

*Short communication*

## **Elevated serum levels of an advanced glycated end-product N<sup>ε</sup>-carboxymethyl-lysine (CML) are associated with proliferative diabetic retinopathy and macular edema**

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**Running Head:** CML and diabetic retinopathy

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## **ABSTRACT**

**Aims/hypothesis** Diabetic retinopathy is a frequent microvascular complication. In search for novel risk markers, we analysed the association of serum levels of the major advanced glycation end product N<sup>ε</sup>-carboxymethyl-lysine with prevalence of advanced stages of retinopathy in Type 2 diabetic patients without nephropathy.

**Methods** In a case control approach we have studied Type 2 patients with and without advanced stages of diabetic retinopathy. Retinopathy and macular edema were defined according to standard criteria. Serum levels of N<sup>ε</sup>-carboxymethyl-lysine were estimated by means of a novel competition based ELISA assay.

**Results** Serum levels of CML were significantly different between age matched controls (N=792; mean value: 521 ng/ml [ $\pm$  1SD: 134 ng/ml]), Type 2 patients without severe retinopathy (821 ng/ml  $\pm$  141; p<0.0001), and patients with proliferative retinopathy (1182 ng/ml  $\pm$  346; p<0.0001). CML levels greater than 1000 ng/ml defined a 25-fold increase in risk for proliferative retinopathy. ROC analysis revealed a CML-threshold of 1087 ng/ml (100% sensitivity, 93% specificity) for clinically significant macular edema.

**Conclusion/interpretation** High serum levels of CML were associated with advanced stages of retinopathy. CML serum levels provided a progressive risk marker, whereby a level of more than 1000 ng/ml gave a 25-fold increase in risk for proliferative retinopathy and clinically significant macular edema. Our data suggest that serum levels of CML may provide a novel risk marker for advanced stages of diabetic retinopathy in Type 2 diabetic patients.

**Keywords:** advanced glycation end products, carboxymethyl-lysine, diabetic retinopathy, proliferative diabetic retinopathy, macular edema, risk marker

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**Abbreviations:**

ABTS	2,2-amino-di-3-ethylbenzthiazoline-sulphonic acid
CML	N <sup>ε</sup> -carboxymethyl-lysine
DR	diabetic retinopathy

## **INTRODUCTION**

Diabetic retinopathy (DR) is a common microvascular complication seen in chronic hyperglycemic states. DR represents a major threat to eyesight in Western countries (1). Two large-scale prospective trials (DCCT, UKPDS) have emphasised the important role of glucose levels in development and in progression of preexisting microvascular complications. Although strong evidence was found that intensive treatment lowers the propensity for development and progression of microvascular complications, no clear-cut evidence for a threshold effect for HbA1c and progression of retinopathy could be found. Therefore one can speculate that other factors besides glycemia reflected by HbA1c levels may influence the presentation of retinopathy (1,2).

Advanced glycation end products (AGE) have been implicated as causal factors in the complications of diabetes mellitus (2). Therefore we reasoned that AGE products may play an important role in diabetic retinopathy. To test this hypothesis we have determined N<sup>ε</sup>-carboxymethyl-lysine [CML] serum levels in a carefully selected cohort of Type 2 diabetic patients without overt nephropathy. The late oxidative product CML was chosen since there is ample of experimental evidence that CML is a biomarker of glycation and oxidation reactions. CML levels may thus faithfully reflect the

(patho)biochemical milieu seen in patients with Type 2 diabetes (2-4). In the present study we took a case-control approach. Diabetic patients with and without advanced stages of retinopathy were studied and their corresponding serum CML levels were compared to an age-matched cohort in search for novel risk markers of diabetic retinopathy.

## **MATERIALS and METHODS**

### *Subjects*

We carefully selected 136 Type 2 diabetic patients with Type 2 diabetes from a larger cohort (N=1,346). Exclusion criteria were uncontrolled glycemia (HbA1c > 9.5%), uncontrolled hypertension, an increased serum creatinine (> 130  $\mu\text{mol/l}$ ). All Type 2 patients had a creatinine clearance  $\geq 80$  ml/min. To exclude exogenous sources of AGE as well as CML, current smoking was an exclusion criterion. 792 matched controls without diabetes mellitus and kidney disease were recruited from a population-based cohort out of the Alb-Donau area. Table 1 gives baseline data of the cohorts studied. Ophthalmological criteria for recruitment were, standard eye examination as described (1,5). Macular edema, including clinically significant macular edema was defined as described (1,5). Grading of retinopathy was done according to the ETDRS scale. Retinopathy was classified as non-proliferative ("without" severe retinopathy) diabetic retinopathy (DR) provided that the ETDRS scale was  $\leq 35$  ( $\leq$  mild nonproliferative DR) or as proliferative DR, provided that ETDRS scale was greater than ETDRS scale 65 (> moderate proliferative diabetic retinopathy).

### *N<sup>ε</sup>-carboxymethyl- lysine [CML] assay.*

AGE-CML was determined with a novel competition based ELISA assay using a N<sup>ε</sup>-carboxymethyl-lysine specific monoclonal antibody (mouse monoclonal 4G9 (Alteon Inc., Ramsey, NY, USA). The assay is CML specific and shows no crossreactivity. The

assay was calibrated with 6-(N-carboxymethylamino) caproate, which refers to the epitope recognized by the mouse monoclonal antibody 4G9. Streptavidin coated 96 well microtiter plates (Roche Diagnostics, GmbH, Penzberg, Germany) were incubated with biotin-labelled AGE-bovine serum albumin (100 ng/well in 100  $\mu$ l) for 1 hour. ELISA plates were washed extensively three times with washing buffer (10 mmol/L TrisHCl, 150 mmol/L NaCl and 0.05% Tween [ICI America Co., Bridgewater, NJ, USA]). Serum samples were preincubated with proteinase K to liberate CML epitopes, protease was inactivated by addition of 1 mmol/L of PMSF. Serum samples as well as CML standards (50  $\mu$ l/well) were then simultaneously incubated with peroxidase-conjugated monoclonal antibody (50  $\mu$ l/well) against CML for 1 hour at room temperature. After three subsequent washing steps, colour reaction was induced by addition of 100  $\mu$ l of ABTS solution/well with 0.3 g/L of 2,2-amino-di-3-ethylbenzthiazoline-sulphonic acid (Roche Diagnostics). Absorbance was read in a microtiter ELISA plate reader (SLT spectra, SLT Labinstruments Inc, Groedig, Austria) at 405 nm. Results show CML levels expressed as the number of single CML epitopes in ng per ml of serum. All samples were run in triplicates. The sensitivity of this competitive ELISA assay was 5 ng CML/ml with intra-assay and inter-assay precision less than 4% and 5%.

#### *HbA1c assay*

HbA1c was determined using ion exchange HPLC (BioRad, Munich, Germany; normal range: 4.3 - 6.1%).

#### *Statistical analysis*

Statistical calculations were performed using the SAS package 6.12 (SAS Institute, Cary, NC). Chi-square test was used for the analysis of categorical data. T-test was used for the comparison of HbA1c-levels.  $P < 0.05$  was accepted as statistically significant.

## RESULTS

Table 1 shows the baseline characteristics and clinical parameters of the diabetic subgroups with and without advanced stages of diabetic retinopathy. Due to criteria used for recruitment no significant differences for variables known to influence both presence and severity of diabetic retinopathy were evident.

Figure 1a gives serum CML levels of the cohorts studied. Serum CML levels were highest in the group of patients with proliferative retinopathy and significantly different ( $p < 0.0001$ ) when compared to controls. Likewise, a significant difference was seen when these Type 2 patients were compared to patients without severe retinopathy. Also a significant difference of serum CML levels was seen when controls and patients without proliferative retinopathy were compared ( $p < 0.0001$ ). However, in this subset some overlap of the corresponding CML levels was evident (Figure 1a).

Serum CML levels provided a progressive risk marker for proliferative retinopathy. An odds ratio of 24.5 could be defined, provided that the CML level was greater than 915 ng/ml (i.e. mean of CML serum level in controls plus 3 standard deviations). A CML serum level greater than 1000 ng/ml was found to be strongly related to the presence of clinically significant macular edema. Only patients with CML levels higher than 1000 ng/ml of CML showed this sight-threatening complication (23/56 [41%] with advanced stages of retinopathy; Table 1). ROC curves revealed that a threshold level of 1087 ng/ml of CML gave 100% sensitivity and 93% specificity for clinically significant macular edema (Figure 1b,c).

## DISCUSSION

We performed a case-control study and have analysed the association of CML serum levels with advanced stages of diabetic retinopathy. The higher the serum CML level the higher the likelihood for advanced stages of diabetic retinopathy. Therefore, serum CML levels provided a novel progressive risk marker independent of corresponding HbA1c levels and various other factors.

A direct link of advanced glycation end products, including the late oxidative product N<sup>ε</sup>-carboxymethyl-lysine with diabetic microvascular complications has been demonstrated in histological studies (2,6-9). Since CML can engage receptors of signal transduction for AGE (RAGE), therefore CML can directly activate key cell signalling pathways and modulate gene expression. Most importantly RAGE expression has been found in the retina, mesangial compartment concomitant with AGE/CML accumulation and may therefore provide a direct link of CML levels and diabetic complications (2,6,7).

There is no doubt that the HbA1c-levels are closely associated with microvascular disease in patients with diabetes. However, there is marked and sometimes perplexing heterogeneity in the presentation of microvascular complications (1). Thus, no threshold effect (HbA1c levels) for diabetic retinopathy was found in both the DCC trial and in the EURODIAB study report (1). Therefore, one might suggest that microvascular complications of diabetes mellitus are themselves related to pathobiochemical alterations other than elevated levels of HbA1c (2).

Our study suggests that factors influencing levels of protein and lipid glycation and oxidation leading to increased level of late oxidative product CML are of considerable importance in microvascular complications. Factors determining differential CML levels could have a major clinical impact and may lead to a wide range of pathologies including vascular complications (2,3,10). However, our novel data have to be

confirmed in independent studies. The predictive value of serum CML levels should also be evaluated in prospective studies. CML serum levels provide a novel risk marker for microvascular complications but do not replace standard eye examinations.

## **ACKNOWLEDGEMENTS**

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## **CONFLICT OF INTEREST**

Nothing declared.

## **Legend to Figure 1:**

**Figure 1A** gives CML serum levels in various cohorts studied.

**Figure 1B** shows receiver operating characteristics (ROC) curve plots comparing serum CML levels of diabetic patients without advanced stages of retinopathy and patients with advanced stages of retinopathy. The best cutpoint for balancing sensitivity and specificity of the CML test is the point on the curve closest to the upper left-hand corner. This point refers to a CML level of 978 ng/ml which refers to the far left point of the ROC curve gives a sensitivity of 79% and a specificity of 82% for proliferative retinopathy.

**Figure 1C** shows a comparison of probands with and without clinically significant macular edema. For the presence of macular edema a “decision threshold” CML level of 1087 ng/ml was defined as the far left point in the ROC curve giving 100% sensitivity and 93% specificity.

**Table 1: Characteristics of study cohort**

	<b>controls</b>	<b>without DR</b>	<b>with DR</b>
Number of subjects	792	81	56
Number of females/males	408/384	43/38	33/23
Age (mean, range)	53.8 (50-62)	53.6 (50-62)	54.2 (50-62)
Number of subjects with clinically significant macular edema	0/792	0/81	23/56
Treatment (%)			
- oral agents	n.a.	59.3	60.7
- combination	n.a.	7.4	5.3
- insulin	n.a.	33.3	34.0
Other medication			
- ACE inhibitors	12.1 <sup>&amp;</sup>	59.2	57.1
- HMG-CoA inhibitors	8.3 <sup>&amp;</sup>	30.8	32.1
Hypertension (%)	21.1 <sup>&amp;</sup>	67.9	71.4
Nephropathy (%)			
- microalbuminuria	n.a.	51.8	57.1
- macroalbuminuria	n.a.	3.7	3.6
- serum creatinine > 130 µmol/l	0	0	0
- creatinine clearance <sup>#</sup> < 80 ml/min	n.a.	0	0
HbA1c (%)	4.8 (4.0-5.4) <sup>&amp;</sup>	9.1 (8.0-9.4)	9.3 (8.1-9.4)
CML (ng/ml; mean, SD)	521 (134)	821.5 (141)	1182 (346)
CML > 789 ng/ml (%) <sup>*</sup>	2.8	46.9	92.8
CML > 915 ng/ml (%) <sup>§</sup>	1.1	24.6	82.2

Mean values are given when appropriate, ranges given in brackets. #) Creatinine clearance was estimated using the Cockcroft-Gault formula. Normal renal function was defined as a clearance greater than 80ml/min. \*) CML level given refers to mean CML of controls plus 2-times standard deviations; or §) plus 3-times standard deviations. No significant differences between the two diabetic cohorts were found for age, gender, current treatment, medication, presence of hypertension, and nephropathy stages. Compared to controls significant differences were seen for prevalence of hypertension, HbA1c levels, and treatment modalities (<sup>&</sup>).

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Figure 1 A

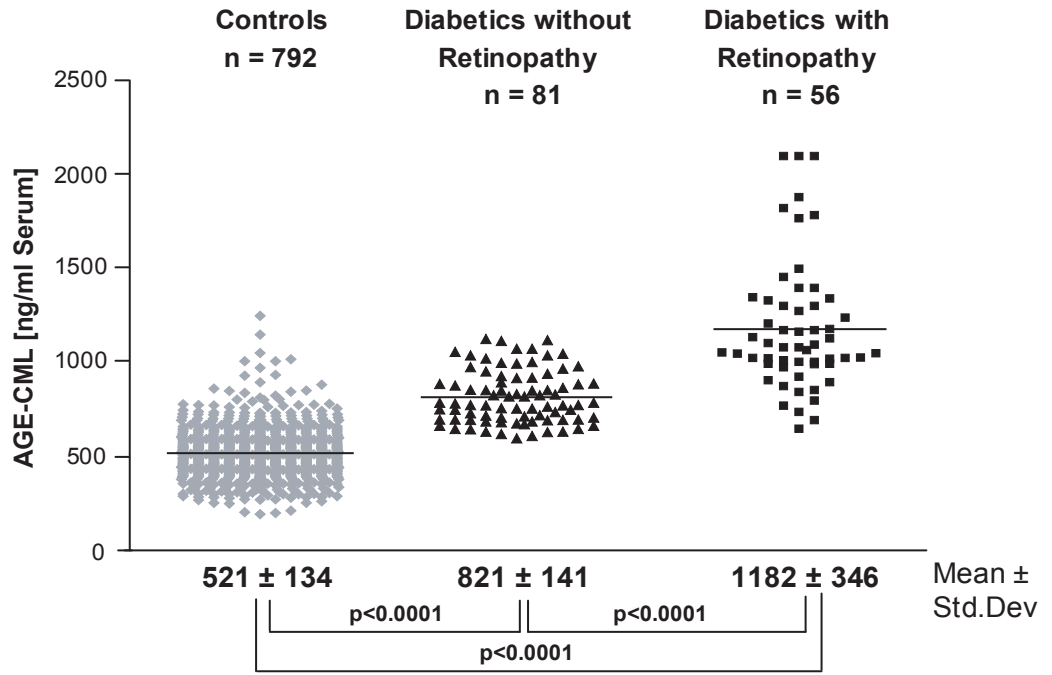


Figure 1 B

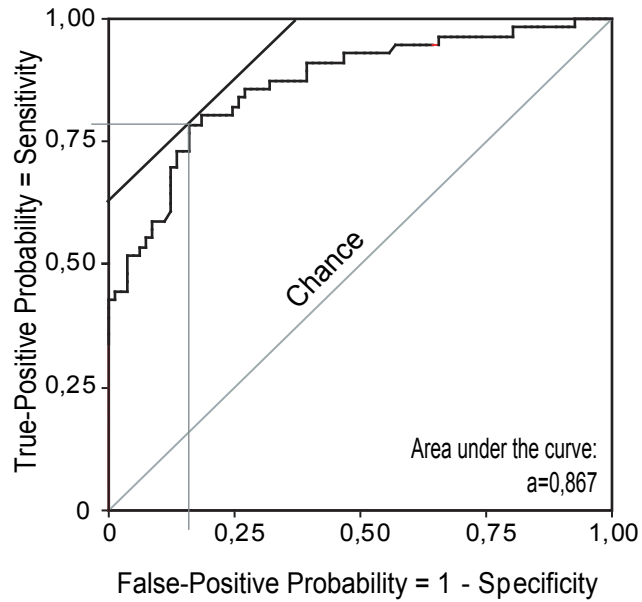


Figure 1 C

