

Role of N^{ϵ} -(Carboxymethyl)Lysine in the Development of Ischemic Heart Disease in Type 2 Diabetes Mellitus

Khaled A. Ahmed^{1,*}, Sekaran Muniandy¹, and Ikram S. Ismail²

¹Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

²Department of Medicine, University of Malaya Medical Center, University of Malaya, 50603 Kuala Lumpur, Malaysia

Received 20 November, 2006; Accepted 19 January, 2007

Summary This study aims to determine the levels of N^{ϵ} -(carboxymethyl)lysine (CML) in patients with Type 2 diabetic patients with and without ischemic heart disease (IHD) and to find for a possible association between circulating CML and a number of clinical parameters including lipids, hemoglobin A1c (HbA1c) and malondialdehyde (MDA) in Type 2 diabetic IHD patients. Serum CML levels were measured by enzyme-linked immunosorbent assay using polyclonal anti-CML antibodies. Serum levels of CML and MDA were assessed in 60 IHD patients with Type 2 diabetes, 43 IHD patients without Type 2 diabetes, 64 Type 2 diabetics without IHD, and 80 sex- and age-matched healthy subjects. Correlations studies between CML levels and lipids, HbA1c, and lipid peroxidation were performed in Type 2 diabetes patients with and without IHD. A statistical significance was observed in the levels of serum glucose, lipids (triglyceride, total cholesterol, HDL-cholesterol), MDA, HbA1c, CML and LDL-cholesterol ($p < 0.05$) between the groups of the study. CML levels were significantly increased in diabetic IHD patients compared with Type 2 diabetes patients but without IHD (537.1 ± 86.1 vs 449.7 ± 54.9 , $p < 0.001$). A positive correlation was observed between serum levels of CML and MDA, $r = 0.338$ ($p = 0.008$) in Type 2 diabetes patients with IHD. However, age, HbA1c and lipids had no significant influence on CML levels among diabetics ($p > 0.05$). In conclusion, this study demonstrates the effect of both diabetes and oxidative stress on the higher levels of circulating CML. These results showed that increased serum levels of CML are associated with the development of IHD in Type 2 diabetes mellitus.

Key Words: advanced glycation end products, N^{ϵ} -(carboxymethyl)lysine, Type 2 diabetes mellitus, ischemic heart disease, oxidative stress

Introduction

Diabetes mellitus (DM) represents a range of metabolic disorders characterized by hyperglycaemia resulting from insulin deficiency or insulin resistance or both. However,

it is well known established that in diabetes, long-term complications ensue from abnormal regulation of glucose metabolism. Type 2 (non-insulin dependent) diabetes mellitus, the most prevalent form of the disease, is associated with chronic macrovascular and microvascular complications and it imposes a significant burden for both the diabetic population and the health system. In macrovasculature, for example, diabetes-accelerated atherosclerosis leads to the development of heart attacks and stroke [1, 2]. In fact, all manifestations of cardiovascular disease, coronary heart disease (CHD),

*To whom correspondence should be addressed.

Tel: +603 7967 4717 Fax: +603 7967 4957

E-mail: khaaah@gmail.com

stroke and peripheral vascular disease are substantially more common in patients with Type 2 diabetes than in non-diabetic individuals [3, 4]. Patients with Type 2 (non-insulin dependent) diabetes mellitus (NIDDM) have a two- to fourfold increased risk of fatal and non-fatal coronary events [5]. CHD, another term given to ischemic heart disease (IHD), can ultimately lead to heart attack. However, the most common cause of death among individuals with diabetes mellitus is ischemic heart disease [6]. Hyperglycaemia, the primary clinical manifestation of diabetes, is strongly associated with development of the diabetic complications [7].

Chronic hyperglycemia results in short-term metabolic changes in lipid and protein metabolism and long-term irreversible vascular and connective tissue changes. These changes include diabetes-specific complications such as retinopathy, nephropathy and neuropathy and complications of the macrovasculature such as atherosclerosis [8]. Hyperglycemia drives non-enzymatic glycation and oxidation of proteins and lipids leading to enhancement the formation of advanced glycation end products (AGEs), which may be involved in the pathogenesis of diabetic vascular diseases. Recently, it has been documented that, among various factors, advanced glycation end products (AGEs), a heterogeneous group of irreversibly modified products formed in excess during aging and diabetes mellitus, play a crucial role in this process [9]. It has been reported that the process of AGE formation is accelerated by hyperglycemia [10, 11]. When accelerated by hyperglycemia, AGE accumulation is believed to contribute to the gradual development of diabetic complications. Accumulation of AGEs with structural alterations result in altered tissue properties that contribute to the reduced susceptibility to catabolism [9, 12]. Several interrelations have been shown between oxidative stress and AGEs. Reactive oxygen species generated in oxidative stress can in turn accelerate the AGE formation. Yan *et al.* [13] showed that interaction of AGEs with endothelial cells leads to oxidative stress by a receptor-mediated process. Therefore, new definition arose, such as glycooxidation which has been proposed by Baynes [14], which refers to AGE formation through an oxidative pathway.

Several advanced glycation end products structures have been structurally identified including *N*^ε-(carboxymethyl) lysine (CML) [15, 16], *N*^ε-(carboxyethyl)lysine (CEL) [17], crossline [18], pentosidine [19], pyralline [20], and imidazolone [21]. The two most commonly measured AGEs are CML and pentosidine, which are glycooxidation products, formed by sequential glycation and oxidation reactions [22]. CML modification of proteins is one of the major glycooxidation products formed *in vitro* by the reaction between glucose and protein [16]. Schleicher and co-workers [23] have shown by immunohistochemistry and chemical analysis that CML was found in atheromas. Furthermore, it is established that oxidation plays an important role in CML

formation [24]. CML, a well-characterized and major AGE structure, is known to be a product of both lipoxidation and glycooxidation [25]. Several *in vitro* experimental studies demonstrated that CML [26] is a major antigenic AGE structure *in vivo* and it seems to be generated through oxidative cleavage of Amadori products, cleavage of Schiff base and autoxidation of glucose. Among these, oxidative cleavage of Amadori products into CML is considered a major pathway *in vivo*. Because CML is a major product of oxidative modification of glycated proteins, it has been suggested to represent a general marker of oxidative stress and long-term damage of proteins in aging, atherosclerosis and diabetes [27].

As the global prevalence of diabetes increases, laboratory findings will continue to play a major role in the early diagnosis and subsequently better management of the diabetic vascular complications specifically ischemic heart disease. The aim of the study was to investigate the serum levels of CML in the IHD patients with and without Type 2 diabetes. Correlation between CML values with other clinical parameters including lipid profile tests, lipid peroxidation in term of malondialdehyde (MDA) and glycated hemoglobin A1c (HbA1c) were examined.

Methods

Study population

The study was performed on 60 patients with both Type 2 diabetes and IHD aged (43.7 ± 14.9 years), 43 patients with IHD but without Type 2 diabetes aged (48.4 ± 18.1 years), and 64 diabetics without IHD aged (45.2 ± 17.1 years). Smokers and former smokers were excluded from the study and none of the patients had retinopathy or nephropathy or other complications of diabetes. Subjects of age ≥ 70 years, known hepatic diseases, antioxidants medications, or insulin therapy were also excluded from this study. Furthermore, premenopausal women were not included since estrogens affect plasma lipids and susceptibility of LDL to oxidation [28]. Patient's age, blood pressure and diabetes duration were recorded. Healthy control subjects included in the present study consisted of 80 healthy controls aged 44.0 ± 14.0 years. The age- and sex-matched healthy control subjects were recruited from Klang Valley, Kuala Lumpur by the distribution of questionnaires. Samples were also collected from government bodies such as Police Force and from private centres in Kuala Lumpur. All the healthy control subjects were not diabetic and not on lipid lowering drugs. In addition, those individuals were free from any vascular diseases or even any increase in glucose levels or both. The exclusion criteria for patients were also applied for control group.

A 10 ml blood sample was collected from each participant in two tubes, 5 ml in EDTA (1 mg/ml) tube and 5 ml in anti-

coagulant-free tube. Of the 5 ml EDTA anticoagulated blood, 1 ml was kept for HbA1c analysis. Blood samples were centrifuged at 3000 g for 15 minutes at room temperature within 90 min after collection and the supernatant was stored at -80°C for MDA assay and -20°C for other assays [29]. The blood samples were taken in the morning between 8:00 and 9:00 a.m. after an overnight (12–14 hours) fast from each patient regularly attending the Cardiology and Endocrinology Clinics at the University of Malaya Medical Centre and from healthy control subjects. Written informed consent was obtained from all patients and control subjects prior to their inclusion in the study. The study was performed in accordance to the approval by the Faculty Perubatan University Malaya Medical Ethics Committee (FPUM MEC). The experimental protocol for anti-sera preparation was approved by the local ethics committee for animal experimentation in the Faculty of Medicine, University of Malaya.

Events definitions

Type 2 diabetes mellitus was defined according to World Health Organization diagnostic criteria [30]. The procedures used for evaluation of ischemic heart disease were previously described [31, 32]. Briefly, the clinical diagnosis of angina was based on a characteristic complaint of chest discomfort or chest pain occurring on exertion and relieved by rest. Confirmation was obtained by observing reversible ischemic changes on electrocardiograms (ECGs) during an attack or by giving a test dose of sublingual nitroglycerin that characteristically relieves the pain in 1 to 3 minutes.

Preparation and characterization of CML- and CEL-modified proteins

CML-bovine serum albumin (CML-BSA) and CML-hemoglobin (CML-Hb) were prepared as described previously [16, 26]. CEL-BSA and CEL-Hb were prepared according to the method of Ahmed *et al.* [17]. The preparations were dialyzed in phosphate buffer saline (PBS), pH 7.4 and filter sterilized by passing through 0.45- μm filters and protein concentrations were measured by the Bradford method [33]. CML- and CEL-modified proteins were characterized by agarose gel electrophoresis [34] using 0.1 M Tris and 0.038 M glycine buffer (pH 8.7). The degree of modification of lysine residues was quantitatively monitored by the trinitrobenzenesulfonic acid (TNBS) assay as previously described [35].

Production of polyclonal anti-CML antibodies

For preparation of polyclonal antibody against CML, CML-BSA (0.5 mg/ml) prepared as above was emulsified in an equal volume of 50% Freund's complete adjuvant in total volume of 2 ml and injected intramuscularly into multiple sites in female New Zealand white rabbit (2.5 kg weight).

This procedure was repeated at weekly intervals for 3 weeks. After a 2-week pause, the rabbit was received a booster injection with the same amount of immunogen emulsified in an equal volume of 50% Freund's incomplete adjuvant [36]. The presence of antibodies was monitored by Ouchterlony double diffusion in 1.0% agarose in PBS (pH 7.4). The antiserum showed a single precipitin line with CML-BSA upon a double immunoprecipitation analysis. The rabbit was then bled on the tenth day after the last booster injection and antiserum was obtained to be used in the enzyme-linked immunosorbent assay (ELISA) work.

Antiserum titer was determined by means of non-competitive ELISA using CML-BSA as coating antigen. Antiserum titer was defined as the serum dilution giving a 50% maximum absorbance (405 nm) signal employing horseradish peroxidase (HRP)-conjugated anti-rabbit IgG as secondary antibody [37]. The cross-reactivity of polyclonal anti-CML antiserum with several proteins (CEL-BSA, CEL-Hb, BSA, Hb) and competitors (CML-BSA, CML-Hb) was evaluated by competitive ELISA at room temperature. CML-BSA (used as adsorbed ligand antigen) and the proteins and competitors diluted with buffer B (0.02% Tween 20/PBS) into different concentrations (0.250, 0.500, 1.0, 5.0, 20, 50, 100 and 250 $\mu\text{g}/\text{ml}$), were used in the experiment. Each well of an immunoplate was incubated with 100 μl of CML-BSA (1.0 $\mu\text{g}/\text{ml}$) in 50 mM carbonate buffer (pH 9.6) at room temperature for 1 h. The wells were washed three times with buffer A (0.05% Tween 20/PBS) and blocked with 100 μl of 6% skimmed milk in PBS for 1 h with shaking followed by triple washing as above. Each protein or competitor to be tested (60 μl) was mixed with 60 μl of antiserum (final dilution 1:2000) for 1 h and the mixture (100 μl) was added to the wells of the microtiter plate followed by shaking for 1 h. The wells were washed again, and then 100 μl HRP-conjugated anti-rabbit IgG (0.1 $\mu\text{g}/\text{ml}$) was added to each well. After incubation for 1 h at room temperature, the wells were washed and color was developed after incubation with 50 μl substrate solution containing 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid (ABTS). The wells were incubated for 20–30 min at room temperature in the dark followed by addition of 100 μl of 1.0 N H_2SO_4 to stop the reaction. The absorbance at 405 nm was read using an ELISA reader (MRX Microplate Reader, Dynatech Laboratories Ltd, UK).

CML measurement by competitive ELISA

CML in the serum of the patients and healthy control subjects were measured by competitive ELISA as described above except that after incubation of the wells with the blocking solution (6% skimmed milk), 60 μl serum to be tested or CML-BSA standard with serial dilutions (0.100, 0.250, 0.500, 0.750, 1.0, 2.5, 5.0 and 7.5 $\mu\text{g}/\text{ml}$) were mixed with an equal volume of antiserum (final dilution 1:2000)

for 1 h and the mixture (100 μ l) was added to the wells and the microtiter plate was shaken for 1 h. The remaining steps were performed in the same manner to that described above and the CML concentrations in the samples were determined from the standard curve. The CML concentration was expressed as ng CML/ml. All ELISA work was performed in duplicates.

Detection of malondialdehyde

The amount of plasma malondialdehyde (MDA), an index of lipid peroxidation, was determined by the thiobarbituric acid reactive substances (TBARS) method as described by Ohkawa *et al.* [38]. Briefly, 100 μ l plasma or standard was combined with an equal volume of 8.1% sodium dodecyl sulfate. Then, 2.5 ml TBA/buffer (prepared by dissolving of 0.53% thiobarbituric acid in 20% acetic acid as adjusted to pH 3.5 with NaOH) were added to the reaction mixture. The tubes were covered with caps and incubated at 95°C for 60 min. The reaction was stopped by placing tubes on ice followed by centrifugation at 4000 rpm for 10 min. The optical density of pink color developed in the supernatant was measured against distilled water as blank at 532 nm. The measurements were performed in duplicates and the concentration of plasma MDA was expressed in μ mol/l. MDA standards (1.25, 2.5, 5, 8 and 10 μ mol/l) were prepared from acid hydrolysis of 1,1,3,3-tetraethoxypropane (TEP) according to the method of Tsaknis and co-workers with modifications [39].

Other measurements

Glucose levels were measured by using the hexokinase-glucose-6-phosphate dehydrogenase UV-method of Kunst *et al.* [40]. HDL cholesterol, triglyceride and total cholesterol levels were measured by enzymatic methods using Dimension Clinical Chemistry System (Dade Behring Inc., Newark, DE). LDL cholesterol was calculated with the Friedewald formula [41]. Percent HbA1c was determined by COBAS INTEGRA systems (Roche diagnostics, F. Hoffmann-La Roche Ltd, Basel, Switzerland) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as the mean \pm standard deviation (SD). Chi-square (X^2) test was used for the analysis of categorical variables. Differences among the four groups (Type 2 diabetes with IHD, IHD without Type 2 diabetes, Type 2 diabetes without IHD, and healthy control subjects) were examined by one-way analysis of variance (ANOVA). If analysis of variance indicated that $p < 0.05$, Bonferroni's Post Hoc test was applied to adjust the multiple comparisons. Coefficients of correlation of CML with other parameters of Type 2 diabetes including serum glucose, diabetes duration, LDL-cholesterol, and MDA were calculated using Pearson's correlation analysis. Statistical computations were calculated

using SPSS 11.5 for Windows software (SPSS Inc, Chicago, IL).

Results

Characterization of CML- and CEL-modified proteins

Agarose gel electrophoresis was used to evaluate the purity of the modified proteins employing 1.5% agarose in Tris-glycine buffer. Figure 1 showed that CML- and CEL-glycated proteins preparations revealed a faster anodic migration than native BSA or Hb, indicating that significant glycation had occurred. Quantitatively, the modified proteins were subjected to TNBS assay to estimate the amount of glycation. The degree of glycation was calculated from the reduction of absorbance compared with native proteins and expressed as percentage of modification. The percentages of lysine residues modifications in CML-BSA, CEL-BSA, CML-Hb, and CEL-Hb CML- and CEL-glycated proteins versus total lysine residues in the native proteins are 66, 48, 62, and 39%, respectively.

Preparation of polyclonal anti-CML antibodies and characterization by ELISA

To develop an antiserum that would selectively and specifically recognize CML adducts, but not epitopes of native proteins, the rabbit was immunized with CML-BSA. The anti-CML-BSA titer was determined to be greater than 36000 in a non-competitive ELISA. The specificity of the antiserum was tested by a competitive ELISA and results are shown in Fig. 2. CML-BSA and CML-Hb were highly effective competitors and significantly reacted with anti-CML

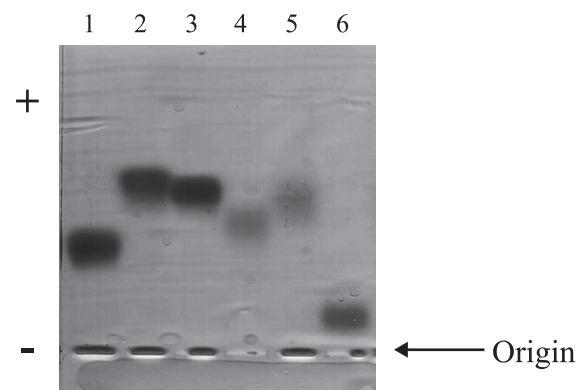


Fig. 1. Agarose gel electrophoresis of glycated and nonglycated proteins. Native BSA (lane 1), CML-BSA (lane 2), CEL-BSA (lane 3), CEL-Hb (lane 4), CML-Hb (lane 5) and Hb (lane 6). Samples in loading buffer (bromophenol blue, 1.5 M Tris and 20% glycerol, pH 9.2) were loaded into agarose gel (1.5%) in 0.1 M Tris and 0.038 M glycine buffer (pH 8.7). The gel tray was run for 40–60 min at 100 V. The gel was stained with 1% Coomassie blue (1 h) followed by overnight destaining.

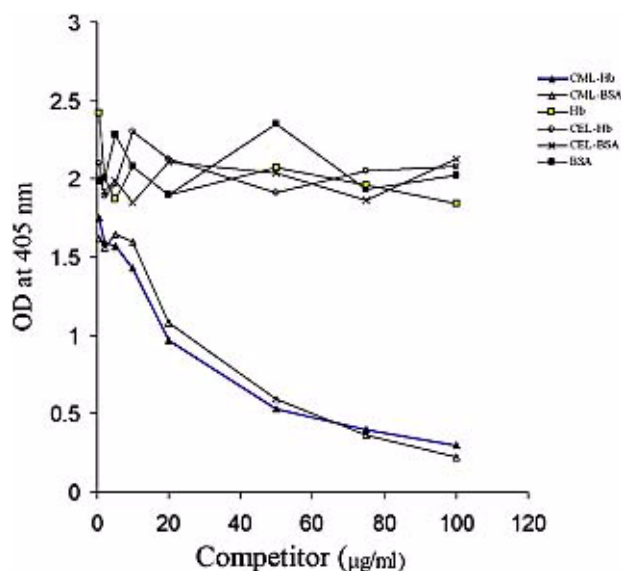


Fig. 2. Immunoreactivity of the polyclonal anti-CML antibody to CML-proteins. The immunoreactivity of the polyclonal anti-CML antiserum was determined by competitive ELISA. The microplate wells were coated with 100 μ l of CML-BSA (1.0 μ g/ml) in 50 mM carbonate buffer (pH 9.6) at room temperature for 1 h. Wells were washed three times with buffer A and blocked with 6% skimmed milk for 1 h followed by triple washing. Each protein or competitor to be tested with different concentrations (60 μ l) was mixed with equal volume of antiserum (final dilution 1:2000) for 1 h and a portion (100 μ l) of the mixture was then added to the wells followed by incubation for 1 h. After triple washing, HRP-conjugated anti-rabbit IgG antibody (0.1 μ g/ml) was used to detect the antibodies bound to wells as described in the methods section.

antibodies, whereas the native (BSA, Hb) and CEL-modified proteins (CEL-BSA, CEL-Hb) were not recognized by the antibody. Thus, this monospecific antiserum is specifically directed to the CML epitope, able to recognize it on any of a variety of different proteins.

Clinical characteristics of study populations

Table 1 shows the clinical characteristics of the study groups. The study populations were well matched for age and gender with their respective control groups. The mean ages in these groups, healthy control subjects, Type 2 diabetes patients with IHD, IHD patients without Type 2 diabetes, and Type 2 diabetics without IHD were 44.0 ± 14.0 , 43.7 ± 14.9 , 48.4 ± 18.1 , and 45.2 ± 17.1 years respectively and the age difference between these groups was not significant ($p = 0.48$). The four groups were comparable with respect to gender as tested by Chi-square ($p = 0.56$). When the four groups were compared, a statistical significance was observed in the levels of serum fasting blood glucose, lipids (triglyceride, total cholesterol, HDL-cholesterol), MDA, HbA1c and CML, and to a lesser extent in LDL-cholesterol. Fig. 3 shows that CML levels were markedly higher in diabetics with IHD when compared to other groups of the study. There was no significant difference in systolic blood pressure and diastolic blood pressure among the study groups. For all variables in the study groups that showed statistical significance by analysis of variance, post-hoc comparisons of means were made using the Bonferroni test. Clinical parameters were also compared between diabetic patients (Type 2 diabetes with and without IHD) groups. The durations of diabetes in the Type 2 diabetics with IHD and without IHD were 9.3 ± 4.0 and 9.9 ± 5.0 years respectively.

Table 1. Clinical and laboratory characteristics of the study groups

	Healthy controls	Diabetes with IHD	IHD without diabetes	Diabetes without IHD	<i>p</i> value*
Sex (Male/Female)	80 (36/44)	60 (32/28)	43 (22/21)	64 (29/35)	NS
Age (years)	44.0 ± 14.0	43.7 ± 14.9	48.4 ± 18.1	45.2 ± 17.1	NS
Systolic blood pressure (mmHg)	123.8 ± 18.1	131.5 ± 19.8	125.4 ± 21.1	123.6 ± 19.8	NS
Diastolic blood pressure (mmHg)	77.6 ± 9.5	77.8 ± 8.0	78.4 ± 7.6	77.4 ± 7.7	NS
Fasting blood glucose (mmol/l)	4.94 ± 0.74	7.88 ± 3.23	5.25 ± 0.96	7.02 ± 2.09	<0.001
HbA1c (%)	5.80 ± 0.69	7.00 ± 1.28	6.06 ± 0.96	6.58 ± 1.50	<0.001
CML (ng/ml)	416.6 ± 71.5	$537.1 \pm 86.1^{**}$	410.0 ± 76.3	449.7 ± 54.9	<0.001
MDA (μ mol/l)	1.80 ± 0.50	$2.73 \pm 1.01^{**}$	2.19 ± 0.65	2.21 ± 0.72	<0.001
Triglyceride (mmol/l)	1.34 ± 0.83	1.81 ± 1.23	1.23 ± 0.57	1.28 ± 0.75	0.002
Total cholesterol (mmol/l) [†]	5.42 ± 0.86	4.81 ± 0.89	5.06 ± 1.00	5.02 ± 0.87	0.001
HDL-Cholesterol (mmol/l) [†]	1.24 ± 0.38	0.99 ± 0.34	1.05 ± 0.36	1.06 ± 0.37	<0.001
LDL-Cholesterol (mmol/l) [†]	3.58 ± 0.76	3.14 ± 0.84	3.45 ± 0.98	3.42 ± 0.86	0.028

ANOVA followed by Bonferroni-corrected repeated pair comparisons were used for statistical analysis; values are mean \pm SD. *Significantly different between groups if $p < 0.05$. NS; the difference is not significant. ** $p < 0.005$ vs Diabetes without IHD, IHD without diabetes, and Healthy subjects. [†]Lower values in patients than healthy controls due to using lipid-lowering drugs.

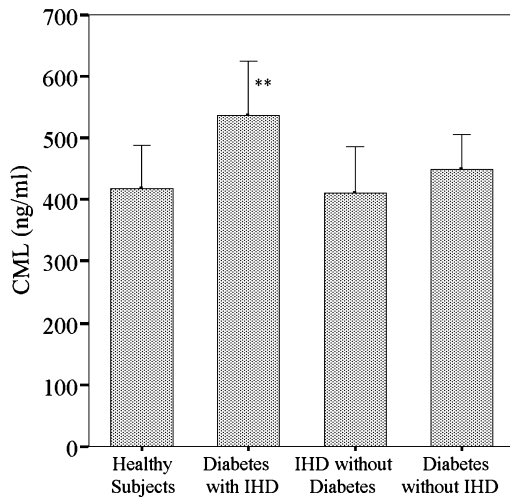


Fig. 3. Concentrations of *N*^ε-(carboxymethyl)lysine (CML) in the study groups. Data represent means \pm SD. ** $p < 0.001$ versus healthy controls, IHD patients without Type 2 diabetes, and Type 2 diabetics without IHD.

Although triglyceride levels were significantly higher in Type 2 diabetes patients with IHD than diabetics without IHD ($p = 0.007$), all lipid profiles including total cholesterol, HDL-cholesterol and LDL-cholesterol were not significant. The difference in the fasting blood glucose levels was not significant between diabetic patients groups. However, patients with Type 2 diabetes and IHD had significantly higher levels of MDA and CML levels than diabetic patients without IHD; 2.73 ± 1.01 vs 2.21 ± 0.72 , $p = 0.001$ and 537.1 ± 86.1 vs 449.7 ± 54.9 , $p < 0.001$, respectively (Table 1). Correlation studies showed that serum CML levels in Type 2 diabetes with IHD were positively and significantly correlated with

MDA ($r = 0.338$, $p = 0.008$) (Fig. 4A) and such correlations were not found between the serum levels of CML and age ($r = 0.168$, $p = 0.19$), HbA1c ($r = 0.067$, $p = 0.61$) and LDL-cholesterol ($r = -0.097$, $p = 0.46$). However, MDA was not correlated with the CML concentrations in Type 2 diabetes but without IHD ($r = 0.035$, $p = 0.78$) (Fig. 4B).

Discussion

It is well established that diabetes is a serious and costly disease that is becoming increasingly common in many countries and the level of mortality and morbidity from coronary heart disease is higher in diabetic patients than nondiabetic individuals [42]. Furthermore, it is well known that diabetes is associated with silent ischemia [43, 44]. This fact should be taken into account to develop a suitable determinant for the early detection of these complications and subsequently reduce the adverse effect of diabetes. Indeed, it is critical that the focus of the development of new markers targeting the prediction and early detection of macrovascular complications, specifically IHD, in Type 2 diabetes remains an important research and clinical objectives. Investigations have shown that serum AGEs concentrations were observed in Type 2 diabetes with coronary artery disease [45, 46] but these studies failed to show the importance of the glycoxidation product, *N*^ε-(carboxymethyl)lysine, in the development of heart diseases in the respective groups of the studies. In fact, the data regarding the importance of CML in the development of IHD in patients with diabetes are scarce so far.

The results of this study demonstrates that both CML and MDA were significantly elevated in Type 2 diabetes mellitus accompanied with ischemic heart disease when compared to

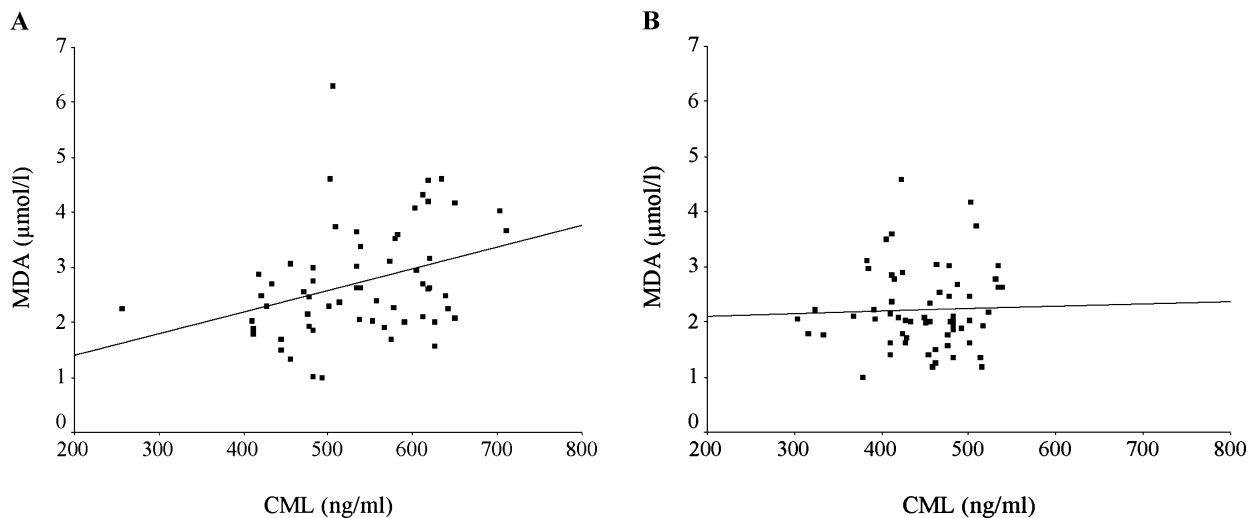


Fig. 4. Correlation between CML and MDA in the two diabetic groups. CML levels were significantly correlated with MDA ($r = 0.338$, $p = 0.008$) in diabetic IHD patients (A) but not in Type 2 diabetes patients without IHD (B) ($r = 0.035$, $p = 0.78$).

that of Type 2 diabetes mellitus without IHD, healthy control subjects, or IHD patients without diabetes. Furthermore, MDA levels showed that the significant difference between means in age- and sex-matched healthy control subjects and nondiabetic IHD patients ($p = 0.035$) was seen but to a much lesser extent in comparison of the Type 2 diabetes with IHD group with either healthy individuals, diabetics without IHD, or nondiabetic IHD patients. The findings reported in this study explain that this slight increase in MDA concentrations in the patients with IHD alone may be derived from oxidative stress, imbalance between antioxidants defence mechanisms and oxidants, rather than carbohydrates or hyperglycemia. Our results are in a good agreement with the previous investigation that formation of MDA on LDL, an end product of lipid peroxidation, may indicate that the circulating MDA-LDL level is increased in coronary artery disease (CAD) compared with the levels in the patients without CAD [47].

Recently, it has been shown that CML may be formed by the copper-ion catalyzed oxidation of LDL in presence of glucose and CML was not detected in LDL incubated with copper ion alone indicating the synergetic effect of glucose on lipid peroxidation and subsequently accumulation of glycoxidized LDL in the macrophage- derived foam cells [48].

Bivariate correlation studies show that CML levels in Type 2 diabetic patients with IHD were positively and significantly affected by MDA levels and these correlations were not seen in Type 2 diabetes patients without IHD, indicating that the extent of LDL-cholesterol oxidation plays a crucial role in the formation and subsequent increase in CML levels in Type 2 diabetes accompanied by macrovascular complications including IHD. With regard to lipids and lipoproteins, it has been demonstrated that the lack relationship between CML and LDL-cholesterol and other lipid profile (data not shown) was observed among diabetics. The main reason for these unusual findings is that most of these patients are on lipid-lowering drugs. Since it has been established that CML levels are increased in the serum with aging [36], our findings exclude the effect of age on CML levels in Type 2 diabetic patients with IHD as explained by correlation analysis ($r = 0.05$, $p = 0.69$).

In conclusion, our study showed a significant association between serum CML levels and Type 2 diabetic complications, especially ischemic heart disease. These findings reported in the present study show that raised CML levels reflect the enhanced oxidative stress due to higher blood glucose levels in diabetic IHD patients and may be used as a useful independent biomarker for the ischemic heart disease resulting from Type 2 diabetes mellitus.

Acknowledgments

This study was financially supported by Intensive Research in Priorities Area (IRPA), Ministry of Science and Technology, Malaysia. (Project grant IRPA No. 36-02-03-6020).

Abbreviations

ABTS, 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid; AGEs, Advanced glycation end products; ANOVA, Analysis of variance; BSA, Bovine serum albumin; CAD, Coronary artery disease; CEL, *N*^ε-(Carboxyethyl)lysine; CHD, Coronary heart disease; CML, *N*^ε-(Carboxymethyl)lysine; ELISA, Enzyme-linked immunosorbent assay; HbA1c, Hemoglobin A1c; HDL, High density lipoprotein; HRP, Horseradish peroxidase; IHD, Ischemic heart disease; LDL, Low density lipoprotein; MDA, Malondialdehyde; NIDDM, Non-insulin-dependent diabetes mellitus; PBS, Phosphate buffer saline; TBARS, Thiobarbituric acid reactive substances; TEP, 1,1,3,3-tetraethoxypropane; TNBS, Trinitrobenzenesulfonic acid.

References

- [1] Schwartz, C.J., Valente, A.J., Sprague, E.A., Kelley, J.L., Cayatte, A.J., and Rozek, M.M.: Pathogenesis of the atherosclerotic lesion: Implications for diabetes mellitus. *Diabetes Care*, **15**, 1156–1160, 1992.
- [2] Manson, J.E., Colditz, G.A., Stampfer, M.J., Willett, W.C., Krolewski, A.S., Rosner, B., Arky, R.A., Speizer, F.E., and Hennekens, C.H.: A prospective study of maturity-onset diabetes mellitus and risk of coronary heart disease and stroke in women. *Arch. Intern. Med.*, **151**, 1141–1147, 1991.
- [3] Laakso, M. and Lehto, S.: Epidemiology of macrovascular disease in diabetes. *Diabetes Res.*, **5**, 294–315, 1997.
- [4] Haffner, S.M., Lehto, S., Rönneaa, T., Pyörälä, K., and Laakso, M.: Mortality from coronary heart disease in subjects with Type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N. Engl. J. Med.*, **339**, 229–234, 1998.
- [5] Pyörälä, K., Laakso, M., and Uusitupa, M.: Diabetes and atherosclerosis: an epidemiologic view. *Diabetes Metab. Rev.*, **3**, 463–524, 1987.
- [6] Gu, K., Cowie, C.C., and Harris, M.I.: Mortality in adults with and without diabetes in a national cohort of the U.S. population, 1971–1993. *Diabetes Care*, **21**, 1138–1145, 1998.
- [7] Assal, J.P.: Hyperglycemia and diabetic complications. *Schweizerische Medizinische Wochenschrift*, **109**, 1791–1801, 1979.
- [8] Hudson, B.J., Hofmann, M.A., Bucciarelli, L., Wendt, T., Moser, B., Yan, L., Wu, Q., Stern, D.M., D'Agati, V., Yan, S.D., Yan, S.F., Grant, P.J., and Schmidt, A.M.: Glycation and diabetes: The RAGE connection. *Current Science*, **83**, 1515–1521, 2002.

- [9] Wautier, J.L. and Guillausseau, P.J.: Advanced glycation end products, their receptors and diabetic angiopathy. *Diabetes Metab.*, **27**, 535–542, 2001.
- [10] Dyer, D.G.: Accumulation of Maillard reaction products in skin collagen in diabetes and aging. *J. Clin. Invest.*, **91**, 2463–2469, 1993.
- [11] Price, D.L., Rhett, P.M., Thorpe, S.R., and Baynes J.W.: Chelating activity of advanced glycation end-product inhibitors. *J. Biol. Chem.*, **276**, 48967–48972, 2001.
- [12] Monnier, V.M., Kohn, R.R., and Cerami, A.: Accelerated age-related browning of human collagen in diabetes mellitus. *Proc. Nat. Acad. Sci. USA*, **81**, 583–587, 1984.
- [13] Yan, S.D., Schmidt, A.M., Anderson, G.M., Zhang, J., Brett, J., Zou, Y.S., Pinsky, D., and Stern, D.: Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J. Biol. Chem.*, **269**, 9889–9897, 1994.
- [14] Baynes, J.W.: Role of oxidative stress in development of complications in diabetes. *Diabetes*, **40**, 405–412, 1991.
- [15] Ahmed, M.U., Thorpe, S.R., and Baynes, J.W.: Identification of N^ε-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *J. Biol. Chem.*, **261**, 4889–4894, 1986.
- [16] Reddy, S., Bichler, J., Wells-Knecht, K.J., Thorpe, S.R., and Baynes, J.W.: N^ε-(carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins. *Biochemistry*, **34**, 10872–10878, 1995.
- [17] Ahmed, M.U., Brinkmann, F.E., Degenhardt, T.P., Thorpe, S.R., and Baynes, J.W.: N^ε-(carboxyethyl)lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. *Biochem. J.*, **324**, 565–570, 1997.
- [18] Ienaga, K., Nakamura, K., Hochi, T., Nakazawa, Y., Fukunaga, Y., Kakita, H., and Nakano, K.: Crosslines, fluorophores in the AGE-related crosslinked proteins. *Contributions Nephrol.*, **112**, 42–51, 1995.
- [19] Sell, D.R. and Monnier, V.M.: Structure elucidation of a senescence cross-link from human extracellular matrix: Implication of pentoses in the aging process. *J. Biol. Chem.*, **264**, 21597–21602, 1989.
- [20] Njoroge, F.G., Sayre, L.M., and Monnier, V.M.: Detection of glucose-derived pyrrole compounds during Maillard reaction under physiological conditions. *Carbohydr. Res.*, **167**, 211–220, 1987.
- [21] Niwa, T., Katsuzaki, T., Miyazaki, S., Miyazaki, T., Ishizaki, Y., Hayase, F., Tatemichi, N., and Takei, Y.: Immunohistochemical detection of imidazolone, a novel advanced glycation end product, in kidneys and aortas of diabetic patients. *J. Clin. Invest.*, **99**, 1272–1280, 1997.
- [22] Booth, A., Khalifah, R.G., Todd, P., and Hudson, B.G.: *In vitro* kinetic studies of formation of antigenic advanced glycation end products (AGEs). *J. Biol. Chem.*, **272**, 5430–5437, 1997.
- [23] Schleicher, E.D., Wagner, E., and Nerlich, A.G.: Increased accumulation of the glycoxidation product N^ε-(carboxymethyl) lysine in human tissues in diabetes and ageing. *J. Clin. Invest.*, **99**, 457–468, 1997.
- [24] Nagai, R., Ikeda, K., Higashi, T., Sano, H., Jinnouchi, Y., Araki, T., and Horiuchi, S.: Hydroxyl radical mediates N^ε-(carboxymethyl)lysine formation from Amadori product. *Biochem. Biophys. Res. Comm.*, **234**, 167–172, 1997.
- [25] Fu, M.X., Requena, J.R., Jenkins, A.J., Lyons, T.J., Baynes, J.W., and Thorpe, S.R.: The advanced glycation end product, N^ε-(carboxymethyl)lysine is a product of both lipid peroxidation and glycoxidation reactions. *J. Biol. Chem.*, **271**, 9982–9986, 1996.
- [26] Ikeda, K., Higash, T., Sano, H., Jinnouchi, Y., Yoshida, M., Araki, T., Ueda, S. and Horiuchi, S.: N^ε-(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.
- [27] Ledl, F. and Schleicher, E.: The Maillard reaction in food and in human body, new results in chemistry, biochemistry and medicine. *Angew. Chem. Int. Ed. Engl.*, **29**, 565–706, 1999.
- [28] Maziere, C., Auclair, M., Ronveaux, M.F., Salmon, S., Santus, R., and Maziere, J.C.: Estrogens inhibit copper and cell-mediated modification of low density lipoprotein. *Atherosclerosis*, **89**, 175–182, 1991.
- [29] Holvoet, P., Stassen, J.M., Cleemput, J.V., Collen, D., and Vanhaecke, J.: Oxidized low density lipoproteins in patients with transplant-associated coronary artery disease. *Arterioscler. Thromb. Vasc. Biol.*, **18**, 100–107, 1998.
- [30] World Health Organization, WHO Study Group on Diabetes Mellitus, Geneva, World Health Org., (Tech. Rep. Ser., no. 727), 1985.
- [31] Dagenais, G.R., Robitaille, N.M., Lupien, P.J., Christen, A., Gingras, S., Moorjani, S., Meyer, F., and Rochon, J.: First coronary heart disease event rates in relation to major risk factors: Quebec cardiovascular study. *Can. J. Cardiol.*, **6**, 274–280, 1990.
- [32] Lamarche, B., Després, J.P., Moorjani, S., Cantin, B., Dagenais, G.R., and Lupien, P.J.: Prevalence of dyslipidemic phenotypes in ischemic heart disease (prospective results from the Québec Cardiovascular Study). *Am. J. Cardiol.*, **75**, 1189–1195, 1995.
- [33] Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254, 1976.
- [34] Bjerrum, O.J., Ingild, A., Løwenstein, H., and Weeke, B.: Quantitation of human IgG by rocket immunoelectrophoresis at pH 5 by use of carbamylated antibodies. A routine laboratory method. *Clin. Chem. Acta*, **46**, 337–343, 1973.
- [35] Habeeb, A.F.: Determination of free amino groups in proteins by trinitrobenzene-sulfonic acid. *Anal. Biochem.*, **14**, 328–336, 1966.
- [36] Hamelin, M., Borot-Laloi, C., Friguet, B., and Bakala, H.: Increased level of glycoxidation product N^ε-(carboxymethyl) lysine in rat serum and urine proteins with aging: Link with glycoxidative damage accumulation in kidney. *Arch. Biochem. Biophys.*, **411**, 215–222, 2003.
- [37] Makita, Z., Vlassara, H., Cerami, A., and Bucala, R.: Immunochemical detection of advanced glycosylation end products *in vivo*. *J. Biol. Chem.*, **267**, 5133–5138, 1992.
- [38] Ohkawa, H., Ohishi, N., and Yagi, K.: Assay for lipid

- peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.*, **95**, 351–358, 1979.
- [39] Tsaknis, J, Lalas, S., Tychopoulos, V., Hole, M., and Smith, G.: Rapid high-performance liquid chromatographic method of determining malondialdehyde for evaluation of rancidity in edible oils. *Analyst*, **123**, 325–327, 1998.
- [40] Kunst, A., Draeger, B., and Ziegenhorn, J.: UV-method with hixokinase and glucose-6-phosphate dehydrogenase, in *Methods of Enzymatic Analysis*, ed. By Bergmeyer H.U., Vol 4, Verlag Chemie, Deerfield, FL, 3rd Ed., pp. 163–172, 1983.
- [41] Friedewald, W.T., Levy, R.I., and Fredrickson, D.S.: Estimation of the concentration of low density lipoprotein cholesterol in plasma without use of the preparative ultracentrifuge. *Clinic. Chem.*, **18**, 499–502, 1972.
- [42] Bierman, E.L.: George lyman duff memorial lecture: atherogenesis in diabetes. *Arterioscler. Thromb. Vasc. Biol.*, **12**, 647–656, 1992.
- [43] Nesto, R.W.: Screening for asymptomatic coronary artery disease in diabetes. *Diabetes Care*, **22**, 1393–1395, 1999.
- [44] Paillole, C., Ruiz, J., Juliard, J.M., Leblanc, H., Gourgon, R., and Passa, P.: Detection of coronary artery disease in diabetic patients. *Diabetologia*, **38**, 726–731, 1995.
- [45] Kilhovd, B.K., Berg, T.J., Birkeland, K.I., Thorsby, P., and Hanssen, K.F.: Serum levels of advanced glycation end products are increased in patients with Type 2 diabetes and coronary heart disease. *Diabetes Care*, **22**, 1543–1548, 1999.
- [46] Kiuchi, K., Nejima, J., Takano, T., Ohta, M., and Hashimoto, H.: Increased serum concentrations of advanced glycation end products: a marker of coronary artery disease activity in Type 2 diabetic patients. *Heart*, **85**, 87–91, 2001.
- [47] Tanaga, K., Bujo, H., Inoue, M., Mikami, K., Kotani, K., Takahashi, K., Kanno, T., and Saito, Y.: Increased circulating malondialdehyde-modified LDL levels in patients with coronary artery diseases and their association with peak sizes of LDL particles. *Arterioscler. Thromb. Vasc. Biol.*, **22**, 662–666, 2002.
- [48] Imanaga, Y., Sakata, N., Takebayashi, S., Matsunaga, A., Sasaki, J., Arakawa, K., Nagai, R., Horiuchi, S., Itabe, H., and Takano, T.: *In vivo* and *in vitro* evidence for the glycooxidation of low density lipoprotein in human atherosclerotic plaques. *Atherosclerosis*, **150**, 343–355, 2000.