurements with either heterozygous or homozygous genotype resulted in higher ORs for AMD.

Sensitivity and specificity measures were determined from ROC curves for all AMD cases and controls for the CEP markers alone, the four genomic markers alone, and the combined markers (Table V). Calculated to maximize the sum of the two values, sensitivity (~73%) was greater for the CEP markers than for any of the genomic markers alone (31–60%), whereas specificity was greater for the genomic markers (77–94%). The combined markers exhibited 63–75% sensitivity and 67–81% specificity. The areas under the ROC curves (c-statistics) were verified by bootstrap sampling and by 10-fold cross-validation and were greater for the combined markers (0.76–0.80) and CEP alone (0.76) than for the genomic markers alone (0.62–0.69).

To probe for possible associations between CEP marker levels and AMD risk genotypes, we evaluated ORs for elevated CEP markers in the advanced AMD cohort and in the

TABLE III

AMD risk for all AMD based on genotype and CEP markers

Odds ratios were determined for all AMD risk based on genotype alone or based on the joint effect of genotype plus elevated levels of both CEP markers relative to median control levels (≥19 pmol/ml CEP adducts and ≥1.3 CEP autoantibody titer). Odds ratios and 95% CI involving CEP markers are based on log-transformed concentrations.

AMD risk genotype	Genotype frequency (%)		Odds ratio (95% CI)		
	Control	AMD	Genotype only	Joint effect CEP adducts and autoantibodies	
				Not both elevated	Both elevated
ARMS2	*n* = 404	*n* = 708			
GG	246 (60.9%)	278 (39.3%)	1.00 (reference)	1.00 (reference)	2.75 (1.92, 3.95)
GT	143 (35.4%)	306 (43.2%)	1.89 (1.46, 2.46)	1.87 (1.32, 2.65)	4.82 (3.26, 7.14)
TT	15 (3.7%)	124 (17.5%)	7.32 (4.17, 12.84)	6.16 (3.00, 12.66)	20.88 (8.22, 53.04)
HTRA1	*n* = 229	*n* = 747			
GG	134 (58.5%)	294 (39.4%)	1.00 (reference)	1.00 (reference)	2.90 (1.84, 4.57)
GA	78 (34.1%)	279 (37.3%)	1.63 (1.18, 2.25)	1.90 (1.26, 2.86)	3.23 (2.01, 5.18)
AA	17 (7.4%)	174 (23.3%)	4.66 (2.72, 7.99)	4.21 (2.06, 8.60)	10.61 (4.74, 23.71)
CFH	*n* = 381	*n* = 788			
TT	161 (42.3%)	160 (20.3%)	1.00 (reference)	1.00 (reference)	2.55 (1.62, 4.02)
TC	172 (45.1%)	384 (48.7%)	2.25 (1.69, 2.98)	2.19 (1.50, 3.20)	6.17 (4.03, 9.45)
CC	48 (12.6%)	244 (31.0%)	5.12 (3.50, 7.47)	6.13 (3.74, 10.06)	10.65 (5.90, 19.20)
C3	*n* = 343	*n* = 769			
CC	198 (57.7%)	317 (41.2%)	1.00 (reference)	1.00 (reference)	2.84 (1.96, 4.13)
CG	114 (33.2%)	270 (35.1%)	1.48 (1.12, 1.96)	1.62 (1.11, 2.34)	3.71 (2.43, 5.68)
GG	31 (9.1%)	182 (23.7%)	3.67 (2.41, 5.58)	4.52 (2.69, 7.60)	8.87 (4.28, 18.37)

TABLE IV

AMD risk for advanced AMD based on genotype and CEP markers

Odds ratios were determined for advanced AMD risk based on genotype alone or based on the joint effect of genotype plus elevated levels of both CEP markers relative to median control levels (≥19 pmol/ml CEP adducts and ≥1.3 CEP autoantibody titer). Odds ratios and 95% CI involving CEP markers are based on log-transformed concentrations.

AMD risk genotype	Genotype frequency (%)		Odds ratio (95% CI)		
	Control	Advanced AMD	Genotype only	Joint effect CEP adducts and autoantibodies	
				Not both elevated	Both elevated
ARMS2	*n* = 404	*n* = 457			
GG	246 (60.9%)	151 (33.1%)	1.00 (reference)	1.00 (reference)	2.51 (1.65, 3.83)
GT	143 (35.4%)	209 (45.7%)	2.38 (1.78, 3.19)	2.35 (1.59, 3.49)	5.58 (3.62, 8.61)
TT	15 (3.7%)	97 (21.2%)	10.54 (5.90, 18.82)	8.94 (4.23, 18.87)	27.74 (10.70, 71.93)
HTRA1	*n* = 229	*n* = 511			
GG	134 (58.5%)	175 (34.2%)	1.00 (reference)	1.00 (reference)	2.75 (1.68, 4.52)
GA	78 (34.1%)	204 (39.9%)	2.05 (1.45, 2.89)	2.43 (1.56, 3.78)	3.79 (2.29, 6.27)
AA	17 (7.4%)	132 (25.8%)	6.08 (3.50, 10.58)	5.84 (2.80, 12.16)	12.82 (5.63, 29.21)
CFH	*n* = 381	*n* = 526			
TT	161 (42.3%)	96 (18.3%)	1.00 (reference)	1.00 (reference)	2.12 (1.26, 3.56)
TC	172 (45.1%)	261 (49.6%)	2.54 (1.85, 3.50)	2.28 (1.49, 3.49)	6.28 (3.93, 10.01)
CC	48 (2.6%)	169 (32.1%)	5.90 (3.92, 8.88)	6.74 (3.96, 11.49)	10.54 (5.63, 19.73)
C3	*n* = 343	*n* = 509			
CC	198 (57.7%)	195 (38.3%)	1.00 (reference)	1.00 (reference)	2.72 (1.80, 4.11)
CG	114 (33.2%)	190 (37.3%)	1.69 (1.25, 2.30)	2.13 (1.42, 3.18)	4.05 (2.56, 6.39)
GG	31 (9.1%)	124 (24.4%)	4.06 (2.61, 6.31)	5.19 (3.00, 8.97)	8.86 (4.15, 18.90)

entire AMD study population. The results (Fig. 3*B*) show an ~2-fold significant increase in the OR for elevated CEP markers over the non-risk genotype for AMD donors carrying homozygous risk alleles for *ARMS2* and *HTRA1* ($p \leq 0.03$) but not for *CFH* and *C3*. Genotype frequencies, *p* values, and ORs with 95% CI for these analyses are presented in Table VI.

The Influence of Demographic and Health Factors on Plasma CEP Markers—Study population characteristics, including age, gender, race, and health history, are summarized in Table VII. Comparison of plasma CEP marker levels by donor age (Fig. 4) revealed that CEP adduct concentrations are relatively stable with age and that AMD patients had significantly higher mean levels than controls at all ages. Mean CEP autoantibody titer remained stable with age in AMD patients and was higher than in controls but increased gradually with age in control donors (Fig. 4). Comparison of log-transformed CEP marker concentrations (not shown) confirmed these results.

Plasma CEP marker concentrations were also compared by gender, race, and health history, including smoking, hyper-

TABLE V

Sensitivity and specificity of CEP markers and genomic markers

Sensitivity and specificity were determined from ROC curves to maximize the sum of the two values and constructed from the output of logistic regression analysis fit with either CEP adduct concentrations plus autoantibody titers, homozygous risk genotype, or the combination of the CEP biomarkers and the risk genotype. c-statistics, 95% CI, and *p* values derived from single and joint markers were determined with SAS 9.1 based on log-transformed CEP marker concentrations. Verification of c-statistics and 95% CI was performed by bootstrap resampling and 10-fold cross-validation. The c-statistic is a measure of the area under the ROC curve and the accuracy of the markers to discriminate between AMD cases and controls with 1.0 equivalent to 100% accuracy and 0.5 equal to no discrimination. Combining the CEP and genomic markers significantly improved the c-statistics for all the genomic markers.

Markers alone	CEP	*ARMS2*	*HTRA1*	*CFH*	*C3*
Sensitivity (%)	73	31	37	60	36
Specificity (%)	65	94	89	77	86
c-statistic	0.76	0.62	0.63	0.69	0.62
95% CI	0.73, 0.79	0.58, 0.67	0.58, 0.68	0.64, 0.73	0.57, 0.66
c-statistic (bootstrap)	0.76	0.62	0.63	0.69	0.61
95% CI (bootstrap)	0.73, 0.79	0.60, 0.65	0.60, 0.66	0.65, 0.72	0.58, 0.62
c-statistic (cross-validation)	0.76	0.58	0.55	0.64	0.58
95% CI (cross-validation)	0.73, 0.78	0.54, 0.62	0.50, 0.60	0.60, 0.69	0.54, 0.62
Joint effect of markers	**CEP + *ARMS2***	**CEP + *HTRA1***	**CEP + *CFH***	**CEP + *C3***	
Sensitivity (%)	63	71	75	71	
Specificity (%)	81	67	76	74	
c-statistic	0.79	0.76	0.80	0.80	
95% CI	0.76, 0.82	0.72, 0.80	0.77, 0.84	0.77, 0.84	
c-statistic (bootstrap)	0.79	0.76	0.81	0.80	
95% CI (bootstrap)	0.76, 0.82	0.71, 0.80	0.77, 0.84	0.77, 0.84	
c-statistic (cross-validation)	0.79	0.75	0.80	0.80	
95% CI (cross-validation)	0.75, 0.82	0.70, 0.79	0.76, 0.83	0.76, 0.83	

TABLE VI

Association of elevated CEP markers with AMD risk genotype

Odds ratios were determined for elevated levels of both CEP markers relative to median AMD levels (≥33 pmol/ml CEP adducts and ≥1.6 CEP autoantibody titer, $n = 916$ AMD plasma samples). The statistical significance (*p* values) of differences in CEP marker concentrations between homozygous risk and non-risk donors was determined by the Fisher exact test. Odds ratios, 95% CI, and *p* values are based on log-transformed CEP marker concentrations.

AMD risk genotype	All AMD			Advanced AMD		
	n	Odds ratio (95% CI)	*p* value	*n*	Odds ratio (95% CI)	*p* value
ARMS2						
GG	278	1.00 (reference)		141	1.00 (reference)	
GT	306	1.39 (0.93, 2.07)	0.1	209	1.43 (0.84, 2.44)	0.2
TT	124	1.99 (1.23, 3.24)	0.007	97	2.05 (1.12, 3.76)	0.03
HTRA1						
GG	294	1.00 (reference)		175	1.00 (reference)	
GA	279	1.59 (1.05, 2.40)	0.04	204	1.62 (0.95, 2.76)	0.09
AA	174	2.07 (1.32, 3.24)	0.002	132	2.17 (1.24, 3.825)	0.01
CFH						
TT	160	1.00 (reference)		96	1.00 (reference)	
TC	384	0.78 (0.50, 1.21)	0.3	261	0.78 (0.45, 1.401)	0.4
CC	244	1.14 (0.72, 1.81)	0.6	169	1.11 (0.62, 2.01)	0.8
C3						
CC	317	1.00 (reference)		195	1.00 (reference)	
CG	270	0.92 (0.63, 1.36)	0.5	190	0.92 (0.56, 1.48)	0.8
GG	182	0.85 (0.55, 1.32)	0.7	124	0.91 (0.53, 1.57)	0.8

tension, hyperlipidemia, diabetes, and cardiovascular disease (Fig. 5). For each comparison, significant differences in CEP adduct and autoantibody concentrations were observed between AMD and control donors. For several of the comparisons, small but significant differences were also detected within the AMD or control cohorts. Specifically in AMD patients, mean CEP adduct levels were higher in Caucasians relative to African-Americans, in females relative to males, and in donors exhibiting hypertension and hyperlipidemia. Within the control cohorts, no significant differences were detected in mean amounts of CEP adducts. However, mean CEP autoantibody titers were slightly higher in control males, control smokers, and controls with diabetes or cardiovascular disease. AMD donors with hypertension and hyperlip-

idemia also exhibited slightly higher amounts of CEP autoantibodies (Fig. 5).

DISCUSSION

CEP modifications are generated by covalent adduction of primary amino groups (*e.g.* protein ε-lysyl NH_2) with 4-hydroxy-7-oxohept-5-enoic acid, an oxidation fragment derived uniquely from DHA-containing phospholipids (15, 25). DHA is the most oxidizable of all fatty acids, and although rare, its highest abundance in human tissues is found in retinal photoreceptor outer segments (26). The high oxygen tension in the retina coupled with light provides a permissive environment for the generation of oxidative post-translational modifications. For example, rodents exposed to intense light accumulate elevated CEP adducts in the retina and elevated CEP adducts and autoantibodies in plasma (27, 28). Of course other oxidative protein modifications accumulate in the eye with age and possibly contribute to AMD pathogenesis. Such modifications include advanced glycation end products in the choriocapillaris, Bruch membrane, and CNV membranes (29, 30) and nitrotyrosine and iso[4]levuglandin E_2 adducts (as well as CEP adducts) in RPE lipofuscin granules (31). Why ocular tissues in AMD patients are more susceptible to oxidative damage than normal eye tissue remains to be determined, but oxidative markers formed from retina-rich components like DHA lipids offer a potential early warning system for predicting AMD susceptibility.

We characterized plasma CEP markers in a large study population ($n = 1404$) and confirmed that mean levels of CEP adducts and autoantibodies are elevated in AMD plasma. The present study found mean CEP adduct concentrations to be ~60% higher in AMD plasma ($p < 0.0001$), which is similar to the ~50% mean elevation previously reported for 19 AMD patients (15). Western analysis also supported higher levels of CEP adducts in AMD plasma and clearly associated adducts with proteins. These data do not exclude CEP adduction of small molecules, *e.g.* phosphatidylethanolamines, which also

TABLE VII
Characteristics of the study population

An additional 98 young control donors (mean age ± S.D., 42 ± 6 years; range, 27–50 years) are included in the category ≤50 in Fig. 4. Values in parentheses reflect percent of total cohort.

Property/category	Control donors $n = 488$	AMD donors $n = 916$
Age (years)*		
Mean ± S.D.	67 ± 15	78 ± 8
Range	51–89	51–97
Gender		
Male	286 (58.6%)	463 (50.5%)
Female	202 (41.4%)	453 (49.5%)
Race		
Caucasian	401 (82.2%)	884 (96.5%)
African-American	79 (16.2%)	20 (2.2%)
Other	8 (1.6%)	12 (1.3%)
Smoking status		
Non-smoker	281 (57.6%)	443 (48.4%)
Smoker	207 (42.4%)	473 (51.6%)
Health history		
Hypertension	251 (51.4%)	509 (55.6%)
Hyperlipidemia	196 (40.2%)	331 (36.1%)
Diabetes	76 (15.6%)	130 (14.2%)
Cardiovascular disease	86 (17.6%)	247 (27.0%)

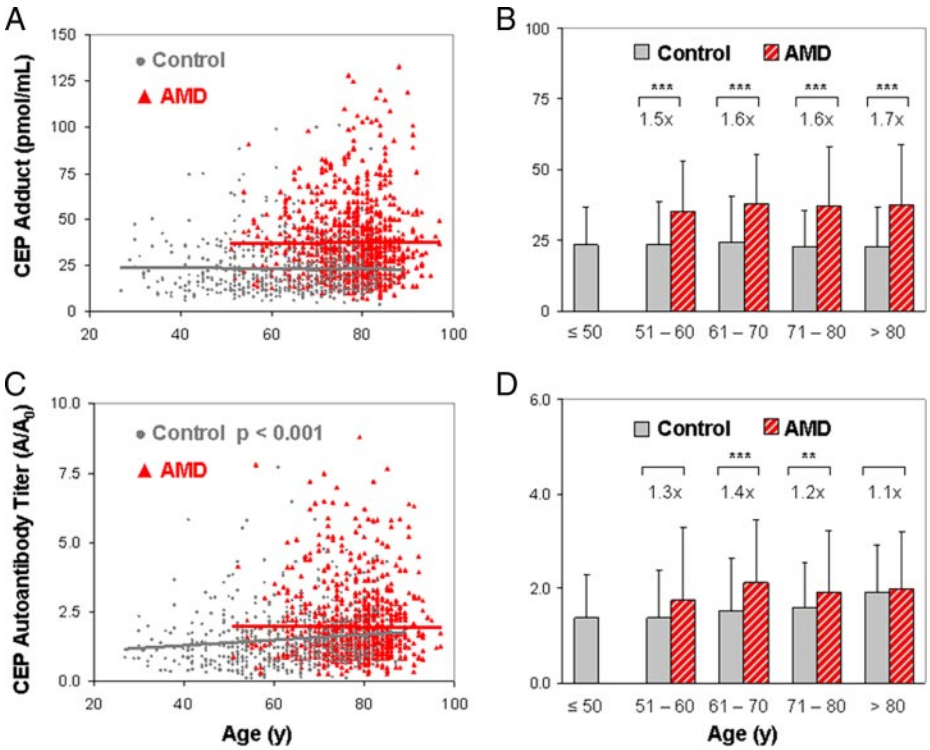

FIG. 4. **Plasma CEP adducts and autoantibodies by donor age.** Plasma CEP adduct (*A*) and CEP autoantibody levels (*C*) in the AMD (△) and control (●) cohorts are shown plotted by donor age. Pearson's correlation analysis (*horizontal color-coded lines* and *p* value from log-transformed data) revealed little change in mean CEP marker concentrations with age except for a gradual increase in CEP autoantibody titer in the control cohort. CEP adduct (*B*) and CEP autoantibody levels (*D*) in AMD and control donors are plotted by age group, including controls ≤50 years (*y*) ($n = 98$), 51–60 years ($n = 138$ control, $n = 26$ AMD), 61–70 years ($n = 153$ control, $n = 123$ AMD), 71–80 years ($n = 154$ control, $n = 389$ AMD), and >80 years ($n = 43$ control, $n = 378$ AMD). -Fold difference in CEP marker concentrations is indicated between the control and AMD groups. *Asterisks* reflect *p* values from a two-sided *t* test (***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$). *Error bars* reflect standard deviation.

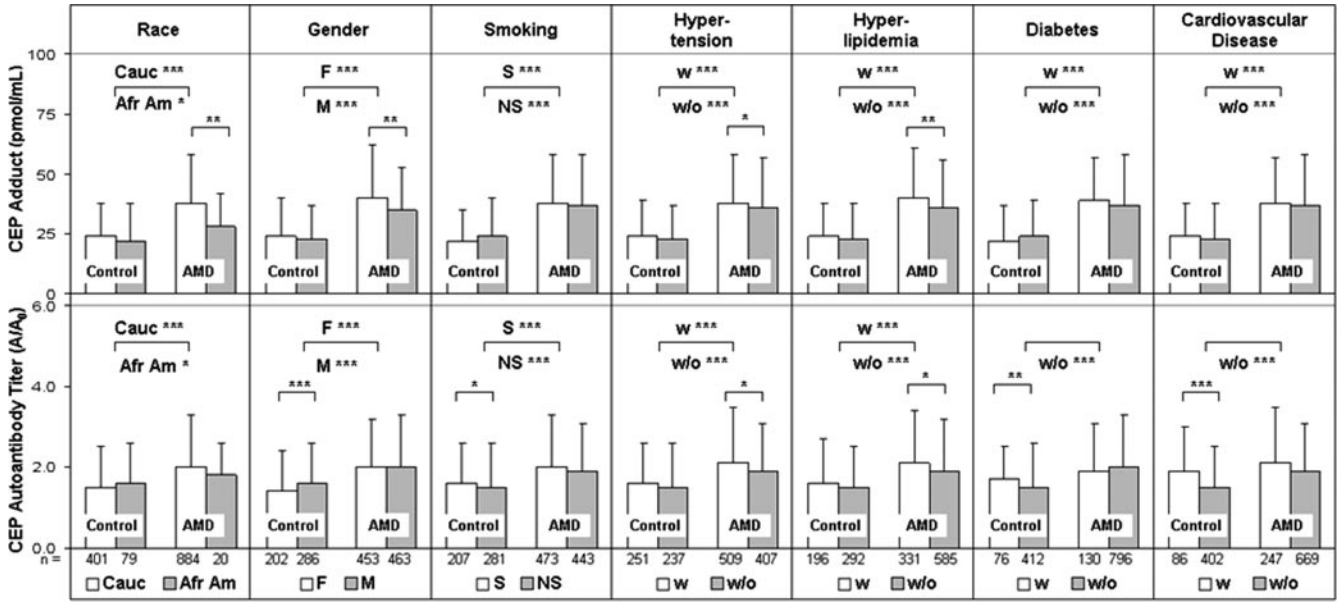

FIG. 5. **Plasma CEP markers stratified by demographic and health factors.** Plasma CEP adduct and CEP autoantibody levels in the AMD and control study populations are plotted based on donor status with regard to race, gender, smoking status, hypertension, hyperlipidemia, diabetes, and cardiovascular diseases. Sample size per group is indicated, and *asterisks* reflect *p* values from a two-sided *t* test of log-transformed CEP marker concentrations (***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$). *Cauc*, Caucasian; *Afr Am*, African-American; *F*, female; *M*, male; *S*, smoking; *NS*, non-smoking; *w*, with; *w/o*, without. *Error bars* reflect standard deviation.

likely occurs. Significant variability in CEP adduct concentrations was observed among both normal and AMD donors as reflected by differences in mean and median levels; however, mean plasma levels changed little with age for either AMD or normal donors. Notably mean CEP adduct concentrations were already elevated in the youngest AMD age group (*i.e.* 51–60 years) and in plasma from those with early stage dry AMD. Interestingly in AMD patients, significantly higher CEP adduct levels were observed in females, consistent with the higher incidence of AMD in females, and in hyperlipidemic individuals, consistent with the lipid source of these adducts. African-Americans with AMD exhibited significantly lower CEP adduct levels than Caucasians, consistent with the lower incidence of AMD in African-Americans and consistent with a greater resistance to oxidative damage perhaps because of the higher levels of ocular melanin in darkly pigmented races and the antioxidant effects of melanin (32). Surprisingly smoking had little impact on plasma CEP adduct levels within either the control or AMD cohorts presumably because of relatively low amounts of DHA in the pulmonary system. The present analyses also detected no confounding influences on plasma CEP adduct concentrations from diabetes and cardiovascular disease, although AMD donors with hypertension exhibited slightly higher levels. Epidemiological studies have inconsistently associated hypertension and cardiovascular disease with AMD (33).

This study found mean CEP autoantibody titers to be ~30% higher in AMD plasma ($p < 0.0001$) and to remain relatively constant with age in AMD plasma but to gradually increase with age in control plasma. It is important to note that the mean CEP autoantibody titers in AMD plasma were above mean control levels over a broad age range (*i.e.* 51–80 years) and for those with early stage dry AMD. In contrast to CEP adducts, mean CEP autoantibody titers were elevated slightly with statistical significance in control males, control smokers, and controls with diabetes or cardiovascular disease. These differences warrant consideration as possible factors that might impact CEP marker predictions of AMD, although their biochemical basis and clinical relevance remain to be determined. In AMD cohorts, mean CEP autoantibody titer exhibited no detectable differences with regard to race, gender, smoking, diabetes, and cardiovascular disease but were elevated in those with hypertension or hyperlipidemia.

Mean CEP adduct and autoantibody levels were elevated in AMD *versus* control plasma donors, but the individual values for both markers were spread across a broad range and exhibited standard deviations up to 67% of the mean. The 30% higher mean CEP autoantibody titer in AMD patients observed here differed from the 2.3-fold elevation previously estimated from 19 AMD and control donors (15). We credit this difference in part to the greater variability of the previous antibody values and the smaller sample size of the previous study. However, although genetic diversity certainly contributed to the range of determined CEP marker values, interassay variability over the 5-year study period also contributed to variability. For broad clinical usefulness as biomarkers, it will be important to further standardize the CEP marker assay conditions. For example, a fasting blood specimen that is assayed without freezing or significant storage time, as in clinical lipid analyses for cholesterol, could significantly de-

crease variability. Nevertheless the present results compellingly demonstrate higher CEP marker levels in AMD plasma, and the areas under the ROC curves (c-statistics) suggest that alone these markers can discriminate between AMD and control plasma donors with ~76% accuracy.

About a 3-fold higher risk of AMD was predicted for plasma donors with both CEP adducts and autoantibodies elevated above median control levels. Although the risk of AMD due to the *ARMS2*, *HTRA1*, or *CFH* homozygous risk polymorphisms was about 2-fold higher than that due to elevated CEP markers alone, that due to the *C3* risk allele approximated that associated with elevated CEP. More importantly, the risk for AMD predicted for individuals carrying any of the four characterized risk genotypes and exhibiting elevated CEP marker concentrations was ~2–3-fold greater than that predicted by genotype alone. Combining the CEP and genomic markers significantly improved the discrimination accuracy (c-statistics) of all the genomic markers. The frequency of the *ARMS2*, *HTRA1*, *CFH*, and *C3* homozygous AMD risk alleles in this study population was in reasonable agreement with the range of values previously reported as were the determined odds ratios for AMD risk based on genotype alone. Within the AMD population, individuals carrying the *ARMS2* or *HTRA1* homozygous risk genotypes, but not those carrying the *CFH* or *C3* risk genotypes, were approximately twice as likely to exhibit elevated CEP markers than those carrying the homozygous non-risk alleles. The significance of these associations is not known but may be related to the sensitivity of the gene products to oxidative stress: *ARMS2* encodes a mitochondrial protein of unknown function (19), and *HTRA1* encodes a heat shock serine protease activated by stress (23, 24).

Systemic biomarkers of inflammation have been associated with AMD, but published reports are inconsistent. For example, elevated levels of C-reactive protein, homocysteine, and interleukin 6 have been implicated in the development of AMD (34–37), but several other case-control studies have found no association with AMD for these and other inflammatory markers (38–40). Studies evaluating the combined effects of the *ARMS2* (39) and *CFH* (41) risk genotypes and inflammatory markers on increased risk for AMD have also been reported, but no studies have yet addressed the sensitivity and specificity of systemic markers for AMD. Sample preparation, specimen storage, and assay methods varied in these studies and likely contributed to the inconsistency of the reports. Although systemic biomarkers of inflammation warrant consideration as tools for monitoring this multifactorial disease, CEP biomarkers offer potentially greater sensitivity and specificity for AMD because of their unique derivation from abundant components of the outer retina namely DHA phospholipids.

Taken together, this study supports the potential utility of CEP proteomic biomarkers for predicting AMD susceptibility, particularly in combination with genomic markers. The statistical analyses suggest that plasma CEP marker levels in combination with genomic markers discriminate between AMD and control patients with up to ~80% accuracy. Standardization of the CEP marker assay could further improve discrimination accuracy. The current results warrant additional prospective and longitudinal investigation of clinical applications. Notably a recent study showed in a mouse model of dry AMD that CEP autoantibody titer increases in direct proportion to the severity of RPE lesions (18). This observation suggests that CEP biomarkers may have utility in monitoring the efficacy of therapeutics that prevent or limit the progression of dry AMD.

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[g] Has a license for CEP as an inventor with Frantz Biomarkers, LLC.

[h] To whom correspondence may be addressed: Cole Eye Inst. i31, Lerner Research Inst., Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195. Tel.: 216-445-4133; Fax: 216-445-3670; E-mail: hagstrs@ccf.org.

[i] Has a license for CEP as an inventor with Frantz Biomarkers, LLC; is a consultant for Alcon Research Ltd. and Allergan, Inc.; and has received funding for this research from Merck & Co. and Johnson and Johnson. To whom correspondence should be addressed: Cole Eye Inst. i31, Lerner Research Inst., Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195. Tel.: 216-445-0425; Fax: 216-445-3670; E-mail: crabbj@ccf.org.

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