

# Assessing Susceptibility to Age-Related Macular Degeneration with Genomic and Proteomic Biomarkers

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## Abstract

**Purpose:** Toward developing methods to predict susceptibility to age-related macular degeneration (AMD), we correlated plasma carboxyethylpyrrole (CEP) adducts and autoantibody levels in AMD patients with AMD risk genotypes for *Y402H* in complement factor H (*CFH*), *R69G* in complement component 3 (*C3*), *A68S* in *ARMS2* (*LOC387716*) and *r1120G88* in *HTRA1*.  
**Methods:** Plasma CEP and CEP autoantibody levels were determined by ELISA. DNA was genotyped by direct genomic sequencing or restriction analysis. Logistic regression modeling for c-statistics, odds ratios and *p* values was performed with SAS 9.1. Sensitivity and specificity were calculated to maximize the sum of the two values using receiver operating characteristic (ROC) curves. Odds ratios for elevated CEP adducts and autoantibodies were calculated relative to control donors for homozygous and heterozygous genotypes. The risk for AMD was predicted based on genotype alone or in combination with the CEP markers.  
**Results:** Mean CEP adduct and autoantibody levels in AMD plasma (*n* = 916) were found to be elevated by ~60% and ~20%, respectively, relative to control plasma (*n* = 488). The odds ratio for both CEP markers to be elevated was at least 3-fold greater in AMD than in control patients. Higher mean CEP marker levels were observed in AMD plasma over a broad age range. 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. C-statistics and ROC curves suggest that CEP markers alone can discriminate between AMD and control plasma with ~75% accuracy and, in combination with genomic markers, provide up to ~90% discrimination accuracy.  
**Conclusions:** CEP plasma biomarkers offer a potential early warning system for predicting AMD susceptibility, especially in combination with genomic biomarkers.

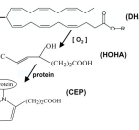
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## Background and Experimental Design

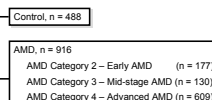
### What are Carboxyethylpyrrole (CEP) adducts?

CEP = Protein Modifications from Diacetylcholine (DHA)

1. Retina-High Oxygen Tension
2. Retina-High Light Exposure
3. Retina-Rich in DHA Phospholipids



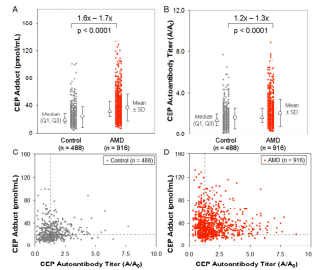
### Case-Control Study



### Characteristics of Study Population

Property	Category	Control n = 488	AMD n = 916
Age (years)	Mean ± SD	67 ± 15	78 ± 8
	Range	51 - 89	51 - 97
Gender	Male	268 (55.6%)	463 (50.6%)
	Female	202 (41.4%)	453 (49.4%)
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%)
	Hypertension	186 (38.2%)	331 (36.1%)
	Diabetes	76 (15.6%)	130 (14.2%)
	Cardiovascular disease	88 (17.8%)	247 (27.0%)

## CEP Adducts and Autoantibodies are Elevated in AMD Plasma



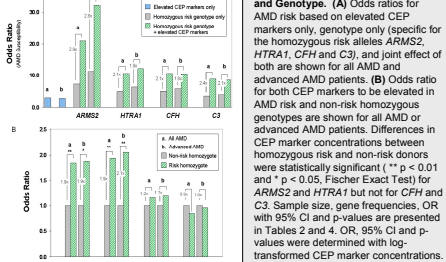
**CEP Adducts and Autoantibodies are Elevated in AMD Plasma.** CEP adduct concentrations (A) and autoantibody titers (B) quantified by ELISA from control (*n* = 488) and AMD (*n* = 916) plasma donors are shown with median (Δ) results in first and third quartiles (Q1, Q3) and mean (•) results ± standard deviation (SD) indicated. *P*-values (two sided *t*-Test) were determined from log-transformed concentrations. This data is presented in Table 1 by category of AMD progression. Correlation between CEP adduct levels and autoantibody titers are 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).

### CEP Markers in AMD and Control Plasma

	<i>n</i>	Mean ± SD	Median (Q1, Q3)	Mean ± SD	Median (Q1, Q3)	Odds Ratio	95% CI	<i>P</i> value
Control	488	23 ± 15	15 (14, 25)	1.5 ± 1.0	1.0 (0.8, 1.5)	1.00	(Reference)	
AMD	916	37 ± 20	33 (23, 46)	2.0 ± 1.3	1.6 (1.1, 2.6)	3.16	2.50 - 4.00	< 0.001
AMD Category 2 - Early AMD	177	35 ± 19	31 (22, 40)	2.2 ± 1.3	1.0 (1.2, 2.6)	4.24	2.95 - 6.10	< 0.001
AMD Category 3 - Mid-stage AMD	130	32 ± 17	28 (20, 38)	2.2 ± 1.4	1.7 (1.2, 2.4)	3.26	2.18 - 4.84	< 0.001
AMD Category 4 - Advanced AMD	609	38 ± 21	34 (24, 48)	1.9 ± 1.2	1.6 (1.0, 2.3)	2.89	2.26 - 3.72	< 0.001

CEP adduct concentration and autoantibody titer were determined by ELISA. Odds ratios for the AMD risk for donors carrying elevated levels of both CEP markers relative to median control levels are shown for the AMD risk for donors carrying elevated levels of both CEP markers relative to median control levels. *P* values were determined using the Fisher Exact Test. AMD categories are based on the AREDS classification system.

### AMD Risk Predicted by CEP Markers and Genotype.

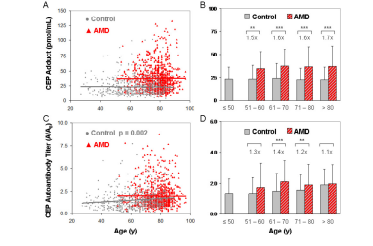


**AMD Risk Predicted by CEP Markers and Genotype.** (A) Odds ratios for AMD risk based on elevated CEP markers only, genotype only (specific for the homozygous risk alleles *ARMS2*, *HTRA1*, *CFH* and *C3*), and joint effect of both are shown for all AMD and advanced AMD patients. (B) Odds ratio for 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 ( $^{*}p < 0.01$  and  $^{*}p < 0.05$ , 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 2 and 4. OR, 95% CI and *p*-values were determined with log-transformed CEP marker concentrations.

## AMD Risk Predicted by CEP Markers and Genotype

AMD Risk Genotype	Genotype Frequency (%)		Odds Ratio (95% CI)			
	Control	AMD	Genotype Only	CEP Adducts and Autoantibodies	Joint Effect	
			Not Both Elevated	Both Elevated		
<b>ARMS2</b>	(404)	(708)				
GT	748 (91.9%)	779 (94.1%)	1.00 (Reference)	1.00 (Reference)	2.76 (1.40 - 5.06)	
TT	143 (35.4%)	308 (43.2%)	1.89 (1.46 - 2.46)	2.44 (1.74 - 3.42)	3.71 (2.48 - 5.54)	
TT	163 (37.7%)	324 (45.8%)	2.32 (1.47 - 12.84)	4.16 (3.00 - 12.66)	20.88 (2.32 - 63.94)	
<b>HTRA1</b>	(573)	(747)				
GG	134 (27.5%)	234 (32.4%)	1.00 (Reference)	1.00 (Reference)	2.88 (1.81 - 4.51)	
GA	82 (35.2%)	279 (37.3%)	1.67 (1.21 - 2.29)	2.15 (1.49 - 3.20)	3.21 (2.00 - 5.15)	
AA	17 (7.3%)	174 (23.3%)	6.01 (2.03 - 8.87)	4.16 (2.06 - 8.44)	10.64 (4.71 - 23.65)	
<b>CFH</b>	(381)	(788)				
TT	16 (3.9%)	160 (20.3%)	1.00 (Reference)	1.00 (Reference)	2.55 (1.62 - 4.02)	
TC	172 (45.1%)	384 (49.7%)	2.25 (1.60 - 2.95)	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)	6.18 (3.77 - 10.13)	10.58 (6.85 - 16.05)	
<b>C3</b>	(343)	(769)				
CC	189 (57.3%)	317 (41.2%)	1.00 (Reference)	1.00 (Reference)	2.81 (1.93 - 4.09)	
CG	114 (33.2%)	270 (35.1%)	1.48 (1.12 - 1.96)	1.60 (1.11 - 2.33)	3.69 (2.41 - 5.64)	
GG	31 (8.1%)	182 (23.7%)	3.67 (2.41 - 5.56)	4.48 (2.86 - 7.54)	9.81 (6.28 - 15.24)	

Odds ratios were determined for AMD risk based on genotype alone or in combination with CEP adducts and autoantibodies. *P* values were determined using the Fisher Exact Test. CEP markers relative to median control levels are shown for the AMD risk for donors carrying elevated levels of both CEP markers relative to median control levels. *P* values were determined using the Fisher Exact Test. CI, confidence interval.



**Plasma CEP Adducts and Autoantibodies by Donor Age.** (A) Plasma CEP adduct and (B) CEP autoantibody levels in the AMD (Δ) 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. (C) CEP adduct and (D) CEP autoantibody levels in AMD and control donors are plotted by age group, including controls ≤ 50 y (*n* = 98), 51 - 60 y (*n* = 138 control, *n* = 26 AMD), 61 - 70 y (*n* = 153 control, *n* = 123 AMD), 71 - 80 y (*n* = 154 control, *n* = 389 AMD) and > 80 y (*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  $^{*}p < 0.05$ ).

### Sensitivity and Specificity of CEP Markers and Genomic Markers

Markers Alone	CEP	ARMS2	HTRA1	CFH	C3
Sensitivity (%)	73%	31%	37%	60%	36%
Specificity (%)	65%	94%	89%	77%	86%
C-Statistic	0.75	0.63	0.63	0.69	0.62
C-Statistic (bootstrap)	0.75	0.63	0.63	0.69	0.62
95% CI	(0.73-0.76)	(0.59-0.67)	(0.59-0.66)	(0.64-0.73)	(0.57-0.66)
95% CI (bootstrap)	(0.73-0.76)	(0.60-0.62)	(0.60-0.66)	(0.62-0.72)	(0.58-0.64)
Joint Effect of Markers					
Sensitivity (%)	60%	69%	73%	72%	72%
Specificity (%)	74%	69%	72%	77%	79%
C-Statistic	0.78	0.76	0.80	0.79	0.79
C-Statistic (bootstrap)	0.79	0.76	0.80	0.79	0.79
95% CI	(0.75-0.82)	(0.73-0.79)	(0.76-0.84)	(0.76-0.83)	(0.76-0.83)
95% CI (bootstrap)	(0.75-0.82)	(0.71-0.79)	(0.76-0.84)	(0.76-0.83)	(0.76-0.83)
<i>P</i> value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Estimated and specificity were determined from ROC analysis. C-statistics are the sum of the area under the curve and area above the curve of the logistic regression analysis fit with either CEP adduct concentrations plus autoantibody titers, or homozygous risk genotype, or the combination of CEP adducts and autoantibody titers, or homozygous risk genotype. C-statistics are calculated from the area under the curve and the accuracy of the markers to discriminate between AMD cases and controls, with 1.0 equating to 100% accuracy and 0.5 equal to no discrimination. Combining the 13 *p* gene markers significantly improved the c-statistic for the genomic markers.

## AMD Risk by Genotype, Demographic and Health Factors

AMD Risk Genotype	All AMD			Advanced AMD		
	<i>n</i>	Odds Ratio (95% CI)	<i>P</i> value	<i>n</i>	Odds Ratio (95% CI)	<i>P</i> value
<b>ARMS2</b>						
GG	278	1.00 (Reference)		141	1.00 (Reference)	
GT	306	1.30 (0.88 - 1.92)	0.1	200	1.32 (0.79 - 2.21)	0.2
TT	124	1.85 (1.16 - 2.97)	0.009	97	1.86 (1.04 - 3.40)	0.03
<b>HTRA1</b>						
GG	294	1.00 (Reference)		175	1.00 (Reference)	
GA	279	1.40 (0.94 - 2.09)	0.06	204	1.47 (0.85 - 2.45)	0.09
AA	174	1.94 (1.25 - 3.01)	0.002	132	2.06 (1.19 - 3.58)	0.007
<b>CFH</b>						
TT	180	1.00 (Reference)		96	1.00 (Reference)	
TC	384	0.85 (0.64 - 1.28)	0.2	261	0.88 (0.60 - 1.61)	0.4
CC	244	1.16 (0.73 - 1.82)	0.3	169	1.18 (0.68 - 1.91)	0.3
<b>C3</b>						
CC	317	1.00 (Reference)		168	1.00 (Reference)	
CG	270	1.01 (0.69 - 1.47)	0.6	190	1.10 (0.66 - 1.76)	0.4
GG	182	0.85 (0.55 - 1.30)	0.3	124	0.86 (0.56 - 1.61)	0.6

Odds ratios were determined for elevated levels of both CEP markers relative to median AMD levels (13 homozygous CEP adducts and 1.6 CEP autoantibody titer) in AMD (control). The values are significant (*p* values) of elevated levels of CEP marker concentrations between homozygous risk and non-risk donors were determined by the Fisher Exact Test. CI, confidence interval.

### 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  $^{*}p < 0.05$ ). Cau, Caucasian; Afr Am, African American; F, female; M, male; S, smoking; NS, nonsmoking; w, with; w/o, without.

### Conclusions

1. Plasma CEP biomarkers in a large study population (*n* = 1444) were correlated with AMD risk genotypes (*ARMS2*, *HTRA1*, *CFH* and *C3*) in up to 86% of the AMD donors and 83% of the control donors.
2. Mean CEP adduct levels were ~60% higher and mean CEP autoantibody titers were ~20% higher in AMD plasma (*p* < 0.0001). Higher mean CEP biomarker levels exist in AMD plasma over a broad age range.
3. 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.
4. 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.
5. Combined plasma CEP proteomic and genomic biomarker measurements are more effective in assessing AMD risk than either method alone. CEP biomarkers alone discriminated between AMD and control patients with ~76% accuracy, the genomic markers alone with ~62-69% accuracy, and the combined biomarkers with ~76-80% accuracy.  
(See CEP-related ARVO 09 poster 2330, board D1063, Monday May 4<sup>th</sup>)