

# Pentosidine and Carboxymethyllysine, Plasma Biomarkers for Age-Related Macular Degeneration

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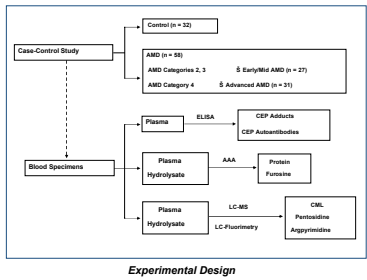
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## Abstract

**Purpose:** To quantify plasma protein carboxymethyllysine (CML) and pentosidine (advanced glycation end products) as possible biomarkers for age-related macular degeneration (AMD).  
**Methods:** Blood was collected from clinically documented AMD and age-matched normal, healthy donors at the Cole Eye Institute, Cleveland Clinic Foundation. Plasma proteins were precipitated with acetone and hydrolyzed in 6N HCl to determine amounts of protein, CML, and pentosidine. Protein and lysine were quantified by amino acid analysis. CML was quantified by LC-MS/MS using a Sciex API 3000 triple quadrupole mass spectrometer. Pentosidine was measured by LC fluorescence monitoring (excitation = 335 nm, emission = 385 nm) using a Waters 474 scanning fluorimeter. Carboxymethyllysine (CEP) adducts were quantified in plasma by ELISA. 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.  
**Results:** Quantitative analyses of plasma from AMD (n = 58) and normal control (n = 32) donors showed that mean CML and pentosidine levels were elevated in AMD plasma by ~60% and ~80%, respectively, while lysine was largely unchanged. In these samples, CEP adducts were 2-fold higher in AMD (n = 58) than in control (n = 32). The odds ratio for both CML and pentosidine elevated was 1.5 fold greater in AMD than in control patients. For these plasma samples, c-statistics and ROC curves indicate that CML discriminated between AMD and control donors with ~79% accuracy, CEP adducts with 81% and pentosidine with 82% accuracy. In combination with CEP adduct levels, CML provided 89% accuracy and pentosidine provided ~96% discrimination accuracy.  
**Conclusions:** CML and pentosidine levels are elevated in AMD plasma and may prove useful for predicting AMD susceptibility and for monitoring therapeutic efficacy, especially in combination with CEP biomarkers.  
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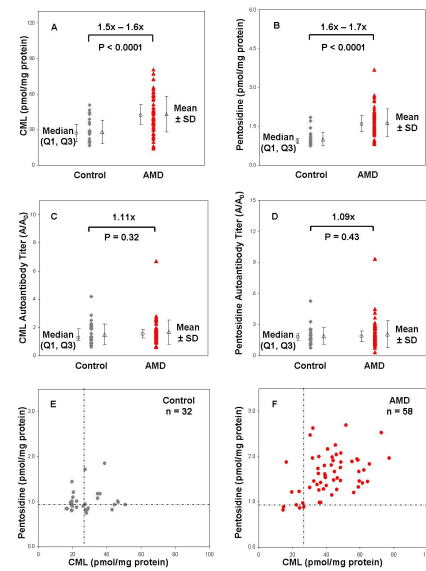
## Background and Experimental Design



## Characteristics of the Study Population

| Property       | Category               | Control<br>n=32 | Early/Mid Stage<br>AMD<br>n=27 | Advanced<br>AMD<br>n=31 |
|----------------|------------------------|-----------------|--------------------------------|-------------------------|
| Age (year)     | Mean ± SD              | 72.6 ± 6        | 73.9 ± 9                       | 74.6 ± 6                |
|                | Range                  | 56-84           | 57-91                          | 64-89                   |
|                |                        |                 |                                |                         |
| Gender         | Male                   | 15 (46.9%)      | 14 (51.9%)                     | 16 (51.6%)              |
|                | Female                 | 17 (53.1%)      | 13 (48.1%)                     | 15 (48.4%)              |
| Race           | Caucasian              | 32 (100%)       | 27 (100%)                      | 31 (100%)               |
|                | Non-smoker             | 30 (93.8%)      | 21 (77.8%)                     | 30 (96.8%)              |
|                | Smoker                 | 2 (6.2%)        | 6 (22.2%)                      | 1 (3.2%)                |
| Health History | Hypertension           | 21 (65.6%)      | 14 (51.9%)                     | 20 (64.5%)              |
|                | Hyperlipidemia         | 15 (46.9%)      | 14 (51.9%)                     | 10 (32.3%)              |
|                | Diabetes               | 0 (0.0%)        | 3 (11.1%)                      | 2 (6.5%)                |
|                | Cardiovascular disease | 6 (18.8%)       | 7 (25.9%)                      | 5 (16.1%)               |
|                |                        |                 |                                |                         |

## CML and Pentosidine in AMD Plasma



## CML and Pentosidine Are Elevated in AMD Plasma Proteins.

CML (A) and pentosidine concentrations (B) quantified by LC-MS/MS and LC-fluorimetry from control (n = 32) and AMD (n = 58) plasma donors and antibody titers for CML (C) and pentosidine (D) quantified by ELISA (32 control and 57 AMD plasma) are shown with median (horizontal line) and first and third quartiles (Q1, Q3) and mean (Δ) results a standard deviation (SD). P-values (two sided T-test) were determined from log-transformed concentrations. Correlation between CML and pentosidine concentrations are shown for the control (E) and AMD (F) cohorts with horizontal and vertical dashed lines indicating median control values.

These results show that mean levels of CML and pentosidine are elevated in AMD plasma proteins (A, B) and that significantly more donors with both CML and pentosidine elevated are apparent in AMD patients than in the controls (upper right quadrants in E and F).

## CML and Pentosidine Markers in Control and AMD Plasma

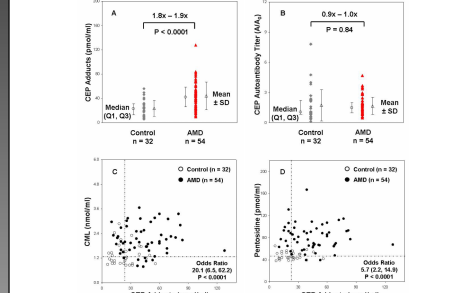
|                     | n  | CML (pmol/mg protein) |                   |            | CML Elevated Above Control Median |          |  |
|---------------------|----|-----------------------|-------------------|------------|-----------------------------------|----------|--|
|                     |    | Mean ± SD             | Median (Q1, Q3)   | Odds Ratio | 95% CI                            | P-value  |  |
| Control             | 32 | 27.9 ± 10.0           | 26.7 (19.7, 34.7) | 1          | (Reference)                       |          |  |
| Early/Mid Stage AMD | 27 | 41.0 ± 18.1           | 39.6 (28.4, 57.3) | 2.9        | 1.0, 8.6                          | 0.068    |  |
| Advanced AMD        | 31 | 44.8 ± 14.0           | 43.3 (35.5, 48.6) | 30.0       | 3.6, 247.3                        | < 0.0001 |  |
| All AMD             | 58 | 43.0 ± 15.0           | 42.0 (34.7, 51.0) | 6.3        | 2.3, 17.3                         | < 0.0001 |  |

|                     | n  | Pentosidine (pmol/mg protein) |                   |            | Pentosidine Elevated Above Control Median |          |  |
|---------------------|----|-------------------------------|-------------------|------------|---|----------|--|
|                     |    | Mean ± SD                     | Median (Q1, Q3)   | Odds Ratio | 95% CI                                    | P-value  |  |
| Control             | 32 | 1.03 ± 0.25                   | 0.83 (0.84, 1.02) | 1          | (Reference)                               |          |  |
| Early/Mid Stage AMD | 27 | 1.51 ± 0.41                   | 1.48 (1.22, 1.62) | 8.0        | 2.0, 32.0                                 | 0.002    |  |
| Advanced AMD        | 31 | 1.78 ± 0.59                   | 1.71 (1.34, 1.99) | 14.5       | 3.0, 71.2                                 | < 0.0001 |  |
| All AMD             | 58 | 1.64 ± 0.53                   | 1.59 (1.29, 1.92) | 10.6       | 3.4, 33.5                                 | < 0.0001 |  |

CML and pentosidine concentrations were determined by LC-MS/MS and LC-fluorimetry, protein was quantified by amino acid analysis. Determined mean concentrations of CML expressed in total CML (nmol lysine) were: control, 39.8 ± 11.9; early/mid-stage AMD, 49.8 ± 19.8; advanced AMD, 54.6 ± 17.4; and all AMD 52.3 ± 18.6. Determined mean concentrations of pentosidine expressed in total pentosidine (nmol lysine) were: control, 1.2 ± 0.3; early/mid-stage AMD, 1.8 ± 0.5; advanced AMD, 2.2 ± 0.8; and all AMD 2.0 ± 0.7. The odds ratio (OR) reflects the AMD risk for donors exhibiting elevated levels of either CML or pentosidine markers relative to median control levels. P-values were determined using the Fisher's Exact Test. Odds ratios, 95% CI and p-values are based on log-transformed CML and pentosidine concentrations.

## AMD Risk Predicted by CML, Pentosidine and CEP



## Correlation Between CML, Pentosidine and CEP Adducts in AMD Plasma.

Plasma CEP adduct concentrations (A) and CEP antibody titers (B) quantified by ELISA from control (n = 32) and AMD donors (n = 54) are shown with median (horizontal line) and first and third quartiles (Q1, Q3) and mean (Δ) results a standard deviation (SD). P-values (two sided T-test) were determined from log-transformed concentrations. Correlation between CML and CEP adduct concentrations (C) and between pentosidine and CEP adduct concentrations (D) are shown with horizontal and vertical dashed lines indicating median control values. Odds ratios for AMD risk and 95% confidence intervals were determined based on two markers elevated relative to the median control values. P-values were determined using the Fisher's Exact Test.

These results show that plasma mean CEP adduct levels were elevated in the AMD cohort and that plasma mean CML and pentosidine levels correlate well with the CEP adducts. (See ARVO09 poster 2342, board D1075, Monday May 4)

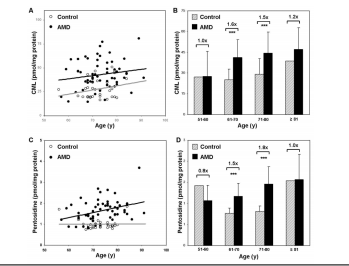
## Sensitivity and Specificity of CML, Pentosidine and CEP Adducts

| Markers Alone                          | CEP adducts           | CML           | Pentosidine   |
|--|-----------------------|---------------|---------------|
| Sensitivity (%)                        | 87%                   | 94%           | 84%           |
| Specificity (%)                        | 81%                   | 72%           | 88%           |
| C-statistic                            | 0.78                  | 0.79          | 0.88          |
| 95% CI                                 | (0.68, 0.88)          | (0.70, 0.89)  | (0.81, 0.95)  |
| C-statistic (Bootstrap Validation)     | 0.67 (0.47)           | 0.80          | 0.80          |
| 95% CI (Bootstrap Validation)          | (0.49, 0.89)          | (0.60, 0.95)  | (0.60, 0.95)  |
| C-statistic (10-Fold Cross-Validation) | 0.78                  | 0.78          | 0.87          |
| 95% CI (10-Fold Cross-Validation)      | (0.68, 0.88)          | (0.68, 0.88)  | (0.78, 0.94)  |
| P-value                                | 0.05 (vs Pentosidine) | 0.10 (vs CEP) | 0.02 (vs CEP) |

| Joint Effect of Markers                | 95% CML + Pentosidine | 95% CEP + CML         | 95% CEP + Pentosidine |
|--|-----------------------|-----------------------|-----------------------|
| Sensitivity (%)                        | 92%                   | 91%                   | 91%                   |
| Specificity (%)                        | 97%                   | 91%                   | 91%                   |
| C-statistic                            | 0.90 (0.89)           | 0.90 (0.89)           | 0.90 (0.89)           |
| 95% CI                                 | (0.80, 0.99)          | (0.80, 0.99)          | (0.80, 0.99)          |
| C-statistic (Bootstrap Validation)     | 0.90                  | 0.90                  | 0.90                  |
| 95% CI (Bootstrap Validation)          | (0.80, 0.94)          | (0.80, 0.94)          | (0.80, 0.94)          |
| C-statistic (10-Fold Cross-Validation) | 0.88                  | 0.88                  | 0.90                  |
| 95% CI (10-Fold Cross-Validation)      | (0.80, 0.95)          | (0.78, 0.93)          | (0.83, 0.96)          |
| P-value                                | 0.06 (vs CEP)         | 0.21 (vs Pentosidine) | 0.12 (vs CEP)         |

Sensitivity and specificity were determined from ROC curves to maximize the sum of the two values and constructed out of the output of logistic regression analysis fit with either CEP adduct, CML, or pentosidine concentrations alone, or in combination. The concentration of CML was expressed in nmol/mg, and pentosidine and CEP adducts were expressed in pmol/mg. C-statistics, 95% CI and p-values derived from single and joint markers were determined with SAS 9.1 based on log-transformed 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 of 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.

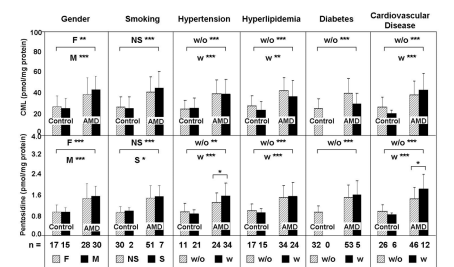


## Plasma CML and Pentosidine by Donor Age.

Plasma protein CML (A) and pentosidine (C) levels in the control (n = 32) and AMD (n = 58) cohorts are shown plotted by donor age. Pearson's correlation analysis revealed gradual increases with age for CML in both control and AMD donors and for pentosidine in AMD donors. Control donors exhibited little change in pentosidine levels with age. Plasma protein CML (B) and pentosidine (D) levels in control and AMD donors are plotted by age group, including 51-60 yr (control, n = 1; AMD, n = 3); 61-70 yr (control, n = 12; AMD, n = 18); 71-80 yr (control, n = 18; AMD, n = 28); ≥ 81 yr (control, n = 1; AMD, n = 9). Fold differences in CML and pentosidine concentrations are indicated between control and AMD groups. Asterisks reflect p-values from a two sample T-test (\*\*\*) p < 0.001.

These results show plasma protein mean CML and pentosidine levels elevated in AMD donors over a broad age range.

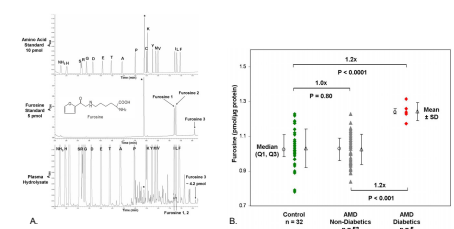
## AMD Risk by Genotype, Demographic and Health Factors



## Plasma CML and Pentosidine Concentrations Stratified by Demographic and Health Factors.

Plasma protein CML and pentosidine levels in the AMD and control cohorts are plotted based on donor status with regard to gender, status of smoking, hypertension, hyperlipidemia, diabetes and cardiovascular diseases. Sample size per group is indicated and asterisks reflect p-values from a two sample T-test of log-transformed marker concentrations (\*\*\*) p < 0.001, \*\* p < 0.01, \* p < 0.05, F, female, M, male, S, smokers, NS, non-smoker, w, with, w/o, without.

These results show pentosidine levels were significantly higher in AMD donors with hypertension or cardiovascular disease. We have also found CEP adducts elevated in AMD patients with hypertension. Epidemiological studies have inconsistently associated hypertension and cardiovascular disease with AMD.



## Furosin in AMD and Control Plasma.

Fructose-lysine, an early marker of glycation, was quantified as furosin by AccQ-TAG<sup>TM</sup> amino acid analysis (A). Furosin peak 3, the putative di-derivatized form of the amino acid, was used for quantification of plasma protein bound furosin. \* Derivatization by products. (B) Furosin results are shown with median (horizontal line) and first and third quartiles (Q1, Q3) and mean (Δ) ± SD. P-values (two sided T-test) were determined from log-transformed concentrations.

These results show that control and non-diabetic AMD plasma protein samples exhibited about the same mean furosin concentrations, in excellent agreement with literature values for nondiabetic plasma donors (< 1 pmol/mg protein). Diabetic AMD plasma donors exhibited ~20% higher mean furosin levels than non-diabetic AMD donors and confound the use of CML and pentosidine as AMD biomarkers. However, CEP adducts could help rule out diabetic complications since plasma CEP adducts are not elevated in diabetes but are elevated in AMD (F) and provide an additional parameter to predict AMD susceptibility.

## Conclusions

- This study supports the potential utility of plasma protein CML and pentosidine as biomarkers for assessing AMD susceptibility, particularly in combination with CEP biomarkers. The statistical analyses suggest that plasma levels of CML together with pentosidine discriminate between AMD and control patients with 89% accuracy and that pentosidine in combination with CEP adducts can discriminate with 92% accuracy.
- Factors potentially confounding the use of CML and pentosidine as AMD biomarkers should not be ignored. Monitoring furosin along with CML and pentosidine provides an effective method to narrow the causes of increased AGEs to possibly AMD susceptibility. When plasma furosin, CML, and pentosidine are all elevated, an accurate clinical assessment will require more information. In such cases, monitoring CEP adducts could help rule out diabetic complications since plasma CEP adducts are not elevated in diabetes but are elevated in AMD (F) and provide an additional parameter to predict AMD susceptibility.
- The present results warrant a much larger investigation, both prospective and longitudinal, of clinical applications of CML, pentosidine and CEP adducts as AMD biomarkers.

## Acknowledgements

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## LC MS/MS and LC Fluorimetry

