ORIGINAL ARTICLE

The Level of N-Carboxymethyllysine and C-Reactive Protein in Type 2 Diabetes Mellitus and it's Association with HbA1c in Diabetic Nephropathy

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ABSTRACT

Introduction: N-Carboxymethyllysine (CML) is involved in diabetic nephropathy (DN) via production of oxidative stress, growth factors and cytokines. C-reactive protein (CRP) is an inflammatory marker associated with diabetes risk. This study is to determine the level of serum CML and CRP in Type 2 diabetes mellitus (T2DM) patients and healthy subjects and to determine the correlation between CML and CRP with glycated haemoglobin (HbA1c) in T2DM patients. **Methods:** This is a case-control study on 73 T2DM patients without nephropathy, 74 T2DM patients with nephropathy and 73 healthy subjects, aged from 18 to 65 years old. Fasting venous blood was taken and analysed for CML, CRP, HbA1c, and creatinine. The comparisons of serum CML and CRP among the three groups and the correlation between CML and CRP with HbA1c (in T2DM patients) were determined. **Results:** The differences in CML [median (Interquartile Range) (IQR)] between healthy subjects [131.80 (73.56) ng/ml] and T2DM patients without nephropathy [188.80 (55.95) ng/ml]; between healthy subjects and T2DM patients with nephropathy [237.70 (439.04) ng/ml] were statistically significant (P<0.001). The differences in CRP [median (IQR)] between healthy subjects [1.64 (1.91) ng/ml] and T2DM patients without nephropathy [2.15 (5.64) ng/ml]; between healthy subjects and T2DM patients with nephropathy [4.75 (6.91) ng/ml] were statistically significant (P<0.001). Logistic regression showed CML and CRP are independent predictors of diabetic groups. There was no correlation between HbA1c with CML and CRP in T2DM groups. **Conclusion:** Since serum CML and CRP are independent predictors of DN, their levels can be used to identify high-risk diabetic patients prone to developing DN.
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Keywords: Carboxymethyllysine, C-reactive protein, HbA1c, Type 2 diabetes mellitus, Diabetic nephropathy

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INTRODUCTION

Chronic hyperglycaemia in Type 2 diabetes mellitus (T2DM) produces oxidative stress, aberrant glycosylation, lipid peroxidation, and inflammatory components, which are mediated by cytokines and growth factors (1). This hyperglycaemia is associated with the production of advanced glycation end products (AGEs) through a non-enzymatic Maillard reaction between amino groups from the protein and carbonyl groups from the glucose (2, 3). AGEs that are produced after protein glycation play an essential role in the pathogenesis of diabetic complications such as nephropathy (4, 5), retinopathy, and neuropathy (2, 6). An AGE can provide a strong and independent predictor for microvascular complications, such as nephropathy in T2DM patients (7). Diabetic nephropathy (DN) is defined by increased urinary albumin excretion in the absence of other renal diseases, affecting 40% of diabetic patients and is the leading cause of kidney disease requiring renal replacement therapy (8).

N-carboxymethyllysine (CML) is one of the AGEs.

Enhanced CML was found to accumulate in the mesangial matrix and thickened glomerular capillary wall of advanced DN cases but not in normal kidneys (9). The mechanism of AGE accumulation in DN is complicated, either due to the increased formation or decreased removal of AGE-modified proteins (10). An early study by Berg et al. showed that serum AGE level was correlated to kidney histology and can predict the progression of kidney damage (11). Hirata and Kubo also found that serum CML levels in chronic kidney disease (CKD) patients were higher than in diabetic patients with and without proteinuria, with greater levels in end-stage kidney disease (12). Due to the high level of CML and its correlation with the severity of DN, it has been reported that CML is one of the predictors for DN (13).

C-reactive protein (CRP) is a pentameric, acute-phase reactant protein synthesised by the liver (14). CRP is a general marker of systemic inflammation and is associated with the risk of diabetes (15, 16). Compared to other inflammatory markers, CRP is the most sensitive marker for systemic inflammation and the most studied marker associated with an increased risk of T2DM (17). High cost, limited availability, and lack of standardisation may limit the clinical use of other inflammatory markers. Although the exact role of CRP in diabetes is unclear, CRP has been shown to enhance the development of diabetes, as diabetes risk was found to be significantly higher in haplotype with high serum CRP levels compared with the most common haplotype (18). CRP has also been shown to be associated with DN, as inflammation is involved in its development (19). Besides that, CRP was found to be positively correlated with CML in diabetic with decreased glomerular filtration rate (GFR) (20).

Glycated haemoglobin (HbA1c) is widely used as a marker of glycaemic index worldwide. As hyperglycaemia contributes to CML formation and increases in CRP, HbA1c levels are postulated to show a correlation with serum CML and CRP. Galler et al. showed a correlation between serum levels of fluorescent AGE and HbA1c (21). Another study demonstrated that in adults with diabetes, a higher HbA1c is associated with a greater likelihood of higher CRP (22). Studies have proven that HbA1c can be used as an early predictor of DN, but it has shortcomings in that the measurement of average blood glucose is only for a relatively short period (120 days) and it is unable to reflect the pathways that produce complications. As a product of early chemical reaction, HbA1c is also unable to identify more complex advanced glycation/oxidation products that induce vascular damage (23).

This study was conducted to determine the level of serum CML and CRP among the T2DM with nephropathy, T2DM without nephropathy and in healthy subjects. This study also investigates the correlation between HbA1c with the level of serum CML and CRP in T2DM patients with and without nephropathy.

MATERIALS AND METHODS

Study Design and Subjects

A case-control study was conducted at the Diabetic Clinic, Hospital Universiti Sains Malaysia Kelantan, Malaysia from June 2017 to June 2018. The study participants were divided into three groups: healthy subjects, T2DM patients with nephropathy and T2DM patients without nephropathy.

After reviewing patient records, participants that fulfilled the inclusion and exclusion criteria, were selected from the list of diabetic patients attending the clinic who came for blood collection prior to a follow-up appointment. Groups of T2DM patients aged between 18 and 65 years old were randomly selected from those who were not on dialysis or experiencing severe anaemia or poorly controlled hypertension. Participants who met the following exclusion criteria were excluded from the study: smokers, alcohol consumers, pregnant or breast feeding patients, patients taking antioxidants or received blood transfusion less than a year ago. Healthy controls were subjects who were not diagnosed with a medical illness after clinical and laboratory assessments.

Based on urine dipstick examination for albumin, T2DM patients were divided into two groups: T2DM with nephropathy and T2DM without nephropathy. Diabetic with nephropathy is diagnosed by the presence of albumin in the urine, reduced GFR, or both (24). Diabetic without nephropathy is diagnosed when urine albumin is <30 µg/min or <30 mg/day or if the urine dipstick examination for albumin is negative (25).

Participants were given a description of this study and informed consent was obtained from those who agreed to participate in the study. After calculating using G-power software considering type I error of 5% and type II error of 80% and 20% anticipated dropout rate, the estimated sample size was 73 for each group.

Specimen Collection and Analysis

A total of seven ml fasting venous blood samples were collected from the participants: two ml in EDTA for HbA1c and five ml in a plain tube for creatinine, CML and CRP measurements. HbA1c was analysed within same day of sample collection using a Biorad D10 analyser (Hercules, United States) based on the high-performance liquid chromatography (HPLC) principle. The serum was taken after separation from the blood clot after centrifugation and was analysed for creatinine and CRP levels. Analysis of creatinine and CRP was performed with the chemistry analyser Architect c8000 (Illinois, United States) using the Jaffe and the immunoturbidimetric methods, respectively. The reference range for creatinine is 62 to 115 µmol/L (male), 53 to 97 µmol/L (female), and ≤ 5 mg/L for CRP.

The remaining serum was then stored at -70 °C for further analysis of CML. The CML was analysed in batches using a one-step sandwich enzyme-linked immunosorbent assay (ELISA) kit from QAYEE-BIO (Shanghai, China). The assay range for CML is 15.6 ng/ml – 500.0 ng/ml.

Statistical Analysis

The data were analysed using SPSS Statistics Version 26.0. Categorical data were presented as frequencies and percentages (%), and all numerical data were presented as mean and standard deviation (SD), except for CML and CRP, which were presented as the median and interquartile range (IQR). The comparison of serum CML and CRP between the groups was accomplished using the Kruskal-Wallis test. A Post hoc Mann-Whitney test with Bonferroni's correction was used to identify the significance for group comparison. Logistic regression analysis was performed to determine the association between CML and CRP in diabetic patients with and without nephropathy in relation to healthy subjects. The correlation between HbA1c with CML and CRP was determined using the Spearman correlation. For all tests, a P-value of <0.05 was considered statistically significant.

Ethical Considerations

All aspects of this study complied with the Declaration of Helsinki. This study was approved by the Human Ethics Committee of USM (USM/JEPeM/17010064).

RESULTS

A total of 220 participants were included in this study of which 73 were healthy subjects, 73 were T2DM patients without nephropathy and 74 were T2DM patients with nephropathy. Most of the participants were Malays [n=210 (95.5%)]. The mean ± SD ages for healthy participants were 42.19 ± 8.60 years old, 55.92 ± 7.62 years old for T2DM patients without nephropathy and 55.96 ± 6.98 years old for T2DM patients with nephropathy. The level of serum CML [median (IQR)] was highest in T2DM patients with nephropathy group [237.70 (439.04) ng/ml], followed by T2DM patients without nephropathy [188.80 (55.95) ng/ml] and lowest in healthy subjects [131.80 (73.56) ng/ml]. The CRP level was also highest in T2DM patients with nephropathy (Table I). There were significant differences in serum CML and CRP levels between the three groups (P<0.001) (Table II and Table III). After the model was adjusted for age, sex, BMI and creatinine levels, multiple logistic regression analysis indicated that CML and CRP are independent predictors of both diabetes and diabetes with nephropathy (Table IV). Table V show the correlation between HbA1c with CML and CRP in T2DM patients with and without nephropathy. There was no correlation found between HbA1c and CML or CRP in those patients.

Table II: The levels of serum CML in T2DM patients without nephropathy, T2DM patients with nephropathy and healthy participants

	N	Median (IQR)	χ^2 stat (df)	P-value*
Healthy subjects	73	137.84 (98.67)	69.09 (2)	<0.001
T2DM without nephropathy	73	188.80 (55.95)		
T2DM with nephropathy	74	237.70 (439.04)		

*Kruskal-Wallis test; significant *P*<0.05
Post hoc tests: Healthy vs.T2DM without nephropathy, P= < 0.001; Healthy vs.T2DM with nephropathy, P= < 0.001; T2DM without nephropathy vs. T2DM with nephropathy, P= 0.024

Table I: Baseline characteristics of the participants according to groups

Variables	Healthy subjects (n =73)		T2DM without nephropathy (n = 73)		T2DM with nephropathy (n = 74)	
	Mean ± SD	**n (%)**	**Mean ± SD**	**n (%)**	**Mean ± SD**	**n (%)**
Age (years)	42.19 ± 8.60		55.92 ± 7.62		55.88 ± 6.97	
Sex						
Male		26 (35.6)		38 (52.1)		33 (44.6)
Female		47 (64.4)		35 (47.9)		41 (55.4)
Race						
Malay		73 (100)		67 (91.8)		70 (94.6)
Chinese		0 (0)		6 (8.2)		1 (1.4)
Siamese		0 (0)		0 (0)		3 (4.1)
eGFR (CKD-EPI), (ml/min/1.73m^2)	102.4 ± 17.0		77.7 ± 17.7		74.6 ± 22.1	
CML (ng/ml)	131.80 (73.56)*		188.80 (55.95)*		237.70 (439.04)*	
CRP (mg/L)	1.64 (1.91)*		2.15 (5.64)*		4.75 (6.91)*	

*Median(IQR)
CKD-EPI - Chronic Kidney Disease Epidemiology Collaboration Equation

Table III: The levels of serum CRP in T2DM patients without nephropathy, T2DM patients with nephropathy and healthy participants

	N	Median (IQR)	χ^2 stat (df)	P-value*
Healthy subjects	73	1.64 (1.91)	31.62 (2)	<0.001
T2DM without nephropathy	73	2.15 (5.64)		
T2DM with nephropathy	74	4.75 (6.91)		

*Kruskal-Wallis test; significant *P*<0.05
Post hoc tests: Healthy vs.T2DM without nephropathy, P= < 0.001; Healthy vs.T2DM with nephropathy, P= < 0.001; T2DM without nephropathy vs. T2DM with nephropathy, P= 0.024

Table IV: Logistic regression analysis to determine the association between CML and CRP with diabetes and diabetes with nephropathy adjusted for age, sex, body mass index, and creatinine

Variables	Diabetes without nephropathy		Diabetes with nephropathy	
	Adjusted OR (95% CI)	P-value*	Adjusted OR (95% CI)	P-value*
CML	1.028 (1.015, 1.041)	<0.001	1.019 (1.008, 1.031)	0.001
CRP	1.567 (1.089, 2.256)	0.021	1.680 (1.166, 2.421)	0.005

*Significant P<0.05

Table V: The correlations between HbA1c with CML and CRP in T2DM patients with and without nephropathy group (n=74).

Variables	HbA1c (with nephropathy)		HbA1c (without nephropathy)	
	r	p-value	r	p-value
CML	-0.115	0.329	-0.180	0.128
CRP	0.067	0.569	0.060	0.616

Spearman rho correlation analysis. Correlation is significant at P<0.05

DISCUSSION

CML has been used as a biomarker for the in vivo formation of AGEs (26). A higher level of serum CML in diabetic patients has been demonstrated in studies and its accumulation has been shown to correlate with diabetic complications such as nephropathy (27). CRP, an inflammatory marker has been shown to be elevated in T2DM patients, suggesting that inflammation plays a major role in the pathogenesis of T2DM and its complications (28). In this study, most of the participants were of Malay ethnicity and were predominantly females, except for the T2DM without nephropathy group. The estimated GFR (eGFR) were relatively equal for the T2DM groups and indicated stage two of CKD (eGFR, mean ± SD :77.7 ±17.7 ml/min/1.73m2 for T2DM without nephropathy and 74.6 ± 22.1 ml/min/1.73m2 for T2DM with nephropathy) as outlined in Kidney Disease: Improving Global Outcomes (KDIGO). Although urine albumin:creatinine or urine protein:creatinine ratios have better diagnostic value for the sensitivity and quantification of albumin/protein levels, urine dipstick examination for albumin was used in this study, as it is easier for the recruitment of the patients via point-of-care testing.

This study found that among all three groups, the level of CML was highest in T2DM patients with nephropathy. The serum CML level showed a significant difference between the three groups (P<0.001). These findings were consistent with the study conducted by Wautier et al. which found that the level of CML was higher in diabetics than in healthy patients and that the levels were more elevated in patients with retinopathy and microalbuminuria (29). Okura et al. also demonstrated that CML levels were higher in T2DM than healthy patients and showed CML had a negative correlation with insulin secretion in T2DM patients (30). Plasma levels of CML were found to be four times higher in CKD patients than in healthy persons. The increase could be due to both an increase in the formation of AGE and a decrease in clearance (31).

A systematic review by Bos et al. suggested an association between AGE levels (accessed via skin auto-fluorescence) and most diabetic complications, including nephropathy, with the exception of retinopathy (32). Three major AGEs; CML, carboxyethyllysine (CEL) and methyl glyoxal hydroimidazolone (MGHI) have the potential to become markers of progression in early DN (23). In this study, CML was shown to be an independent predictor of DN. CML with other AGEs such as pentosidine and malondialdehyde-lysine can be used as predictors for DN, as these markers were found deposited in kidneys in the early DN and in nodular lesions of advanced disease (33). Compared to other AGEs, CML is widely studied, often used as an AGE marker and is one of the major AGEs discovered at high levels in vivo and in food products (34). Any factors affecting protein and lipid glycation and oxidation will increase CML levels as seen in diabetic complications. The drawback of CML is its measurement method. Most of the studies used the ELISA method due to its convenience in analysing serum CML. Immunoassays are also frequently used, but the use of antisera for protein-bound AGEs in this assay is questionable. Gas chromatography-mass spectrometry or liquid chromatography-mass spectrometry are the most precise methods; however, high operational costs limit its usage (35).

In this study, the CRP result also showed significant levels between the three groups (P<0.001). The CRP levels were highest in T2DM patients with nephropathy compared to the other groups. High sensitive-CRP (hs-CRP) levels were observed to be higher in T2DM patients compared to the healthy population (36). Evidence has proven that T2DM patients presenting with an inflammatory component has been related to diabetic complications such as nephropathy. This finding is parallel with the study that showed CRP was elevated in DN in which the elevation is due to microinflammation or low-grade inflammation that is involved in the development of DN, although the level is lower compared with classic inflammatory diseases such as rheumatoid arthritis

(37). The level of hs-CRP has also been documented to increase in diabetic patients with microalbuminuria compared to patients without microalbuminuria (38).

We hypothesised that HbA1c would show a significant correlation with CML, as both are the products of glycation in diabetic patients. For CRP, a significant correlation would also be expected. However, our study found no correlation between HbA1c with CML and CRP. The result for CML is similar to a study by Wautier et al., which demonstrated that serum levels of HbA1c were not correlated with CML, indicating the different metabolism and/or turnover rate between these molecules (29). Hirata and Kubo found that CML and pentosidine (another form of AGE) also exhibited no correlation with HbA1c. In addition, their results showed no correlation between blood CML and urinary albumin (12). Another study done by Takeuchi et al. revealed a similar finding in which CML was shown not to have a correlation with HbA1c (39). For CRP, many studies showed a positive correlation with HbA1c as opposed to this study (40-43). However, a study from Iran demonstrated that CRP levels were positively associated with diabetic patients with high HbA1c levels, not with low HbA1c levels (44). A study from Singapore also revealed that CRP was only positively associated with diabetes in Chinese populations with high levels of HbA1c, not with those with low HbA1c (45).

Definite reasons regarding the lack of correlation between HbA1c with CML and CRP remain under study. For CML, one possible reason is due to interference by the exogenous AGE present in food. A study by Vlassara et al. found that circulating serum AGEs would greatly be affected by the content of the AGEs in the diet itself. The subjects who were put on a high AGEs diet showed an increment of serum AGEs up to 64.5%, while the subjects on a low AGEs diet showed a 30% decrease of CML level over a two-week study period (46). This might be one of the reasons we could not find any correlation between the serum CML level and HbA1c, where the diet was not strictly controlled in our study. Henle claimed that the amount of AGE consumed in a conventional diet is relatively higher than the total amount present in plasma (47). Future studies should consider how CML in food affects serum CML, so that proper sampling of serum CML can be done to reduce the interferences from the diet factor.

Another possible reason could also be due to the irreversible nature of the AGE product itself. CML can be regarded as a long-term measurement of glycaemic index compared to HbA1c which is regarded as an intermediate term of glycaemic index measurement, which indicates glycaemic control over the previous 120 days. Gerrits et al. revealed that the assessment of HbA1c over several years only poorly predicts the level of AGE in skin. They suggested that exposure to glucose should be longer or that other factors such as oxidative stress that increase the level of AGE should be controlled (48). A study conducted by Wolffenbuttel et al. concluded that the measurement of haemoglobin-AGE (Hb-AGE) is superior to HbA1c as a long-term glycaemic index. As the half-life and the metabolism differ, it may lead to a lack of association between the two molecules, thus affecting the concentration (49). As CML could serve as a long term glycaemic index marker, a comparison between CML and average HbA1c over a longer duration would be more appropriate compared to a single point comparison HbA1c.

Other than that, serum CML analysis was performed using the manual ELISA method, which depends largely on antibody design. Due to potential differences in the antibodies used for testing, the results obtained in this study were difficult to compare with other studies. The results might also have yielded false positives or negatives due to insufficient blocking of the microtiter plate immobilised with the antigen (50).

For CRP, it is suggested that, perhaps, the results did not show a positive correlation with HbA1c because the levels of CRP are not only determined by hyperglycaemia, but are also influenced by other factors such as age, gender, body weight and lipid level (44).

CONCLUSION

Serum CML and CRP are independent predictors of DN. Hence, their levels can be used to identify high-risk diabetic patients prone to developing DN and early intervention can be initiated to prevent this complication.

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