

# The Advanced Glycation End Product N $\epsilon$ -(carboxymethyl)lysine Is Increased In Serum From Children and Adolescents With Type 1 Diabetes

TOR JULSRUD BERG, MD  
JES THORN CLAUSEN, MSC  
PETER A. TORJESEN, PHD

KNUT DAHL-JØRGENSEN, MD, PHD  
HANS-JACOB BANGSTAD, MD, PHD  
KRISTIAN F. HANSEN, MD, PHD

**OBJECTIVE** — To investigate whether children and adolescents with type 1 diabetes have increased serum levels of the glycoxidation product N $\epsilon$ -(carboxymethyl)lysine (CML) at an early stage of the disease.

**RESEARCH DESIGN AND METHODS** — The serum levels of CML in 38 patients with type 1 diabetes aged  $14 \pm 3.2$  (mean  $\pm$  SD) years were compared with those in 26 control subjects aged  $16 \pm 1.7$  years. The mean duration of diabetes was  $5 \pm 4.7$  years, ranging from 0.5 to 15 years. The mean levels of HbA<sub>1c</sub> were  $10.3 \pm 2.5\%$  in the patient group. The serum levels of CML were measured using a monoclonal anti-CML antibody in a fluoremetric immunoassay. Serum protein levels of advanced glycation end products (AGEs) were assayed using a polyclonal antibody from rabbit immunized with AGE-RNase (pAGE).

**RESULTS** — The serum levels of CML and pAGE were significantly increased in the patient group versus the control group: 1.08 (0.45–2.97) U/ml CML (median 10–90 percentiles) vs. 0.70 (0.36–1.79) U/ml CML,  $P < 0.03$ , and 6.6 (5.1–9.9) U/ml pAGE vs. 5.5 (3.7–8.2) U/ml AGEs,  $P < 0.01$ . A significant relationship between CML and pAGE was found in the IDDM group,  $r = 0.76$ ,  $P < 0.001$ . The CML levels were not associated with the HbA<sub>1c</sub> levels ( $n = 23$ ,  $r = -0.02$ , NS), cholesterol levels ( $n = 21$ ,  $r = 0.07$ , NS), age, sex, or diabetes duration.

**CONCLUSIONS** — Serum levels of CML are increased in patients with type 1 diabetes. This increase precedes the development of micro- and macrovascular complications.

*Diabetes Care* 21:1997–2002, 1998

**A**mino groups of proteins can react nonenzymatically with reducing sugars to form unstable Schiff bases that can then undergo the Amadori rearrangement to irreversible advanced glycation end products (AGEs). AGEs can also be formed without going through Amadori rearrangement (1). These AGEs are thought to be

involved in the etiology of micro- and macrovascular complications seen in type 1 diabetes (2–6).

By chemical characterization, the AGE products have been shown to include N $\epsilon$ -(carboxymethyl)lysine (CML) (1), pentosidine (7), pyrraline (8), crosslines (9), and recently, imidazolone (10). Increased levels

of the glycoxidation product CML have been found in skin collagen of patients with type 1 diabetes (11). CML is associated with the severity of retinopathy and nephropathy (12). It is found in the expanded mesangial cell matrix and the thickened glomerular capillary walls of patients with diabetic nephropathy (7), in atherosclerotic plaques (6), in the human diabetic peripheral nerve (13), and in human diabetic retinal vessels (14). Accordingly, CML may be associated with the development of vascular complications of diabetes.

Based on polyclonal antibodies raised against AGE-RNase (3), we have developed an AGE immunoassay that measures the serum levels of AGEs. Using this immunoassay, we have recently shown that serum levels of AGEs are increased in children with type 1 diabetes even before they show microvascular complications (15). We also reported that serum levels of AGEs are prognostic indicators of the mesangial cell matrix expansion and increased basement membrane thickness in the kidneys of young patients with type 1 diabetes and microalbuminuria (16). These results underline conclusions obtained from several studies using the same polyclonal antibodies against AGE proteins (3,17–21), supporting the importance of AGEs in the development of microvascular complications in diabetes.

Although recent reports have demonstrated that the glycoxidation product CML is a major epitope recognized by polyclonal anti-AGE antibodies in vitro (22–24), the epitope structures recognized by the polyclonal antibody to AGE proteins are still unknown.

The measurement of AGEs and CML have proven to be complex and difficult from a methodological point of view. Because the quantification of CML has so far been based on reversed phase high-performance liquid chromatography (HPLC) or selected ion-monitoring gas chromatography/mass spectrometry, which are not suitable for the measurement of CML in a large number of patient samples, handier methods have been sought to assess CML

From the Aker Diabetes Research Center (T.J.B., K.D.-J., H.-J.B., K.F.H.) and the Hormone Laboratory (P.A.T.), Aker University Hospital, Oslo, Norway; and Novo Nordisk (J.T.C.), Bagsvaerd, Denmark.

Address correspondence and reprint requests to Tore Julsrud Berg, MD, Aker Diabetes Research Center, Aker University Hospital, 0514 Oslo, Norway. E-mail: t.j.berg@ioks.uio.no.

Received for publication 15 April 1998 and accepted in revised form 16 July 1998.

**Abbreviations:** AGE, advanced glycation end product; AGE-BSA, advanced glycation end product–modified bovine serum albumin; BSA, bovine serum albumin; CML, N $\epsilon$ -(carboxymethyl)lysine; CV, coefficient of variation; DELFIA, delayed europium lanthanide fluorescence immunoassay; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; pAGE, advanced glycation end products measured by a polyclonal anti-AGE antibody.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

in clinical studies. Accordingly, the primary aim of the present work was to develop an immunoassay for the measurement of CML in serum and to subsequently examine the presence and levels of CML in children with type 1 diabetes before the development of late complications. A second goal was to access the efficacy of widely used polyclonal antibodies against AGE-RNase to investigate whether they recognize the specific CML protein epitope *in vitro*.

### RESEARCH DESIGN AND METHODS

#### Subjects

There were 38 patients with type 1 diabetes recruited from the pediatric outpatient clinic of Aker University Hospital. The patients had been screened for retinopathy by ophthalmoscopy after the age of 10 years. All had diabetes for at least 5 years' duration. Of the 24 patients who had been screened for retinopathy, one showed background retinopathy. An increased urinary albumin excretion rate ( $>20 \mu\text{g}/\text{min}$ ) was seen in 1 of the 38 patients. Of 38 patients, 4 were smokers. The 26 nondiabetic control subjects were healthy school children taking part in a study of bone mineralization in children. All had normal standard laboratory tests. Mean age of the patients with type 1 diabetes was  $14 \pm 3.2$  years, as compared with  $16 \pm 1.7$  years in the group of control subjects. The mean duration of diabetes was  $5 \pm 4.7$  years, ranging from 0.5 to 15 years, and  $\text{HbA}_{1c}$  was  $10.3 \pm 2.5\%$  ( $n = 23$ ).

#### Preparation of CML-bovine serum albumin

CML-bovine serum albumin (BSA) (080895) was prepared according to Reddy et al. (25). Briefly, the CML-BSA was prepared using 0.2 mol/l phosphate, pH 8.0, as solvent for 1 ml of 175 mg/ml BSA (Fraction V Sigma A-2153; Sigma, St. Louis, MO), which was simultaneously mixed with 0.5 ml 0.3 mol/l glyoxylic acid (Sigma G-4627) and 0.5 ml freshly prepared 0.9 mol/l  $\text{NaBH}_3\text{CN}$ , and allowed to mix for 10 min at  $37^\circ\text{C}$  followed by dialysis against phosphate-buffered saline containing 0.1% (wt/vol)  $\text{NaN}_3$  overnight at  $5^\circ\text{C}$ .

The purity of the CML-BSA preparation was assessed by amino acid composition analysis on a modified Beckman 121 MB apparatus (Beckman Coulter, Fullerton, CA) after hydrolysis in a closed vial with 6 mol/l HCl ( $111^\circ\text{C}$  for 24 h). Lysine was the only amino acid residue modified by CML

formation. The CML-BSA preparation contained 20 CML-modified lysine residues per molecule of albumin, corresponding to 34% of all the available lysine residues (24).

#### Production of BSA-Ribose-AGE

50 mg/ml BSA was mixed with 500 mmol/l ribose in a phosphate buffer at  $37^\circ\text{C}$  in the dark for 90 days. In this AGE preparation, 10% of lysine residues were modified by CML.

#### Preparation of monoclonal CML-2F8AxB antibodies

RBF mice were immunized by injecting subcutaneously  $50 \mu\text{g}$  of CML-BSA emulsified in Freund's complete adjuvant, followed by two injections with  $20 \mu\text{g}$  of CML-BSA emulsified in Freund's incomplete adjuvant. High responder mice were boosted intravenously with  $25 \mu\text{g}$  of CML-BSA, and the spleen was harvested after 3 days. Spleen cells were fused with the myeloma Fox cell line by the polyethyleneglycol method. Supernatants were screened for CML-specific antibody production by enzyme-linked immunosorbent assay (ELISA). Monoclonal antibodies were purified by protein A affinity chromatography.

#### Production of AGE-BSA and polyclonal anti-AGE-RNase antibodies

The AGE-BSA and polyclonal anti-AGE-RNase antibodies were produced according to Makita et al. (3). Briefly, AGE-modified BSA (AGE-BSA) and AGE-modified bovine pancreatic ribonuclease (AGE-RNase) were obtained by incubating the proteins ( $25\text{--}50 \text{ mg/ml}$ ) in 500 mmol/l phosphate buffer at pH 7.40, with 500 mmol/l glucose for 12 weeks at  $37^\circ\text{C}$ . Two female New Zealand white rabbits received four primary and one booster immunization of AGE-RNase emulsified in Freund's complete adjuvant. The specificity of the antisera was examined by a competitive ELISA system (3). The anti-AGE antibodies used in the present studies were identical to those described previously (3).

#### Delayed europium lanthanide fluorescence immunoassays

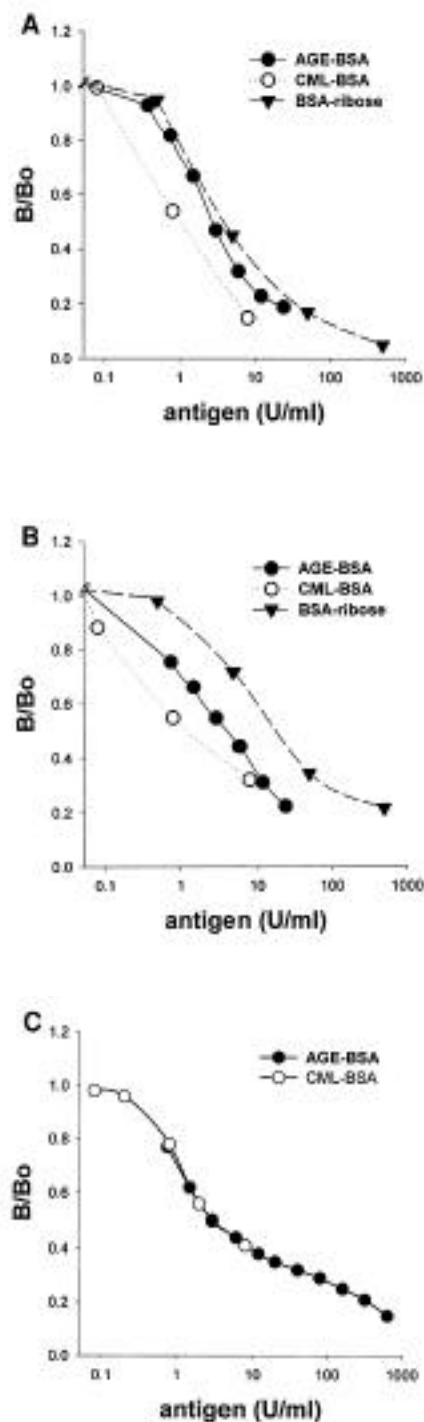
For this procedure, 12-well microtiter strips (Cat. no. 1244-550; Wallac, Turku, Finland) were coated with 0.1 ml of CML-BSA or AGE-BSA ( $25 \mu\text{g}/\text{ml}$ ) diluted in 0.05 mol/l carbonate buffer, pH 9.8, and incubated overnight with shaking at room temperature.

After washing, the wells were blocked overnight with 50 mmol/l Tris buffer containing 100 mmol/l NaCl, 0.1% Germal II (Sutton, Chatham, NJ), and 1% Tween 20 (Boehringer Mannheim, Mannheim, Germany). The strips were covered and stored in this buffer at  $4^\circ\text{C}$ . Immediately before the assay, wells were washed six times in the delayed europium lanthanide fluorescence immunoassay (DELFLIA) washing buffer (Cat. no. B117-100; Wallac). A quantity of 100  $\mu\text{l}$  CML-BSA or AGE-BSA standard (in triplicate) or twofold diluted (CML-assay) or threefold diluted (pAGE assay) serum sample (in duplicate), diluted in assay buffer (50 mmol/l Tris buffer containing 100 mmol/l NaCl, 0.1% Germal II, and 0.1% Tween 20), was added to each well together with 50  $\mu\text{l}$  of anti-CML-BSA antiserum diluted 1:5,000 or anti-AGE-RNase antiserum diluted 1:1,000 in assay buffer. Six standard solutions consisting of 0, 0.75, 1.5, 3, 6, 12, and 24  $\mu\text{g}/\text{ml}$  CML-BSA or AGE-BSA standards were used in each assay. The strips were incubated with shaking at room temperature for 2 h and then washed six times in washing buffer. After washing, 100  $\mu\text{l}/\text{well}$  of Europium-labeled anti-mouse IgG antibodies (Cat. no. 1244-330; Wallac) (for the CML assay) or anti-rabbit IgG antibodies (Cat. no. 1244-100; Wallac) for the polyclonal anti-AGE-RNase antibody in a final concentration of 0.1  $\mu\text{g}/\text{ml}$  in assay buffer were added. The strips were incubated with shaking for 1 h and washed six times. Finally, the strips were incubated with DELFLIA enhancement solution (Cat. no. 1244-105; Wallac) with shaking for 5 min at room temperature, and the Europium ion chelate specific fluorescence was measured in a 1232 DELFLIA Fluorometer (Wallac).

One AGE or CML unit was, according to Makita et al. (17), defined as the competitive activity of 1  $\mu\text{g}$  of AGE-BSA, BSA-ribose, or CML-BSA standard. The final serum concentrations of AGE or CML were corrected for total protein concentration in each serum in the following way: (AGE units per milliliter)  $\times$  (sample protein concentration/mean protein concentration of all the sera measured).

Europium fluorescence was measured by a plate fluorometer (1232 DELFLIA Fluorometer; Wallac).

Serum samples were immediately frozen at  $-40^\circ\text{C}$  (for up to 12 months) until analysis. All serum samples were run in one batch. Data presented are means of triplicates.



**Figure 1**—Effect of varying well coating and antibody in the AGE and CML assays. The figures depict immunoassay displacement curves for the anti-CML antiserum with the modified albumins CML-BSA, AGE-BSA, and BSA-ribose after coating with AGE-BSA (A); the anti-AGE antiserum with the modified albumins CML-BSA, AGE-BSA, and BSA-ribose after coating with AGE-BSA (B); and the anti-CML antiserum with the modified albumins CML-BSA and AGE-BSA after coating with CML-BSA (C). All data points represent the mean of triplicate determinations.

**Table 1**—Serum CML recovery studies (U/ml) in the CML antibody assay

CML initially present (A)	CML-BSA added (B)	Total CML measured (C)	CML recovered (D)	Recovery (%)
0.39*	0.40	0.70	0.31	79
	1.00	1.30	0.91	91
	2.00	2.45	2.07	103
0.34*	0.40	0.72	0.32	81
	1.00	1.47	1.13	113
	2.00	2.64	2.30	115
0.34	0.40	0.71	0.37	93
	1.00	1.55	1.21	121
	2.00	3.18	2.84	142
0.25	0.40	0.64	0.39	98
	0.10	1.41	1.15	115
	2.00	3.34	3.09	155

Recovery (mean  $\pm$  SD): 109  $\pm$  23%

Recovered concentration:  $D = C - A$ . Recovery:  $(D/B) \times 100$ . \*Sera from patients with type 1 diabetes. (A) represents the CML concentration in threefold diluted sera.

### Other analyses

The total protein concentration in each serum sample was determined by the Biuret method (Boehringer Mannheim), which has a coefficient of variation (CV)  $< 2\%$ . HbA<sub>1c</sub> was analyzed with an HPLC method (Diamat analyzer; Biorad, Richmond, CA). Normal range was 4.3–6.1%, with an interassay CV of 3%.

### Statistical analysis

Comparisons between groups were analyzed by two-sided Mann-Whitney *U* test, since data did not show a normal distribution. Linear correlations were tested by least-squares regression to the mean, and regression analyses were tested by linear regression. Data are means  $\pm$  SD, unless otherwise stated. The level of significance was set at  $P < 0.05$ . Calculations were performed using the Number Cruncher Statistical System, (Kaysville, UT) or Sigma Stat (Jandel, Erkrath, Germany). Intra-assay CV was calculated by the MedCalc software (MedCalc Software, Mariakerke, Belgium).

## RESULTS

### Specificity studies

The percentage binding of the anti-CML antibodies to wells coated with AGE-BSA, BSA-ribose, or CML-BSA added (Fig. 1A). Displacement of 50% was reached with 1.0 U/ml CML-BSA, 3.0 U/ml AGE-BSA, and 3.5 U/ml BSA-ribose. Thus, the anti-CML antibodies cross-reacted 100, 33, and 29% against CML-BSA, AGE-BSA, and BSA-

ribose, respectively. This is in accordance with the amino acid analysis (24) showing that 34% of the lysine residues (20 residues/mol) are modified to CML in CML-BSA and 10% (6 residues/mol) in BSA-ribose. As the parallelism between the displacement curves suggests that the anti-CML antibodies recognize identical epitopes on these antigens, the percent cross-reaction indicates that the AGE-BSA contains CML in the same amount as is found in BSA-ribose. Figure 1B indicates that in an AGE assay the anti-AGE antibodies recognize epitopes in CML-BSA and AGE-BSA not found in BSA-ribose. In a homologous CML-assay (Fig. 1C), a higher concentration of CML-BSA was needed (2.5 U/ml) to get a 50% displacement in this assay as compared with the two other assays (Fig. 1A and B).

### Accuracy of the CML-assay

Recovery studies performed by adding CML-BSA in different concentrations to sera from IDDM patients and control subjects showed a mean recovery of 109  $\pm$  23% (Table 1). A close association between the dilution factor and measured levels of CML was observed when serum was diluted one-half, one-fourth, and one-eighth (Table 2). Intra- and interassay CV of the standard curve and serum measurements were 8 and 10%, respectively, for the assay using the polyclonal anti-AGE antibody and 6 and 20%, respectively, for the CML assay.

### The serum levels of CML and pAGE

The serum levels of CML and pAGE were determined by the CML-BSA antibody/CML-

Table 2—Results from the CML antibody assay

	CML concentration		
	One-half	One-fourth	One-eighth
Control			
1	1.58	1.70	1.87
2	1.90	2.20	2.20
Type 1 diabetes			
1	0.78	1.10	1.28
2	0.71	1.04	1.01

The CML concentrations (U/ml) in sera from two control subjects and two patients with type 1 diabetes diluted twofold, fourfold, and eightfold were determined in the CML antibody assay. Results are multiplied up to undiluted concentrations.

BSA and AGE-RNase antibody/AGE-BSA assays. Serum CML and pAGE were significantly increased in the group of diabetic patients in comparison with the control group: 1.08 (0.45–2.97) U/ml CML (median 10th to 90th percentiles) vs. 0.70 (0.36–1.79) U/ml CML,  $P < 0.03$  (Fig. 2), and 6.6 (5.1–9.9) U/ml pAGE vs. 5.5 (3.7–8.2) U/ml AGEs,  $P < 0.01$ . The three patients with either background retinopathy or microalbuminuria had CML and pAGE levels within the 10th to 90th percentiles of the group with diabetes.

A close relationship between the serum levels of CML and pAGE were observed in the IDDM group,  $r = 0.76$ ,  $F = 49$ ,  $R^2 = 0.57$ ,  $P < 0.001$  ( $n = 38$ ) (Fig. 3), showing that 57% of the variation in pAGE could be explained by variations in serum levels of CML. The linear regression analysis, based on analysis of residuals, passed normality ( $P < 0.35$ ) and homoscedasticity ( $P < 0.16$ ) tests. The CML levels were not correlated with HbA<sub>1c</sub> levels,  $r = -0.02$ , NS ( $n = 23$ ), or cholesterol levels,  $r = 0.07$ , NS ( $n = 21$ ). The correlation between pAGE levels and HbA<sub>1c</sub>

failed to reach significance:  $r = 0.35$ , NS ( $n = 23$ ). There was no significant correlation between pAGE and cholesterol:  $r = 0.13$ , NS ( $n = 21$ ). No association between the CML or pAGE levels and age, sex, or diabetes duration was found.

Two newly diagnosed patients with diabetes who showed more than twofold increased levels of both CML (Fig. 2) and pAGE, as compared with the median of the diabetic group, were followed prospectively for 24 months to see whether they had sustained increased serum levels. Their mean levels of CML and pAGE, in five serum samples during 24 months, were 4.2 and 3.5 U/ml and 19.6 and 13.8 U/ml, respectively, despite HbA<sub>1c</sub>  $< 8\%$ . These two patients had normal kidney function and lipid values and had no other disease except for diabetes. Excluding these two patients from the statistical analysis, significant differences were retained between the diabetic group and the control subjects for both CML ( $P < 0.05$ ) and pAGE ( $P < 0.03$ ).

**CONCLUSIONS** — The present investigation demonstrates that the serum levels of CML, a product of hyperglycemia combined with free radical oxidation, are increased in the serum of children and adolescents with type 1 diabetes shortly after the diagnosis of diabetes and before they develop micro- or macrovascular complications. We find a high and significant association between the serum CML levels and the serum AGE levels as determined by a widely used polyclonal antibody. We also demonstrate in vitro that the polyclonal anti-AGE antibody recognizes the specific glycoxidation product CML and uncharacteristic epitope(s) found in BSA-ribose (Fig. 1B) and that AGE-BSA contains CML (Fig. 1A and C).

The monoclonal anti-CML antibody used in the present study has been shown to have a high specificity toward CML-

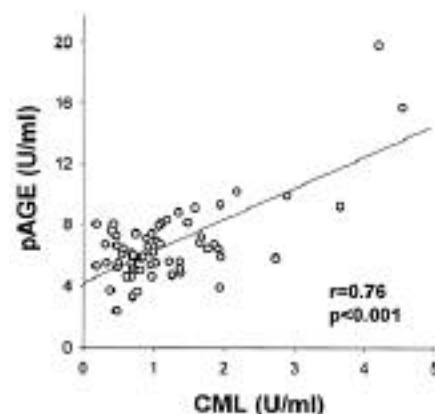


Figure 3—The linear regression between serum levels of AGEs as measured by a polyclonal anti-AGE antibody and serum levels of CML in 38 children and adolescents with type 1 diabetes.

modified proteins (24), and it forms the basis for the CML assay of the present work. Unlike other methods for the determinations of CML, the present CML immunoassay is easy to perform and is, therefore, a suitable tool for the detection of CML levels in clinical studies. The inter-assay CV of the CML assay renders necessary within-assay determination of patient and control samples.

A polyclonal anti-AGE antibody, produced as in the present study, has recently been shown to recognize CML in human serum (26). A high correlation between AGE proteins in serum as measured by a pAGE immunoassay and CML as quantified by gas chromatography–mass spectrometry was found in a small combined group of patients with kidney failure and control subjects (26). The present study shows a positive correlation between the serum levels of pAGE and CML in children with diabetes who have normal kidney function, indicating that our immunoassay specifically measures CML in vivo.

In the present study, 57% of the variation of pAGE could be explained by variations in serum levels of CML, which demonstrates in vivo what has recently been shown in vitro, namely that CML is a major epitope of AGEs (22–24). Because CML is an in vivo epitope recognized by the polyclonal anti-AGE antibody used in the AGE assay of several important animal and clinical studies where the connection between AGEs and microvascular complications in diabetes were reported (3,17–21), the pathological effects of AGEs might partly be mediated by the glycoxidation product CML. On the other hand, it

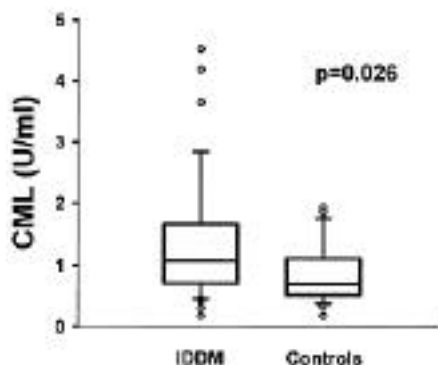


Figure 2—The serum levels of CML in children and adolescents with type 1 diabetes ( $n = 38$ ) compared with age- and sex-matched control subjects ( $n = 26$ ). Data represent 10th, 25th, 75th, and 90th percentiles and median values.

must be emphasized that the polyclonal anti-AGE antibody may recognize other as yet unidentified AGE epitopes in addition to CML (23). This is supported by the methodological and clinical studies of the present work.

CML might be a predictor for the development of microvascular complications in type 1 diabetes because increased levels of CML precede the development of these complications (as seen in this study), and an association between CML and microvascular complications has been demonstrated (12). Although we have shown that the serum levels of pAGE may predict susceptibility to the microvascular complications (16), it remains to be proven whether CML is involved in the etiology of microvascular complications in diabetes or whether increased serum levels of CML predict susceptibility to the development of these complications.

We recently demonstrated a low, but significant, correlation between the serum levels of pAGE and HbA<sub>1c</sub> in children and adolescents with type 1 diabetes (15) and a somewhat higher correlation in patients with type 1 diabetes who had microalbuminuria (16). The same relationship did not reach a significant correlation in the present investigation, probably because the study group was of a smaller size. The present study confirms the increased serum levels of pAGE in children with type 1 diabetes found in our previous study (15).

Few investigators have reported evidence of the presence of CML in children. One study did not identify CML immunohistochemically in fetal or juvenile normal human tissue (6), whereas another study showed small amounts of CML in tissue from newborn stillbirth (11). Using a sensitive assay to avoid the losses in extracting CML from skin collagen, we were able to identify CML in the serum of both children with type 1 diabetes and age-matched control subjects.

At present, the source of CML found in circulation is unknown. CML and pentosidine have only been found in the glomeruli of patients with type 1 diabetes and diabetic nephropathy (7,27). Because CML is increased in the circulation of children with type 1 diabetes who do not have microvascular complications, our observations suggest CML to be associated with the disease as such rather than related to microvascular complications, and furthermore, our findings are not in line with a singular local production of CML in the glomeruli of

patients with diabetic nephropathy, as was recently suggested (28).

In conclusion, we have demonstrated that serum levels of CML are increased in patients with type 1 diabetes. This increase precedes the development of micro- and macrovascular complications.

**Acknowledgments** — This work was supported by the Norwegian Research Council, J.E. Isberg's Foundation, The Norwegian Diabetes Association, The Novo Nordisk Foundation, and The Blix Family Foundation.

Dr. Ole J. Bjerrum is acknowledged for the synthesis of CML-BSA. For the generous gifts of anti-AGE antibodies and AGE-BSA, Dr. Richard Bucala of the Picower Institute for Medical Research, New York, is greatly acknowledged. We thank Turi Arnesen Siegwath for skillful technical assistance.

#### References

- Ahmed MU, Thorpe SR, Baynes JW: Identification of N-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *J Biol Chem* 261:4889-4894, 1986
- Brownlee M, Vlassara H, Cerami A: Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N Engl J Med* 318:1315-1321, 1988
- Makita Z, Vlassara H, Cerami A, Bucala R: Immunochemical detection of advanced glycosylation end products in vivo. *J Biol Chem* 267:5133-5138, 1992
- Vlassara H, Bucala R, Striker L: Pathogenic effects of advanced glycosylation: biochemical, biologic, and clinical implications for diabetes and aging. *Lab Invest* 70:138-151, 1998
- Nakamura Y, Horii Y, Nishino T, Shiiki H, Sakaguchi Y, Kagoshima T, Dohi K, Makita Z, Vlassara H, Bucala R: Immunohistochemical localization of advanced glycosylation endproducts (AGEs) in coronary atheroma and cardiac tissue in diabetes mellitus. *Am J Pathol* 143:1649-1656, 1993
- Schleicher ED, Wagner E, Nerlich AG: Increased accumulation of the glycoxidation product N-(carboxymethyl)lysine in human tissues in diabetes and aging. *J Clin Invest* 99:457-468, 1997
- Sell DR, Monnier VM: Structure elucidation of a senescence cross-link from human extracellular matrix. *J Biol Chem* 264:21597-21602, 1989
- Hayase F, Nagaraj H, Miyata S, Njoroge FG, Monnier V: Aging of proteins: immunological detection of a glucose-derived pyrrole formed during Maillard reaction in vivo. *J Biol Chem* 263:3758-3764, 1989
- Ienaga K, Kakita H, Hoshi T, Nakamura K, Nakazawa Y, Fukunaga Y, Aoki S, Hasegawa G, Tsutsumi Y, Kitagawa Y, Nakano K: Crossline-like structure accumulates as fluorescent advanced glycation end products in renal tissues of rats with diabetic nephropathy. *Proc Jpn Acad* 72:79-84, 1996
- Konishi Y, Hayase F, Kato H: Novel imidazolone compound formed by the advanced Maillard reaction of 3-deoxyglucosone and arginine residues in proteins. *Biosci Biotech Biochem* 58:1953-1955, 1994
- Dyer DG, Dunn JA, Thorpe SR, Bailie KE, Lyons TJ, McCance DR, Baynes JW: Accumulation of Maillard reaction products in skin collagen in diabetes and aging. *J Clin Invest* 91:2463-2469, 1993
- McCance DR, Dyer DG, Dunn JA, Bailie KE, Thorpe SR, Baynes JW, Lyons TJ: Maillard reaction products and their relation to complications in insulin-dependent diabetes mellitus. *J Clin Invest* 91:2470-2478, 1993
- Sugimoto K, Nishizawa Y, Horiuchi S, Yagihashi S: Localization in human diabetic peripheral nerve of N-carboxymethyllysine-protein adducts, an advanced glycation end-product. *Diabetologia* 40:1380-1387, 1997
- Murata T, Nagai R, Ishibashi T, Ikeda K, Horiuchi S: The relationship between accumulation of advanced glycation end products and expression of vascular endothelial growth factor in human diabetic retinas. *Diabetologia* 40:764-769, 1997
- Berg TJ, Dahl-Jørgensen K, Torjesen PA, Hanssen KF: Increased serum levels of advanced glycation end products (AGEs) in children and adolescents with IDDM. *Diabetes Care* 20:1006-1008, 1997
- Berg TJ, Bangstad H-J, Torjesen PA, Østerby R, Bucala R, Hanssen KF: Advanced glycation end products in serum predict changes in the kidney morphology of patients with insulin-dependent diabetes mellitus. *Metabolism* 46:661-665, 1997
- Makita Z, Vlassara H, Rayfield E, Cartwright K, Friedman E, Rodby R, Cerami A, Bucala R: Hemoglobin-AGE: a circulating marker of advanced glycosylation. *Science* 258:651-653, 1992
- Bucala R, Makita Z, Koschinsky T, Cerami A, Vlassara H: Lipid advanced glycosylation: pathway for lipid oxidation in vivo. *Proc Natl Acad Sci U S A* 90:6434-6438, 1993
- Beisswenger PJ, Makita Z, Curphey TJ, Moore LL, Jean S, Brinck-Johnsen T, Bucala R, Vlassara H: Formation of immunohistochemical advanced glycosylation end products precedes and correlates with early manifestations of renal and retinal disease in diabetes. *Diabetes* 44:824-829, 1995
- Nishino T, Horii Y, Shiiki H, Yamamoto H, Makita Z, Bucala R, Dohi K: Immunohistochemical detection of advanced glycosylation end products within the vascular lesions and glomeruli in diabetic nephropathy.

- thy. *Hum Pathol* 26:308–313, 1995
21. Wolfenbittel BHR, Giordano D, Founds HW, Bucala R: Long-term assessment of glucose control by haemoglobin-AGE measurement. *Lancet* 347:513–515, 1996
  22. Reddy S, Bichler J, Wells-Knecht KJ, Thorpe SR, Baynes JW: N-(Carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins. *Biochemistry* 34:10872–10878, 1995
  23. Ikeda K, Higashi T, Sano H, Jinnouchi Y, Yoshida M, Araki T, Ueda S, Horiuchi S: N-(Carboxymethyl)lysine protein adduct is a major immunological epitope in proteins modified with advanced glycation end products of the Maillard reaction. *Biochemistry* 35:8075–8083, 1996
  24. Clausen JT, Christensen M, Skovsted I, Mortensen SB, Bjerrum OJ, Wilken M: Antibodies against in vitro generated AGE: specificity and applicability to assays for in vivo AGE (Abstract). *Diabetologia* 40:A588, 1997
  25. Reddy S, Bichler J, Wells-Knecht KJ, Thorpe SR, Baynes JW: N-(Carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins. *Biochemistry* 34:10873–10878, 1995
  26. Degenhardt TP, Grass L, Reddy S, Thorpe SR, Diamandis EP, Baynes JW: The serum concentration of the advanced glycation end-product N-epsilon-(carboxymethyl)lysine is increased in uremia. *Kidney Int* 52:1064–1067, 1997
  27. Imai N, Nishi S, Suzuki Y, Karasawa R, Ueno M, Shimada H, Kawashima S, Nakamaru T, Miyakawa Y, Araki N, Horiuchi S, Gejyo F, Arakawa M: Histological localization of advanced glycosylation end products in the progression of diabetic nephropathy. *Nephron* 76:153–160, 1997
  28. Horie K, Miyata T, Maeda K, Miyata S, Sugiyama S, Sakai H, van Ypersele de Strihou C, Monnier V, Witztum JL, Kurokawa K: Immunohistochemical colocalization of glycoxidation products and lipid peroxidation products in diabetic renal glomerular lesions. *J Clin Invest* 100:2995–3004, 1997