Diabetologia (2004) 47:1376–1379
DOI 10.1007/s00125-004-1455-y

Diabetologia

Short Communication

Elevated serum levels of N^{ε}-carboxymethyl-lysine, an advanced glycation end product, are associated with proliferative diabetic retinopathy and macular oedema

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Abstract

Aims/hypothesis. Diabetic retinopathy is a frequent microvascular complication. In search of novel risk markers, we analysed the association between serum levels of the major advanced glycation end product N^{ε}-carboxymethyl-lysine (CML) and prevalence of advanced stages of retinopathy in Type 2 diabetic patients without nephropathy.
Methods. We carried out a case-control study of Type 2 diabetic patients with and without advanced stages of diabetic retinopathy. Retinopathy and macular oedema were defined according to standard criteria. Serum levels of CML 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 ± SD: 521±134 ng/ml), Type 2 diabetic patients without severe retinopathy (821±141 ng/ml; p<0.0001) and Type 2 diabetic patients with proliferative retinopathy (1182±346 ng/ml; p<0.0001). Levels of CML greater than 1000 ng/ml represented a 25-fold increase in risk of proliferative retinopathy. Receiver operating characteristics analysis revealed a CML threshold of 1087 ng/ml (100% sensitivity, 93% specificity) for clinically significant macular oedema.
Conclusions/interpretation. High serum levels of CML were associated with advanced stages of retinopathy. Serum levels were shown to be a progressive risk marker, whereby a level of more than 1000 ng/ml induced a 25-fold increase in risk of proliferative retinopathy and clinically significant macular oedema. Our data suggest that serum levels of CML 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 · Macular oedema · Proliferative diabetic retinopathy · Risk marker

Received: 1 March 2004 / Accepted: 4 May 2004
Published online: 17 July 2004

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Abbreviations: CML, N^{ε}-carboxymethyl-lysine · ETDRS, Early Treatment Diabetic Retinopathy Study · RAGE, receptor for advanced glycation end products · ROC, receiver operating characteristics

Introduction

Diabetic retinopathy is a common microvascular complication seen in chronic hyperglycaemic states, representing a major threat to eyesight in western countries [1]. Two large-scale prospective trials (the DCCT and the UKPDS) have emphasised the important role of glucose levels in the development and progression of pre-existing microvascular complications [1]. Although strong evidence has been found to suggest that intensive treatment lowers the propensity for the development and progression of microvascular complications, there is no clear-cut evidence to suggest a threshold effect of HbA_1c and progression of retinopathy. Therefore, it can be speculated that other factors

Table 1. Characteristics of the study cohort

	Controls	Without diabetic retinopathy	With diabetic retinopathy
Number of subjects	792	81	56
Females/males	408/384	43/38	33/23
Age, mean (range)	53.8 (50–62)	53.6 (50–62)	54.2 (50–62)
Clinically significant macular oedema present	0	0	23
Treatment (%)			
Oral agents	na	59.3	60.7
Combination	na	7.4	5.3
Insulin	na	33.3	34.0
On other medication (%)			
ACE inhibitors	12.1[d]	59.2	57.1
HMG-CoA inhibitors	8.3[d]	30.8	32.1
Hypertension present (%)	21.1[d]	67.9	71.4
Nephropathy (%)			
Microalbuminuria	na	51.8	57.1
Macroalbuminuria	na	3.7	3.6
Serum creatinine >130 μmol/l	0	0	0
Creatinine clearance[a] <80 ml/min	na	0	0
HbA_1c (%)	4.8 (4.0–5.4)[d]	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 (%)[b]	2.8	46.9	92.8
CML >923 ng/ml (%)[c]	1.1	24.6	82.2

[a] Creatinine clearance was estimated using the Cockroft–Gault formula. Normal renal function was defined as a clearance rate higher than 80 ml/min; [b] CML level given refers to mean CML of controls plus two times standard deviation, or [c] plus three times standard deviation. No significant differences between the two diabetic cohorts were found for age, sex, current treatment, medication, presence of hypertension, and nephropathy stages. Compared with controls, significant differences were seen for prevalence of hypertension, HbA_1c levels, and treatment modalities [(d)]. na, not applicable

besides glycaemia, reflected by HbA_1c levels, may influence the development 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 may play an important role in diabetic retinopathy. To test this hypothesis we determined serum levels of N^{ε}-carboxymethyl-lysine (CML) in a carefully selected cohort of Type 2 diabetic patients without overt nephropathy. The late oxidative product CML was chosen because there is ample experimental evidence that CML is a biomarker of glycation and oxidation reactions. Increased levels of CML may thus reflect the (patho)biochemical milieu seen in patients with Type 2 diabetes [2, 3, 4]. In the present study we adopted a case-control approach. Diabetic patients with and without advanced stages of retinopathy were studied and their corresponding serum CML levels were compared with those of an age-matched cohort in search of novel risk markers of diabetic retinopathy.

Subjects and methods

Subjects. We carefully selected 136 patients with Type 2 diabetes from a larger cohort (n=1346). Exclusion criteria were uncontrolled glycaemia (HbA_1c >9.5%), uncontrolled hypertension, and increased serum creatinine (>130 μmol/l). All patients had a creatinine clearance rate higher than 80 ml/min. To exclude exogenous sources of AGE as well as CML, current smoking was also an exclusion criterion. A total of 792 matched controls without diabetes mellitus and kidney disease were recruited from a population-based cohort from the Alb–Donau area. Table 1 outlines the baseline data of the cohorts studied. The ophthalmological criteria for recruitment was a standard eye examination as described [1, 5]. Macular oedema, including clinically significant macular oedema, was defined as described [1, 5]. Grading of retinopathy was done according to the scale of the Early Treatment Diabetic Retinopathy Study (ETDRS). Retinopathy was classified as non-proliferative ("without" severe retinopathy) if it was graded level 47 or less on the ETDRS scale (mild and moderate non-proliferative diabetic retinopathy), or as proliferative if it was graded level 65 or more on the scale (moderate and high-risk proliferative diabetic retinopathy).

This study was approved by the local research ethics committee, and subjects gave written informed consent to participate.

N^{ε}-carboxymethyl-lysine assay. Serum levels of CML were determined with a novel competition-based ELISA assay using a CML-specific monoclonal antibody (mouse monoclonal 4G9; Alteon, Ramsey, N.J., USA). As an antigen we used BSA (Calbiochem, Bad Soden, Germany) that had been maximally glycated in vitro for 3 weeks with 0.5 mol/l D-glucose at 35 °C, dialysed and then biotinylated. The assay is CML-specific and shows no cross-reactivity. The assay was cali-

brated with 6-(*N*-carboxymethylamino)caproate, which refers to the epitope recognised by the mouse monoclonal antibody 4G9. Streptavidin-coated 96-well microtitre plates (Roche Diagnostics, Penzberg, Germany) were incubated with biotin-labelled AGE-BSA (100 ng/well in 100 µl) for 1 h. ELISA plates were washed extensively three times with washing buffer (10 mmol/l Tris-HCl, 150 mmol/l NaCl and 0.05% Tween [ICI America, Bridgewater, N.J., USA]). Serum samples were pre-incubated at 37 °C for 3 h with 1 mg/ml proteinase K (Roche Diagnostics, Mannheim, Germany) to liberate CML epitopes. Protease was inactivated by adding 1 mmol/l of phenylmethylsulphonyl fluoride. Serum samples as well as CML standards (50 µl/well), generated using various concentrations of 6-(*N*-carboxymethylamino)caproate, were each simultaneously incubated with peroxidase-conjugated monoclonal antibody (50 µl/well) against CML for 1 h at room temperature. After three subsequent washing steps, colour reaction was induced by adding 100 µl/well of ABTS solution containing 0.3 g/l of 2,2-amino-di-3-ethylbenzthiazoline-sulphonic acid (Roche Diagnostics, Mannheim, Germany). Absorbance was read using a microtitre ELISA plate reader (SLT spectra; SLT Labinstruments, Groedig, Austria) at 405 nm. Results show CML levels expressed as the number of single CML epitopes in ng/ml serum. All samples were run in triplicate. The sensitivity of this competitive ELISA assay was 5 ng CML/ml with intra-assay and inter-assay precision at less than 4% and 5%.

HbA1c assay. HbA_1c 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, N.C., USA). Chi square test was used for the analysis of categorical data. Student's *t* test was used for the comparison of HbA_1c levels. A *p* value of less than 0.05 was considered statistically significant.

Results

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

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

Serum CML levels provided a progressive risk marker for proliferative retinopathy. An odds ratio of 24.5 was recorded, given that the CML level was higher than 915 ng/ml (i.e. the mean of CML serum

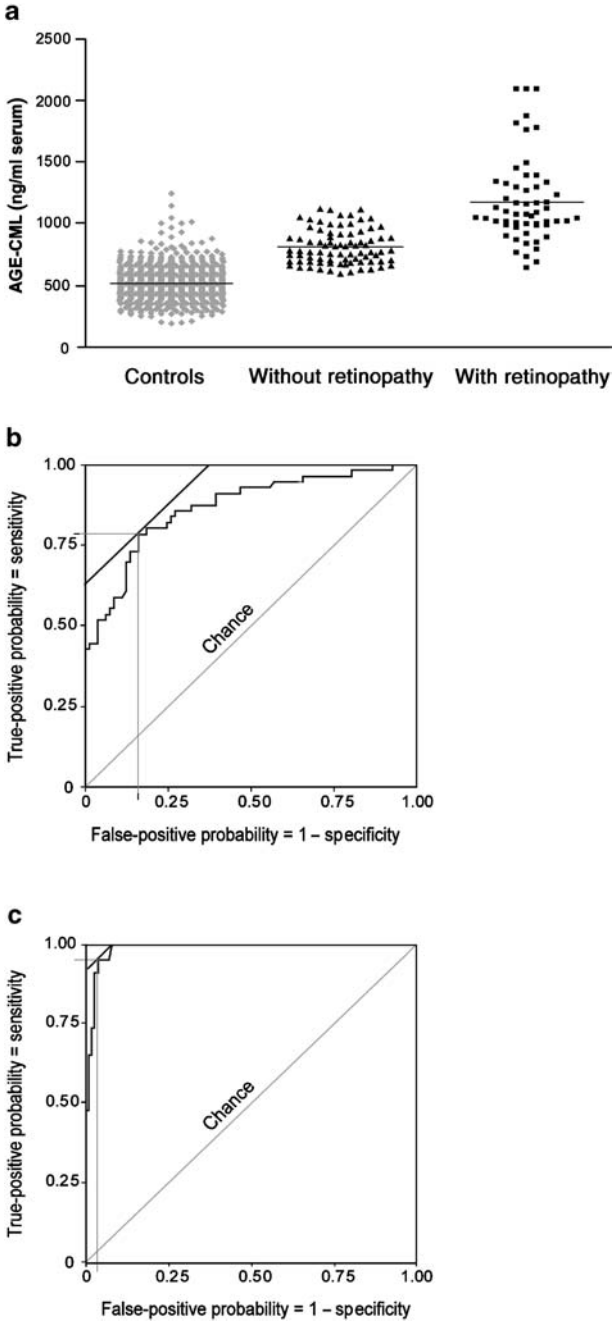

Fig. 1a. CML serum levels in various cohorts studied. **b.** Receiver operating characteristics curve plots comparing serum CML levels of diabetic patients without advanced stages of retinopathy with those of patients with advanced stages of retinopathy. The best cut-point for balancing sensitivity and specificity of the CML test is the point on the curve closest to the upper left-hand corner. This point represents a CML level of 978 ng/ml, which refers to the far left point of the curve, giving a sensitivity of 79% and a specificity of 82% for proliferative retinopathy. AUC=0.867. **c.** A comparison of probands with and without clinically significant macular oedema. For the presence of macular oedema a "decision threshold" CML level of 1087 ng/ml was defined as the far left point in the curve, giving 100% sensitivity and 93% specificity. AUC=0.988

level in controls plus three standard deviations). A CML serum level higher than 1000 ng/ml was found to be strongly related to the presence of clinically significant macular oedema. Only patients with CML levels higher than 1000 ng/ml showed this sight-threatening complication (23/56 [41%] with advanced stages of retinopathy; Table 1). Receiver operating characteristics (ROC) curves revealed that a threshold level of 1087 ng/ml of CML gave 100% sensitivity and 93% specificity for clinically significant macular oedema (Fig. 1b, c).

Discussion

We carried out a case-control study and analysed the association between CML serum levels and advanced stages of diabetic retinopathy. The higher the serum CML level, the higher the likelihood of advanced stages of diabetic retinopathy. Therefore, serum CML levels were shown to be a novel progressive risk marker independent of corresponding HbA_1c levels and various other factors.

A direct link between AGE, including the late oxidative product CML, and diabetic microvascular complications has been demonstrated in histological studies [2, 6, 7, 8, 9]. Since CML can engage receptors of signal transduction for AGE (RAGE), it can directly activate key cell signalling pathways and can 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 between CML levels and diabetic complications [2, 6, 7].

There is no doubt that HbA_1c levels are closely associated with microvascular disease in patients with diabetes. However, there is marked and sometimes perplexing heterogeneity in the development of microvascular complications [1]. Thus, no threshold effect (HbA_1c levels) for diabetic retinopathy was found in either the DCCT study or the EURODIAB study [1]. Therefore, it is possible that microvascular complications of diabetes mellitus are themselves related to pathobiochemical alterations other than elevated levels of HbA_1c [2].

Our study suggests that factors influencing levels of protein and lipid glycation and oxidation leading to increased levels 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]. A limitation of this study is that we adopted a cross-sectional approach to provide clear-cut evidence of an association between CML levels and severity of diabetic eye complications. To address the predictive potential of CML levels in diabetic complications, prospective studies have to be carried out. Since CML levels can be determined in samples stored for a very long time, important observations could be made using samples from clinical trials already performed. In conclusion, CML serum levels may prove to be a novel risk marker for microvascular complications, but this method will not replace standard eye examinations.

Acknowledgements. B. O. Boehm was supported by the German Research Council (DFG), the Collaborative Research Centre (SFB 518) and the Research Training Group (GRK 1041). The authors wish to thank Dr Feldmann and Dr Buchwald for their help with recruitment of patients.

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