

Serum Nε-Carboxymethyllysine as a Biomarker of Severity of Diabetic Retinopathy: A Cross-Sectional Study

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Abstract: Background: Chronic hyperglycaemia accelerates the non-enzymatic glycation of proteins, generating advanced glycation end-products (AGEs) such as Nε-carboxymethyllysine (CML), which have been implicated in the microvascular pathology of diabetic retinopathy (DR). **Aim:** To evaluate and compare serum CML levels across the spectrum of severity of DR and to correlate CML with glycaemic and clinical parameters. **Methods:** This cross-sectional comparative study was conducted at a tertiary care hospital in Northern India. Two hundred age- and sex-matched subjects were divided into four equal groups (n = 50 each): proliferative DR (PDR), non-proliferative DR (NPDR), diabetes mellitus without DR, and healthy controls. Serum CML was estimated by enzyme-linked immunosorbent assay and compared using one-way ANOVA with post-hoc pairwise testing; associations with clinical and biochemical parameters were assessed using Spearman's correlation. **Results:** Serum CML rose progressively across the study groups (Control 433.31 ± 73.04 , DM without DR 640.81 ± 78.48 , NPDR 765.39 ± 69.49 , PDR 861.72 ± 75.98 ng/mL; $F = 309.617$, $p < 0.001$), with every pairwise comparison reaching significance ($p < 0.001$). CML correlated strongly with HbA1c ($r = 0.608-0.771$) and fasting blood sugar ($r = 0.538-0.636$) across diabetic subgroups, and with diabetes duration in PDR ($r = 0.359$, $p = 0.010$). **Conclusion:** Serum CML increases in a stepwise fashion with worsening severity of DR and correlates strongly with glycaemic burden, supporting its candidacy as a biochemical adjunct for risk-stratifying diabetic patients for retinopathy.

Keywords: Carboxymethyllysine, Diabetic Retinopathy, Advanced Glycation, End Products, Glycaemic Control, Biomarker.

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INTRODUCTION

Diabetic retinopathy (DR) remains one of the foremost causes of preventable blindness among the working-age population worldwide, with a global prevalence estimated at 22.27% among individuals with diabetes and an anticipated rise to over 160 million affected persons by 2045 [1]. As the prevalence of type 2 diabetes mellitus continues its global ascent, the burden of DR-related visual disability is projected to increase correspondingly, particularly in low- and middle-income countries where screening infrastructure remains limited [1]. Diabetic retinopathy is a multifactorial microvascular complication driven by chronic hyperglycaemia acting through several convergent biochemical pathways, including increased polyol flux, activation of protein kinase C isoforms, oxidative stress

and, importantly, accelerated formation of advanced glycation end-products (AGEs) [2,3].

AGEs are generated through the non-enzymatic Maillard reaction between reducing sugars and the amino groups of proteins, lipids, and nucleic acids, and their tissue accumulation is proportional to the degree and duration of hyperglycaemic exposure [4]. Among the numerous AGE species identified, Nε-carboxymethyllysine (CML) is the most abundant and best characterised, and is frequently used as a representative marker of total body AGE burden [5]. CML-modified proteins bind the receptor for advanced glycation end-products (RAGE) on vascular endothelial and retinal cells, triggering downstream oxidative and inflammatory cascades that compromise the blood-retinal barrier and promote neovascularisation, the pathological hallmark of

proliferative DR [3]. Early case-control studies demonstrated that serum CML is elevated in patients with proliferative DR and macular oedema compared with diabetic patients without such complications, suggesting a possible role for CML as a circulating biomarker of severity of retinopathy [6].

Despite this evidence, most existing studies have compared broadly diabetic and non-diabetic cohorts, with relatively few systematically examining CML across the full clinical spectrum of DR from diabetes without retinopathy through non-proliferative to proliferative disease within a single, well-matched population, particularly from the Indian subcontinent. Given the disproportionate and growing burden of diabetes in India, biomarker data from this population may have particular clinical relevance. The present study was therefore undertaken to compare serum CML levels across four defined groups spanning the DR severity spectrum and to determine its correlation with clinical, glycaemic, and biochemical parameters, with the aim of clarifying its potential utility as an adjunct biomarker for risk-stratifying diabetic patients for retinopathy.

MATERIALS AND METHODS

Study Design and Setting

This cross-sectional comparative study was carried out at tertiary care hospital in Northern India over a period of two years.

Ethical Considerations

The study protocol was approved by the Institutional Ethics and Research Committee of Sri Guru Ram Das Institute of Medical Sciences and Research, Sri Amritsar (Approval No. SGRD/IEC/2024-285). The study was conducted in accordance with the tenets of the Declaration of Helsinki and written informed consent was obtained from all the participants before enrolment.

Study Population

A total of 200 subjects were enrolled and stratified into four groups of 50 subjects each: Group I — proliferative diabetic retinopathy (PDR); Group II — non-proliferative diabetic retinopathy (NPDR); Group III — type 2 diabetes mellitus without retinopathy; and Group IV — age- and sex-matched healthy, non-diabetic controls. Type 2 diabetes mellitus was diagnosed according to American Diabetes Association criteria [8], and the severity of DR was graded on dilated indirect ophthalmoscopy and fundus examination according to the International Clinical Diabetic Retinopathy Disease Severity Scale [7].

Inclusion criteria: A total of 150 patients of either sex aged ≥ 40 years with a confirmed diagnosis of type 2 diabetes mellitus were included in the study. Fifty

healthy non-diabetic participants were taken as the control group.

Exclusion criteria: Patients with type 1 diabetes mellitus, diabetic nephropathy or deranged renal function, chronic liver disease, active infection or systemic inflammatory disease, other retinal or ocular pathology unrelated to diabetes, pregnancy, and smokers were excluded from the study.

Sample Collection and Biochemical Analysis

Under aseptic precautions, 8 mL of fasting venous blood was collected from each participant. Fasting blood sugar was estimated by the hexokinase enzymatic method, and glycated haemoglobin (HbA1c) was measured by turbidimetric inhibition method. Serum urea and creatinine were estimated by standard enzymatic/kinetic methods, and the fasting lipid profile (total cholesterol, triglycerides, LDL, HDL, VLDL, and non-HDL cholesterol) was assayed using standard enzymatic colorimetric methods on an automated analyser. Serum CML was estimated using a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol, and results were expressed in ng/mL.

Statistical Analysis

Data was entered and analysed using SPSS software (version 26.0; IBM Corp., Armonk, NY). Continuous variables were expressed as mean $\pm$ standard deviation and median (interquartile range), and categorical variables as frequencies and percentages. Comparison of continuous variables across the four groups was performed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for pairwise comparisons. Categorical variables were compared using the chi-square test. Comparison between the combined diabetic and control groups was performed using the Mann-Whitney U test. Correlation between serum CML and clinical/biochemical parameters was assessed using Spearman's rank correlation coefficient. A two-tailed p-value < 0.05 was considered statistically significant throughout.

RESULTS

The study comprised 200 subjects distributed equally across four groups. The four study groups were comparable with respect to age, sex distribution, height, and weight (Table 1). Body mass index differed significantly across groups ($p = 0.008$), being lowest in the control group, and diabetes duration and glycaemic parameters (fasting blood sugar and HbA1c) rose progressively from the DM-without-DR group through NPDR to PDR (all $p < 0.001$), confirming the expected gradient of metabolic derangement with increasing retinopathy severity (Table 1).



Table 1. Baseline Demographic and Glycaemic Characteristics of Study Groups

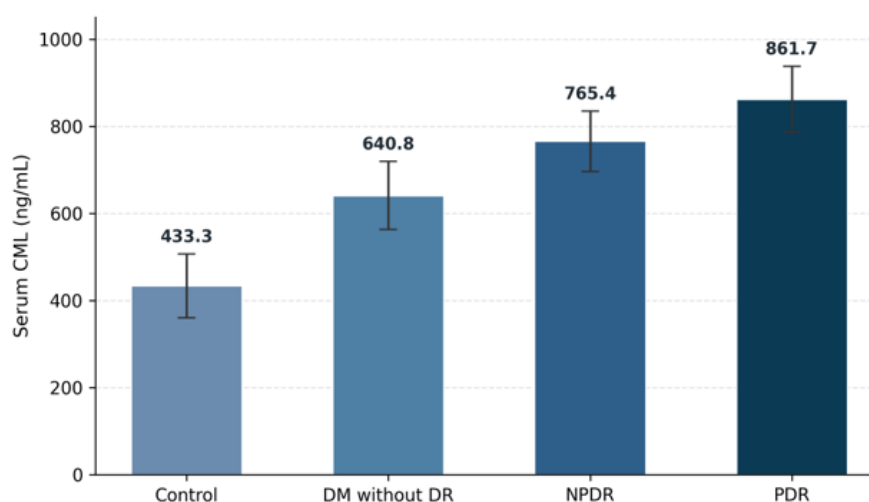
Parameter	PDR (n=50)	NPDR (n=50)	DM without DR (n=50)	Control (n=50)	p-value
Age (years), mean $\pm$ SD	58.62 $\pm$ 8.98	60.08 $\pm$ 9.07	59.36 $\pm$ 9.58	57.08 $\pm$ 6.62	0.349
Sex (Male), n (%)	30 (60.0%)	27 (54.0%)	26 (52.0%)	27 (54.0%)	0.867
Height (feet), mean $\pm$ SD	5.49 $\pm$ 0.27	5.43 $\pm$ 0.45	5.44 $\pm$ 0.36	5.56 $\pm$ 0.31	0.290
Weight (kg), mean $\pm$ SD	71.48 $\pm$ 10.09	71.78 $\pm$ 11.07	73.50 $\pm$ 12.14	69.96 $\pm$ 8.48	0.418
BMI (kg/m ²), mean $\pm$ SD	25.50 $\pm$ 3.20	26.39 $\pm$ 4.61	26.59 $\pm$ 3.08	24.41 $\pm$ 2.79	0.008
Diabetes duration (years)	12.70 $\pm$ 5.28	11.14 $\pm$ 3.52	8.16 $\pm$ 3.48	0.00 $\pm$ 0.00	<0.001
FBS (mg/dL)	167.94 $\pm$ 81.37	151.40 $\pm$ 52.61	132.48 $\pm$ 64.72	87.54 $\pm$ 5.37	<0.001
HbA1c (%)	8.94 $\pm$ 1.88	8.19 $\pm$ 1.71	7.33 $\pm$ 1.45	5.26 $\pm$ 0.23	<0.001

Serum CML levels showed a marked and statistically significant progressive elevation across the four study groups, rising from 433.31 $\pm$ 73.04 ng/mL in controls to 640.81 $\pm$ 78.48 ng/mL in the DM-without-DR group, 765.39 $\pm$ 69.49 ng/mL in the NPDR group, and 861.72 $\pm$ 75.98 ng/mL in the PDR group (one-way ANOVA, F =

309.617, $p < 0.001$; Table 2, Figure 1). When the combined diabetic cohort was compared with controls, serum CML remained substantially and significantly higher in diabetic subjects (755.97 $\pm$ 117.24 vs 433.31 $\pm$ 73.04 ng/mL; Mann-Whitney Z = 18.296, $p < 0.001$).

Table 2. Serum CML Levels Across Study Groups

Group	N	Mean $\pm$ SD (ng/mL)	Median (IQR)	F-value	p-value
PDR	50	861.72 $\pm$ 75.98	868.90 (813.40–926.23)	309.617	<0.001
NPDR	50	765.39 $\pm$ 69.49	773.15 (718.10–815.83)		
DM without DR	50	640.81 $\pm$ 78.48	655.55 (591.83–698.10)		
Control	50	433.31 $\pm$ 73.04	435.05 (387.65–489.90)		

**Figure 1. Mean serum CML levels ($\pm$ SD) across the four study groups. One-way ANOVA, F = 309.617, $p < 0.001$**

Post-hoc pairwise comparison confirmed that serum CML differed significantly between every pair of study groups (all $p < 0.001$), with the largest mean difference observed between the PDR and control groups (428.41

ng/mL, 95% CI 399.10–457.73) and the smallest, though still highly significant, difference between the PDR and NPDR groups (96.32 ng/mL, 95% CI 67.01–125.64) (Table 3).

Table 3. Post-hoc Pairwise Comparison of Serum CML Between Study Groups

Comparison	Mean Difference (ng/mL)	Std. Error	p-value	95% CI
PDR vs. NPDR	96.32	14.86	<0.001	67.01 to 125.64
PDR vs. DM without DR	220.91	14.86	<0.001	191.60 to 250.23
PDR vs. Control	428.41	14.86	<0.001	399.10 to 457.73
NPDR vs. DM without DR	124.59	14.86	<0.001	95.27 to 153.90
NPDR vs. Control	332.09	14.86	<0.001	302.77 to 361.40
DM without DR vs. Control	207.50	14.86	<0.001	178.18 to 236.82

On Spearman's correlation analysis, serum CML correlated positively and significantly with HbA1c in all three diabetic subgroups, most strongly in the DM-without-DR group ($r = 0.771$, $p < 0.001$), followed by the PDR group ($r = 0.608$, $p < 0.001$) and the NPDR group ($r = 0.540$, $p < 0.001$). A similar pattern was observed for

fasting blood sugar ($r = 0.538$ – 0.636 , $p \leq 0.001$ across subgroups). CML correlated significantly with diabetes duration only in the PDR group ($r = 0.359$, $p = 0.010$), and showed no significant correlation with age, BMI, lipid profile, or renal parameters in any group (Table 4, Figure 2).

Table 4. Spearman's Correlation of Serum CML with Clinical and Biochemical Parameters Across Diabetic Subgroups

Parameter	PDR (r, p)	NPDR (r, p)	DM without DR (r, p)
Age	-0.160 (0.267)	-0.359 (0.010)	-0.312 (0.027)
BMI	-0.168 (0.245)	0.106 (0.463)	0.235 (0.101)
Diabetes duration	0.359 (0.010)	0.050 (0.731)	0.078 (0.591)
FBS	0.636 (<0.001)	0.266 (0.062)	0.538 (<0.001)
HbA1c	0.608 (<0.001)	0.540 (<0.001)	0.771 (<0.001)
Urea	-0.278 (0.051)	-0.137 (0.341)	0.089 (0.540)
Creatinine	-0.210 (0.144)	-0.056 (0.700)	-0.080 (0.580)
Total cholesterol	0.203 (0.158)	0.069 (0.633)	0.109 (0.451)
LDL cholesterol	0.238 (0.095)	0.133 (0.361)	0.139 (0.336)
HDL cholesterol	0.016 (0.911)	-0.059 (0.682)	0.034 (0.814)

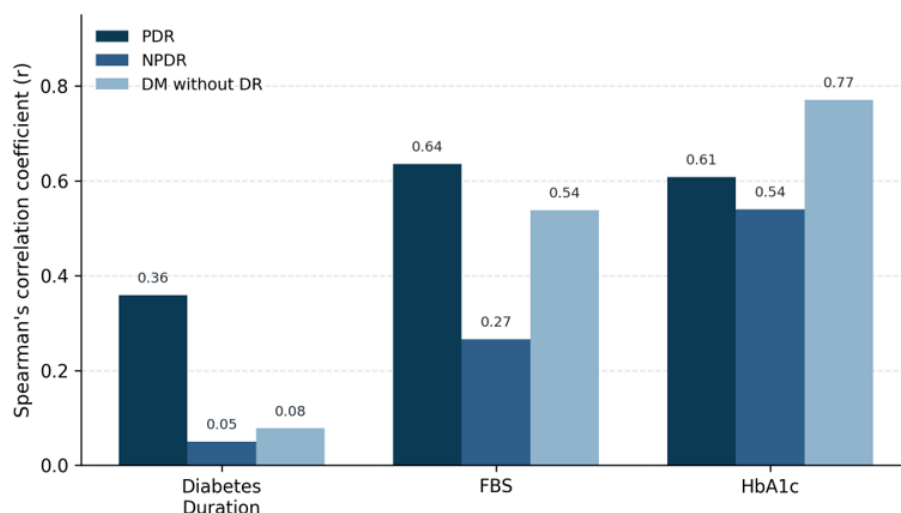


Figure 2. Spearman's correlation coefficients of serum CML with diabetes duration, FBS, and HbA1c across the PDR, NPDR, and DM-without-DR groups

DISCUSSION

This cross-sectional study evaluated serum CML, a major circulating AGE, across the full clinical spectrum of diabetic retinopathy in a well-matched North Indian

cohort. Our principal finding was a consistent, statistically robust, stepwise elevation in serum CML with increasing severity of retinopathy, together with a strong positive correlation between CML and indices of



glycaemic control. These findings reinforce the biological plausibility of AGE accumulation as a contributor to diabetic microvascular injury and lend support to the candidacy of CML as an adjunct biochemical marker of DR severity.

The baseline comparability of the four groups for age, sex, height, and weight (Table 1) indicates that the observed differences in CML are unlikely to be confounded by these demographic variables, strengthening the internal validity of subsequent comparisons. The progressive rise in diabetes duration, fasting blood sugar, and HbA1c from the DM-without-DR group through NPDR to PDR (Table 1) is consistent with the well-established relationship between cumulative glycaemic exposure and retinopathy risk described in large epidemiological analyses [2, 4].

The stepwise elevation of serum CML across study groups (Table 2, Figure 1) closely mirrors the findings of Boehm *et al.* [6], who first reported significantly higher serum CML in patients with proliferative retinopathy and macular oedema compared with diabetic patients without advanced retinopathy, and of Wautier *et al.* [10], who similarly demonstrated CML to be a biomarker of microvascular complications in type 2 diabetes. Our finding of a highly significant overall ANOVA ($F = 309.617$, $p < 0.001$) and, more strikingly, significant pairwise differentiation between every group including the two non-proliferative subgroups (Table 3), extends these earlier observations by showing that CML tracks retinopathy severity along a graded continuum rather than merely distinguishing diabetic from non-diabetic states. This pattern is corroborated by Ghanem *et al.* [11], who reported that both serum and aqueous humour CML increased progressively with worsening retinopathy grade, and by Choudhuri *et al.* [12], who found serum CML and reactive oxygen species markers to rise in parallel with DR severity in an Indian population comparable to the present cohort. The magnitude of pairwise separation observed here, particularly between the PDR and control groups (mean difference 428.41 ng/mL, Table 3), is also concordant with the report of Mishra *et al.* [13], who additionally linked elevated serum CML to structural photoreceptor disruption on optical coherence tomography, suggesting that the biochemical elevation captured in the present study may parallel concurrent structural retinal damage.

The correlation analysis (Table 4, Figure 2) demonstrated that CML tracks most closely with HbA1c and fasting blood sugar rather than with lipid or renal parameters, indicating that glycaemic exposure, rather than dyslipidaemia, is the principal determinant of AGE accumulation in this cohort. This is in keeping with the longitudinal data of Genuth *et al.* [14] from the Diabetes Control and Complications Trial cohort, in which skin collagen CML levels independently predicted the ten-year risk of retinopathy progression, reinforcing the concept that CML functions as a form of biochemical

“metabolic memory” of cumulative hyperglycaemic exposure rather than merely reflecting glycaemic status at a single time point.

Mechanistically, the association between elevated CML and retinopathy severity observed in the present study is best explained by the AGE–receptor for advanced glycation end-products (RAGE) signalling axis. CML-modified proteins bind RAGE expressed on retinal Müller cells, vascular endothelium, and pericytes, activating nuclear factor-kappa B and downstream pro-inflammatory and pro-angiogenic cascades that compromise the blood–retinal barrier and promote pathological neovascularisation, the histological hallmark of proliferative disease [15, 16]. This receptor-mediated signalling additionally amplifies intracellular reactive oxygen species generation, which further accelerates AGE formation in a self-perpetuating cycle of oxidative and glycative stress considered central to the pathogenesis of diabetic microvascular complications [17]. The particularly strong CML–HbA1c correlation observed across all diabetic subgroups in the present study is therefore biologically consistent with this shared upstream driver of both glycation and oxidative injury.

Clinical Significance

From a clinical standpoint, these findings suggest that serum CML estimation could serve as a low-cost, minimally invasive adjunct to conventional glycaemic markers for identifying diabetic patients at higher biochemical risk of retinopathy progression, particularly in resource-limited settings where dilated fundus examination or retinal imaging may not be readily accessible. Incorporation of CML into risk-stratification algorithms alongside HbA1c and diabetes duration could help prioritise patients for closer ophthalmic surveillance, and serial CML measurement may eventually hold value in monitoring the biochemical response to intensified glycaemic control.

Limitations

This study has certain limitations. Its cross-sectional design precludes establishing a temporal or causal relationship between serum CML elevation and retinopathy progression, and the single-centre design may limit generalisability to other populations. Serum CML was quantified by ELISA rather than by mass spectrometry, which, although widely used and validated, is comparatively less precise than chromatographic reference methods. Potential dietary contribution to circulating CML was also not separately accounted for. Longitudinal, multi-centric studies incorporating dietary AGE assessment and adjusted multivariable analysis are warranted to validate these findings further.

CONCLUSION

Serum CML rises progressively and significantly with increasing severity of diabetic retinopathy and correlates strongly with glycaemic burden, particularly HbA1c and



fasting blood sugar. These findings support serum CML as a biologically plausible and clinically promising adjunct biomarker for risk-stratifying diabetic patients for retinopathy, warranting further validation in longitudinal, multi-centric cohorts.

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Conflict of interest: The authors declare that there are no conflicts of interest

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Ethical Statement: Institutional Ethics and Research Committee of Sri Guru Ram Das Institute of Medical Sciences and Research, Sri Amritsar (Approval No. SGRD/IEC/2024-285)

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