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# Increased levels of N$^{\varepsilon}$- Carboxy methyl lysine (N$^{\varepsilon}$-CML) are associated with topographic alterations in retinal pigment epithelium: A preliminary study

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ABSTRACT

*Purpose:* To evaluate the association of serum levels of N$^{\varepsilon}$- Carboxy methyl lysine (N$^{\varepsilon}$-CML), an advanced glycation end product with topographic alterations in retinal pigment epithelium (RPE) in diabetic retinopathy on spectral domain optical coherence tomography (SD-OCT).
*Method:* Consecutive cases of type 2 diabetes mellitus with no retinopathy (n = 20); non-proliferative diabetic retinopathy (n = 20); proliferative diabetic retinopathy (n = 20) and healthy controls (n = 20) between the ages of 40 and 65 years were included. RPE alterations were graded on segmentation map of SD-OCT: grade 0, No RPE alterations; grade 1, RPE alterations in up to two quadrants and grade 2, RPE alterations in more than two quadrants. Serum level of N$^{\varepsilon}$-CML and glycated hemoglobin (HbA1c) was analyzed using the standard protocol. Statistical analysis was done.
*Results:* Significant increase in N$^{\varepsilon}$-CML was observed with increased severity of diabetic retinopathy ($F = 34.1$; $p < 0.0001$). Fisher exact test revealed significant increase in grades of RPE alterations with increased severity of diabetic retinopathy ($p < 0.001$). Univariate ordinal regression analysis was done to calculate the risk of progression in grades of RPE alteration with individual changes in variables like duration of diabetes (odds ratio = 1.37; $p = 0.001$), HbA1c (odds ratio = 1.37; $p = 0.002$) and Nε-CML (odds ratio = 1.37; $p < 0.0001$). Multivariate ordinal regression analysis for predicting progression in grades of RPE alteration revealed Nε-CML to be an independent predictor of increase in grades of RPE alteration (adjusted odds ratio = 1.07; $p < 0.01$) when duration of diabetes and HbA1c were held constant.
*Conclusion:* Increase in serum levels of N$^{\varepsilon}$- Carboxy methyl lysine is significantly associated with topographic alterations in RPE. Grades of RPE alteration increase significantly with increased severity of diabetic retinopathy.



## 1. Introduction

Diabetic retinopathy (DR) is a major microvascular complication of diabetes mellitus (DM) (Chowdhury, Hopkins, Dodson, & Vafidis, 2002). It is an important cause of preventable blindness (King, Aubert, & Herman, 1998). Among the different biochemical pathways implicated in the pathogenesis of DR, the process of formation and accumulation of advanced glycation end-products (AGEs) is considered a major contributor to retinal microvascular complications in type 2 DM (Liu & Qiu, 2013; Sharma et al., 2013). AGEs are nonenzymatically glycated and oxidized proteins that accumulate in

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the vessel wall, where they disturb the capillary pericyte cell structure and function (Chilelli, Burlina, & Lapolla, 2013; Kerkeni et al., 2013; Stróżecki et al., 2013; Yamagishi et al., 2007). Studies have documented Nε-CML, the most prevalent AGE, as a biomarker of DR (Ghanem, Elewa, & Arafa, 2010). The retinal pigment epithelium (RPE) is a mono layer of pigmented cells situated between the neuroretina and the choroid and constitutes the outer blood–retinal barrier (BRB) (Strauss, 2005). Most of the research on the patho-physiology of DR has been focused on the neuroretinal impairment and the breakdown of the inner BRB. By contrast, the effect of diabetes on the RPE has received not as much of consideration (Simó, Carrasco, García-Ramírez, & Hernández, 2006).

Spectral domain optical coherence tomography (SD-OCT) can perform cross sectional imaging of the biological tissues and provides information akin to live *in-vivo* histology of the retina (Saxena & Singh, 2009). Topography of the RPE can be non-invasively and accurately evaluated using segmentation map of RPE on SD-OCT. Our recent study highlighted the topographical alterations in RPE in diabetic retinopathy (Sharma et al., 2015). Our recent study correlated the levels of $N^{\varepsilon}$-CML with severity of diabetic retinopathy and disruption of retinal photoreceptor external limiting membrane and ellipsoid zone in type 2 DM (Mishra et al., 2015).

The purpose of this prospective case control study was to evaluate the association of increase in serum levels of $N^{\varepsilon}$-CML with topographic alterations of RPE in DM, for the first time. The objectives were: (1) to determine the association of grades of RPE alteration with increased severity of DR, (2) to establish the association of increase in serum levels of $N^{\varepsilon}$-CML with logMAR visual acuity, (3) to evaluate the association of grades of RPE alteration with visual acuity.

## 2. Materials and Methods

The authors confirm adherence to the tenets of the Declaration of Helsinki. Study was undertaken after institutional review board clearance and a written informed voluntary consent from all the study subjects. The study was a tertiary care centre based cross sectional study. Sixty consecutive cases of type 2 DM in the age group of 40–65 years were divided into three groups: diabetes patients without retinopathy ($n = 20$), non-proliferative diabetic retinopathy ($n = 20$), and proliferative diabetic retinopathy ($n = 20$) based on the early treatment diabetic retinopathy study (ETDRS) classification (Early treatment diabetic retinopathy study research group, 1991). Twenty healthy controls were also included. Subjects with ocular or systemic diseases affecting the retinal vascular pathology, subjects with previous intravitreal injection(s), ophthalmic surgical or laser interventions, with media haze at any level giving signal strength of less than 5 on SD-OCT were excluded from the study. Also excluded were patients with conditions in which serum level of $N^{\varepsilon}$-CML is raised like Alzheimer's disease, pulmonary fibrosis, atherosclerosis, end stage renal disease, tobacco smoking.

The best-corrected visual acuity was documented on the logMAR scale. Patient's age, gender, and duration of diabetes were documented. All the study subjects underwent detailed fundus evaluation using stereoscopic slit lamp biomicroscopy and indirect ophthalmoscopy. Digital fundus photography and flourescein angiography were done using Zeiss fundus camera FF 450 Plus. Every study subject underwent macular thickness analysis using the macular cube $512 \times 128$ feature of SD-OCT (Cirrus High Definition OCT, Carl Zeiss Meditec Inc., CA, U.S.A). RPE topography was studied on the SD-OCT segmentation map and RPE alterations were graded as: grade 0, No RPE alterations, grade 1, RPE alterations in up to two quadrants and grade 2, RPE alterations in more than two quadrants (Fig. 1) (Sharma et al., 2015). Two experienced observers masked to the status of DR assessed the RPE alteration. The interobserver correlation was computed using Spearman's rank correlation. (See Figs. 2 and 3.)

Blood samples of 7 ml were collected from the study subjects. Glycated hemoglobin (HbA1c) was measured on autoanalyser using standard protocol.

*Assay of $N^{\varepsilon}$-CML*: 5 ml serum was used for assay of $N^{\varepsilon}$-CML using standard protocol. Human $N^{\varepsilon}$-CML ELISA kit procured from USCN, Life Science Inc., Houston, USA and ELISA plate reader (Synergy HT, Biotech, and U.S.A) were utilized. Concentration of Nε-CML in serum sample was calculated based on the standard curve. The values were expressed as ng/ml.

### 2.1. Statistical analysis

Data are summarized and presented as mean ± SD. Chi-square ($\chi 2$) test, one way analysis of variance (ANOVA) followed by post hoc test (Fisher's least significant difference), Fisher exact test, univariate and multivariate ordinal regression and univariate and multivariate linear regression analysis have been utilized for statistical analysis of the study variables among the study groups. $p < 0.05$ was considered statistically significant. All analyses were performed using SPSS software (window version 21.0).

## 3. Results

Table 1 summarizes the mean age, gender distribution, duration of DM, logMAR visual acuity, and serum levels of HbA1c and $N^{\varepsilon}$-CML among the study groups. The mean age among different study groups using one way ANOVA showed no significant difference ($F = 0.51$; $p > 0.05$). No statistically significant difference existed in gender distribution between the cases and controls ($\chi 2 = 0.42$; $p > 0.05$). A significant increase in severity of diabetic retinopathy with increased duration of DM ($F = 21.85$; $p < 0.0001$), increase in HbA1c ($F = 10.97$; $p < 0.001$) and increase in serum levels of $N^{\varepsilon}$-CML ($F = 34.1$; $p < 0.001$) was observed using one way ANOVA. Increased severity of DR was significantly associated with increase in logMAR visual acuity ($F = 23.02$; $p < 0.001$).

No RPE alterations were present in controls and no DR group. Distribution of RPE alterations grade 0/1/2 in NPDR was 12/7/1 respectively and in PDR group 5/8/7 respectively. Inter-observer correlation was observed to be ($\rho = 0.78$; $p < 0.001$). Fisher exact test revealed significant increase in grades of RPE alterations with increased severity of DR (Fisher's exact value = 32.32, $p < 0.001$).

Log-antilog values were taken for adjusting for the skewing of data. Univariate ordinal regression analysis done to calculate the risk of progression in grades of RPE alteration revealed increase in grade of RPE alteration with increase in duration of DM (odds ratio = 1.37; $p = 0.001$), HbA1c (odds ratio = 1.37; $p = 0.002$) and Nε-CML (odds ratio = 1.08; $p < 0.0001$). Multivariate ordinal regression analysis for predicting progression in grades of RPE alteration revealed Nε-CML to be an independent predictor of increase in grades of RPE alteration (adjusted odds ratio = 1.07; $p < 0.01$) when duration of DM and HbA1c were held constant.

Univariate linear regression was done taking logMAR visual acuity as a dependent variable with duration of DM, HbA1c and Nε-CML as independent variables. It revealed increase in logMAR visual acuity with increase in duration of DM ($B = 1.0$; $p = 0.002$), Hba1c ($B = 1.016$; $p = 0.001$) and $N^{\varepsilon}$-CML ($B = 1.07$; $p < 0.001$). Multivariate linear regression, taking logMAR visual acuity as a dependent variable with duration of DM ($B = 1.0$; $p = 0.56$), HbA1c ($B = 1.04$; $p = 0.087$) and Nε-CML as independent variables, revealed $N^{\varepsilon}$-CML ($B = 1.0$; $p = 0.001$), to be an independent predictor of visual acuity ($r^2 = 0.277$; $p < 0.001$).

## 4. Discussion

The present study highlights the association of increased serum levels of $N^{\varepsilon}$-CML with increased grades of RPE alterations in DR.