Ellisras Longitudinal Study 2017: elevated serum levels of carboxymethyl-lysine, an advanced glycation end-product, are associated with higher odds of developing endothelial dysfunction in black South African patients with type 2 diabetes mellitus (ELS 29)

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Abstract

This case-control study investigated the association between major types of serum advanced glycation end-products (AGEs) and selected serum/plasma markers of endothelial dysfunction in black patients with type 2 diabetes mellitus in Ellisras, South Africa. Serum AGEs were measured using either enzyme-linked immunosorbent assay (ELISA) or spectrophotometry. Serum markers of endothelial dysfunction were measured using either ELISA or calometry. The correlation and associations between major types of serum AGEs and markers of endothelial dysfunction were investigated using the Spearman correlation coefficient and bivariate logistic regression analysis, respectively. Although both serum total immunogenic AGEs and serum carboxymethyl-lysine (CML) were moderately and negatively associated with endothelial dysfunction, only serum CML was significantly associated with a higher odds for the development of endothelial dysfunction (low nitric oxide levels) in our diabetic subjects. It can therefore be concluded from this study that high serum levels of CML may predispose to endothelial dysfunction in black South Africans with type 2 diabetes.

Keywords: serum AGEs, endothelial dysfunction, markers of endothelial dysfunction, black South Africans, type 2 diabetes mellitus

Clinical and research-based evidence indicates that both type 1 and type 2 diabetes mellitus are associated with long-term microvascular complications (nephropathy, retinopathy and neuropathy) and macrovascular complications (myocardial infarction and cerebrovascular accident). Available evidence also suggests that the pathogenesis of these vascular complications of diabetes involve endothelial activation or dysfunction. Endothelial dysfunction, defined as impaired biosynthesis of endothelium-derived nitric oxide (NO) or its reduced bioavailability, is an established mediator of the atherosclerotic process. Indeed, most of the traditional and emerging cardiovascular risk factors are known to promote the development and progression of vascular atherosclerosis through their deleterious effect on the endothelium. The development of endothelial dysfunction in diabetes mellitus is attributable, among other factors, to the formation and action of advanced glycation end-products (AGEs).

AGEs are a heterogeneous group of compounds formed by the non-enzymatic reaction between reducing sugars such as glucose and proteins, nucleic acids and lipids. The formation of AGEs is reported to be enhanced by both chronic hyperglycaemia and oxidative stress, two conditions that are closely associated with diabetes mellitus. Available evidence also suggests that in diabetes mellitus, AGEs may promote endothelial dysfunction via a variety of mechanisms. Firstly, collagen cross-linked AGEs in the vascular wall may trap and quench NO on its way from the endothelium to the smooth muscle layer to stimulate their relaxation. Secondly, the interaction of certain serum AGEs with the receptor for advanced glycation end-products (RAGE) on vascular endothelial cells results in the activation and translocation of nuclear factor kappa B (NF-kB) into the nucleus. Once in the nucleus, NF-xB up-regulates several genes whose protein and peptide products are involved in the activation of the endothelium or endothelial dysfunction. Thirdly, serum AGE/RAGE interaction on the vascular endothelium may result in deactivation of the enzyme, endothelial nitric oxide synthase (eNOS), which synthesises NO in the endothelium. Fourthly, the superoxide anion (O2–) generated during the formation of AGEs may react with NO to form the peroxynitrite ion (ONOO–), thereby reducing the bioavailability of NO. Lastly, AGEs may
impair Ca²⁺ signalling in endothelial cells, thereby interfering with several endothelial cell processes, including the biosynthesis of NO.¹³ Racial/ethnic disparities in endothelial dysfunction have been observed in a number of studies. For example, African-Americans are reported to have reduced NO bioavailability compared to their Caucasian counterparts.¹⁴ Also, Tibetan type 2 diabetes patients are reported to have less NO levels than their Chinese Han counterparts.¹⁵ On the other hand, research evidence has also shown that both tissue and serum AGE levels may be influenced by genetics.¹⁶ Taken together, this information suggests that the association between serum (and tissue) AGE levels and endothelial dysfunction may be influenced by the genetic make-up and ethnicity/race of an individual. However, with the exception of a single study that investigated the association between serum AGE levels and endothelial dysfunction among Chinese type 2 diabetes patients,¹⁷ there is no other information in the literature regarding the association between serum levels of AGEs and endothelial dysfunction. In particular, no study has ever been conducted to investigate the association between serum AGE levels and endothelial dysfunction among type 2 diabetes patients of black African descent. Therefore, the aim of this study was to investigate the association between the different types of serum AGEs and circulating markers of endothelial dysfunction among black South African patients with type 2 diabetes mellitus.

**Methods**

A random sample of 138 black type 2 diabetes patients attending the diabetes clinic of Dr George Mukhari Academic Hospital (DGMAH) for medical review, and a convenient sample of 81 age-matched non-diabetic control subjects were recruited into this study. The control subjects were recruited mainly from the orthopaedic wards of DGMAH. Controls were included in the study if they had fasting blood glucose level of < 6.1 mmol/l. Both type 2 diabetes patients and control subjects were excluded from the study if they had any sign of renal impairment, history or evidence of any of the factors known to affect endothelial dysfunction, such as the traditional cardiovascular risk factors, uncontrolled hypertension, dyslipidaemia, cigarette smoking and obesity.

All type 2 diabetes patients and control subjects gave their informed consent after the purpose of the study and their rights were clearly explained to them. The study was conducted in accordance with the requirements of the research and ethics committee of the University of Limpopo (MREC/P/2013/PG).

After an overnight fast, venous blood samples for measurement of levels of the different types of serum AGEs, urea and electrolytes, as well as selected circulating markers of endothelial dysfunction were collected from all participants into blood collection tubes (BD Vacutainer®, Franklin Lakes, NJ, USA). The samples were left to clot for 30 min and then centrifuged at 4 000 rpm for 15 min at 4°C. Aliquots of the resultant serum samples were then stored at −80°C until analysed. For blood glucose and glycated haemoglobin (HbA₁c) measurements, blood samples were collected into citrate and EDTA blood tubes, respectively.

Serum total immunogenic AGEs (TIAGEs), Nε-carboxymethyllysine (CML) and Nε-carboxyethyl-lysine (CEL) were measured using STA-317, STA-316 and STA-300 Oxiselect™ ELISA kits, respectively, (2BScientific, Upper Heyford, UK), according to the manufacturer’s instructions. Fluorescent serum AGEs (FAGEs) were measured according to the method described by Munch et al.¹⁸ In brief, 20 μl of serum was diluted to a volume of 10 ml with 20 mM phosphate buffered saline, pH 7.4. Fluorescence of the diluted sample was then measured spectrophotometrically (excitation at 370 nm and emission at 440 nm) using a GloMaxR multi-detection spectrophotometer (Promega Corp, Madison, WI, USA). Fluorescent readings were expressed as arbitrary units (emission intensity/excitation intensity).

Plasminogen activator inhibitor-1 (PAI-1) was measured using ELISA kits purchased from Cell Biolabs, and NO and endothelin-1 (ET-1) were measured using colorimetric and immunometric kits, respectively, purchased from Cayman Chemical’s ACE. Fasting blood glucose levels were measured using a commercially available glucose oxidase-based kit adapted to the Beckman Coulter® UniCell DXC 800 Synchron® Clinical System available in the National Laboratory Health Services (NLHS) laboratory at the DGMAH. HbA₁c level was measured using the immune chemiluminescent assay kit adapted to the Abbot Architec system Ci 8200 in the NLHS laboratory at DGMAH, in accordance with the manufacturer’s instructions.

**Statistical analysis**

All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software (Version 23.0), SPSS Inc, Chicago, IL, USA. Continuous variables are expressed as mean ± standard deviation (SD) while categorical variables are expressed as percentages. Means of the experimental and control groups were compared using the student’s t-test, and p < 0.05 was regarded as statistically significant differences between the groups. Bivariate logistic regression and the Spearman rank correlation coefficient were used to determine the association and correlation between the major types of serum AGEs and circulating markers of endothelial dysfunction, respectively. Significance level was set at p < 0.05.

**Results**

Table 1 shows the demographic, clinical and laboratory characteristics of the type 2 diabetes patients and the non-diabetic controls. With the exception of the fasting blood glucose and HbA₁c levels, there were no significant differences in any other demographic, clinical or laboratory parameters between the diabetic and the non-diabetic groups.

As shown in Fig. 1, the mean serum levels of TIAGEs, CML and CEL were significantly higher in the diabetic than the non-diabetic group (p < 0.001, p < 0.001 and p < 0.01, respectively). On the other hand, there was no significant difference between serum FAGE levels of the diabetic and the non-diabetic groups.

As shown in Fig. 2, the mean NO serum level of the diabetic patients was significantly lower than that of the non-diabetic control group (p < 0.001). On the other hand, the mean serum ET-1 and PAI-1 levels of the diabetic group were significantly higher than those of the control group (p < 0.05) (Fig. 2).

Gender and age of the study subjects, as well as the different types of serum AGEs (TIAGEs, CML, CEL and FAGEs) measured in the diabetic group were correlated with
the corresponding selected circulating markers of endothelial dysfunction (NO, ET-1 and PAI-1) using the Spearman rank correlation coefficient (r).

Results shown in Table 2 suggest a significant weak negative correlation (p < 0.05) between the age of the study subject and serum levels of NO, as well as a significant moderate negative correlation between serum CML and NO levels (p < 0.05), and between serum CML and NO levels (p < 0.05) (Table 2). Table 2 also shows a significant weak positive correlation between serum TIAGE and ET-1 levels (p < 0.05), as well as between serum CML and ET-1 levels (p < 0.05).

Discussion

As expected, serum levels of TIAGEs, CML and CEL were found to be significantly higher in the diabetic patient group compared with the non-diabetic control group. However, serum FAGE levels of diabetic patients were not significantly different from those of non-diabetic controls. This observation might be attributed to the nature of the control group used in the study.

High serum FAGE levels, in particular high serum levels of FAGEs (Au) 0.050, 0.722, –0.036, 0.802, 0.175, 0.224, and CEL (ng/ml) –0.150, 0.297, –0.145, 0.758, were also shown to be significantly higher in the diabetic patient group compared with the non-diabetic control group. However, serum levels of TIAGEs, CML and CEL were found to be significantly higher in the diabetic patient group compared with the non-diabetic control group.

Bivariate logistic regression analysis of the association between age and gender of the diabetic subjects, as well as serum levels of the major types of serum AGEs with endothelial dysfunction (serum NO levels less than the first quartile) revealed that only higher serum levels of CML were significantly associated with higher crude odds of endothelial dysfunction (COR (95% CI), 1.910 (0.655–0.893) (p < 0.05) (Table 3).
The observation in this study that serum NO levels were negatively and significantly correlated with the age of the study subject is in agreement with the well-documented observation negatively and significantly correlated with the age of the study markers of endothelial dysfunction, levels of NO, ET-1 and PAI-1, among others, as surrogate parameter in this study that was significantly associated with endothelial cell. The finding that serum CML level was the only significant higher serum levels of both ET-1 and PAI-1 are expected to be increased in conditions associated with endothelial dysfunction, such as type 2 diabetes mellitus. Therefore the findings of significantly reduced NO levels and significantly higher serum levels of both ET-1 and PAI-1 are in perfect agreement with the results of these previous studies. However, these findings should be interpreted with caution, since these circulating markers of endothelial dysfunction may come from sources other than the vascular endothelium.

The results of this study showed that serum AGE levels were significantly higher in type 2 diabetes patients than in non-diabetic black South Africans, and with the exception of CEL were not influenced by gender. In addition, serum FAGE levels appeared to be positively associated with increasing age of the subjects in the non-diabetic controls, but not in the diabetic subjects. Furthermore, the findings of this part of the thesis showed that serum TIAGEs, CML, CEL, ET-1 and PAI-1 levels were significantly elevated, whereas serum levels of NO were significantly reduced in black South African patients with type 2 diabetes compared to those in non-diabetic control subjects. Moreover, the findings indicated that serum TIAGE and CML levels, but not CEL and FAGE levels were correlated with endothelial dysfunction in black South African patients with type 2 diabetes mellitus. However, only serum CML levels were associated with a higher odds of developing endothelial dysfunction in these black South African type 2 diabetes patients.

We acknowledge the contribution of the nursing and medical personnel as well as the phlebotomists at the diabetes clinic of Dr George Mukhari Academic Hospital. We are grateful for the research funding obtained from the National Research Foundation (grant no. TP1407187704).

**References**


8. Yan SF, D’Agati V, Schütz AM, Ramasamy R. Receptor for Advanced dietary and smoking-related AGEs on serum AGE levels was not addressed. Fourthly, the control group selected for this study might have confounded the results, particularly those of the FAGEs. Fifthly, we did not concurrently measure serum AGE levels and circulating markers of endothelial dysfunction of other South African race groups for comparison purposes.

Despite these limitations, we believe that the results of this study are of great interest in that they are the first to describe the status of serum AGE levels among black South African patients with type 2 diabetes, as well as the association between serum AGE levels and endothelial dysfunction in black South African patients with type 2 diabetes mellitus.

**Limitations**

There are several limitations that should be taken into consideration when interpreting results of this study. Firstly, the sample size was small and study subjects were recruited from a single health institution, therefore the findings could not be generalised beyond the study samples. Secondly, the study was cross-sectional and therefore cause and effect relationships could not be inferred from the results. Thirdly, the possible confounding effect of exogenous menopausal women. Whether the high level of pentosidine observed in the cited studies was the cause or product of osteoporosis is currently not clear. It is possible that our patient control group, which was recruited from orthopaedic wards at DGM AH, may have included non-diabetic postmenopausal women with osteoporosis-related fractures. While this likelihood was not verified in the current study, it might explain the observed high levels of FAGE in the non-diabetic control group.

Previous studies reported in the literature have used circulating levels of NO, ET-1 and PAI-1, among others, as surrogate markers of endothelial dysfunction *in vivo*. According to these previous studies, serum levels of NO and its metabolites are expected to be decreased, while serum levels of both ET-1 and PAI-1 are expected to be increased in conditions associated with endothelial dysfunction, such as type 2 diabetes mellitus. Therefore the findings of significantly reduced NO levels and significantly higher serum levels of both ET-1 and PAI-1 are in perfect agreement with the results of these previous studies. However, these findings should be interpreted with caution, since these circulating markers of endothelial dysfunction may come from sources other than the vascular endothelium. The findings that serum levels of both TIAGEs and CML were negatively and significantly correlated with serum NO levels and positively and significantly correlated with serum levels of ET-1 were also not unexpected, since high levels of some serum AGEs are known to promote endothelial dysfunction through their interaction with RAGE on the surface of the vascular endothelial cell. The finding that serum CML level was the only parameter in this study that was significantly associated with increased odds of developing endothelial dysfunction suggests that serum CML is the major type of serum AGEs that interacts with RAGE to promote endothelial dysfunction.

### Table 3. Bivariate logistic analysis of the association between gender, age and the major types of serum AGEs with endothelial dysfunction (less than the first quartile of NO levels)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>COR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.040</td>
<td>0.996–1.22</td>
<td>0.296</td>
</tr>
<tr>
<td>Gender</td>
<td>1.040</td>
<td>0.996–1.22</td>
<td>0.296</td>
</tr>
<tr>
<td>TIAGEs (µg/ml)</td>
<td>0.348</td>
<td>0.014–8.916</td>
<td>0.523</td>
</tr>
<tr>
<td>CML (ng/ml)</td>
<td>1.910</td>
<td>0.655–0.893</td>
<td>0.013*</td>
</tr>
<tr>
<td>CEL (ng/ml)</td>
<td>1.172</td>
<td>0.963–1.638</td>
<td>0.112</td>
</tr>
<tr>
<td>FAGEs (Au)</td>
<td>0.991</td>
<td>0.882–1.038</td>
<td>0.141</td>
</tr>
</tbody>
</table>

*Significant at p < 0.05.*


