Association of POC-based Measurement of Skin AGEs with Serum AGEs as well as AGE Biomarkers in Individuals with and without Glucose Intolerance

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ABSTRACT

Aim: Accumulation of advanced glycation end products (AGEs) occurs with aging and in various disease states. There are no reliable screening techniques to measure AGEs in clinical settings. In this study, a point-of-care (POC) device was used to validate skin AGE measurements with serum AGE levels and to assess its usefulness to identify individuals with abnormal glucose tolerance (AGT).

Materials and methods: The study group comprised individuals with normal glucose tolerance (NGT: n = 47) and with AGT, that is, either diabetes or prediabetes (n = 68). Intrinsic AGE fluorescence was measured spectrophluorimetrically using multimode plate reader in the serum by exciting the samples at 370 nm and emission readouts at 440 nm. Skin AGEs were acquired using a CE-marked Scout DS commercial device. Serum levels of biomarkers carboxymethyl lysine (CML), carboxyethyl lysine (CEL), and pentosidine were analyzed by enzyme-linked immunosorbent assay (ELISA).

Results: In subjects with AGT, the skin AGEs [61.3 vs 53.7 arbitrary units (AU), p < 0.0001] and serum AGEs (3.5 vs 2.8 AU, p < 0.0001) were significantly higher than in individuals with NGT. The levels of CML, CEL, and pentosidine were also significantly higher in the subjects with AGT when compared with NGT (138 vs 89 pg/mL; 2.4 vs 1.4 nmol/mL, and 64 vs 48 nmol/mL, p < 0.0001), respectively. Pearson correlation analysis showed a significant positive association of skin AGEs with serum AGEs (r = 0.434) (p < 0.001), CML (r = 0.323) (p < 0.001), CEL (r = 0.308) (p < 0.001), and pentosidine (r = 0.251) (p < 0.001). In addition, it also showed a positive correlation with fasting plasma glucose (FPG) (r = 0.01), 2-hour post-glucose (r = 0.004), glycated hemoglobin (HbA1c) (r = 0.01), and body mass index (BMI) (p < 0.05). Multiple logistic regression analysis using AGT as a dependent variable showed that skin AGE scores were significantly (p < 0.001) associated with AGT (odds ratio: 1.133, confidence intervals: 1.067–1.203).

Conclusion: This study shows that the measurement of skin AGEs using a POC device may be suitable for mass screening of AGT even in low-resource settings.

ORIGINAL ARTICLE

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INTRODUCTION

Hyperglycemia and impaired insulin secretion or action are key characteristics of type 2 diabetes. Based on the 2021 Atlas of International Diabetes Federation, 10th edition, India is second in the prevalence of diabetes globally, next to China, with 74.2 million people being affected and it is projected to increase to 124.9 million in the year 2045.1,2 Screening of high-risk individuals is an imperative step in the primary prevention of diabetes. Conventional diabetes screening program of random capillary glucose needs pricking of skin to obtain blood samples. A noninvasive method of diabetes mass screening has been proposed as a substitute to invasive screening, based on skin intrinsic fluorescence.3–5 The POC device—Scout DS (VeraLight Inc., Albuquerque, NM) measures diabetes biomarkers in the skin, including AGEs and collagen cross-links as well as fluorescent mediators of cellular metabolism and oxidative stress, such as nicotinamide adenine dinucleotide and flavin adenine dinucleotide based on the principle of fluorescence spectroscopy.6,7 This can potentially be used as a first step in the noninvasive mass screening of individuals at risk, especially subjects with prediabetes. The device measures skin fluorescence to assess the absorption of light by melanocytes and hemoglobin in the subject’s skin and integrates this with the measurement algorithm to generate a diabetes risk score. Advanced glycation end products are nonenzymatically glycated and oxidized modifications of proteins, lipids, or nucleic acids that may contribute to micro- and macrovascular disease development.8 The link between the pathogenesis of diabetes and products of AGE is very well established.9,10 In most clinical settings, AGE measurements are not possible because they require high-pressure liquid chromatography or gas or liquid chromatography-mass spectrometry analysis.11,12 In this scenario, our team had already developed an assay to calculate AGEs in serum expressed as advanced glycation index (AGI).13 In this paper, we have validated the skin AGE measurement by Scout DS by comparing it with systemic levels of AGEs in individuals with NGT and AGT.

Materials and Methods

Study Participants

Individuals with NGT (n = 47) and AGT (n = 68) were recruited from Dr Mohan’s Diabetes Specialities Centre, screening from community and referrals from study participants which have been described in detail earlier.14 Institutional Ethics Committee approval was obtained from Madras Diabetes Research Foundation. All participants provided written informed consent for the study. NGT and AGT were defined based on World Health Organization criteria as follows: after 75-gm glucose load during an oral glucose tolerance test, individuals with 2-hour post-plasma glucose value <7.8 mmol/L (140 mg/dL) were characterized as NGT and those with 2-hour
post glucose value ≥7.8 mmol/L (140 mg/dL) were diagnosed to have AGT. AGT included those with either diabetes (n = 37) and prediabetes (n = 31). For the rest of the paper, AGT is considered as one group. Of the diabetic patients, 90% were on oral hypoglycemic agents (OHA) and 10% on OHA plus insulin for control of hyperglycemia. Among the diabetic patients, 90% took OHA and 10% used OHA plus insulin. The inclusion criterion was age ≥20 years of either sex. The exclusion criteria were detailed earlier.¹⁴

Anthropometry and Biochemical Parameters Estimation

Anthropometric measurements were obtained using standardized techniques. Weight (kg)/height (m²) was the formula used to calculate BMI. Venipuncture was performed to collect FPG, HbA1c, hemogram, lipid profile, and liver function tests. Automated analyzer (Beckman Coulter, Brea, CA, USA) and testing system analyzers (VARIANT II TURBO; Bio-Rad, Hercules, CA, USA) were used to perform FPG (hexokinase) and A1c assays. The A1c values were Diabetes Control and Complications Trial-aligned, and the laboratory is certified by the College of American Pathologists and the National Accreditation Board for Testing and Calibration. Beckman Coulter automated analyzer was used to perform lipid profile. The intra- and interassay coefficients of variation (CVs) for the plasma glucose assays were <2.2 and 2.5% and for A1c assay were <0.6 and 1.5%, respectively.

SCOUT DS Measurement

The study participants were measured twice using the SCOUT DS machine, with each measurement consisting of two consecutive positions of the forearm on the SCOUT device [Fig. 1].

SCOUT data were collected using a commercial SCOUT device marked with CE (software revision 1.2). Individuals who did not receive a SCOUT score after two attempts were classified as screen failures and did not proceed with blood testing at the first visit. A follow-up phase recalled screen failures for SCOUT remeasurement and reacquisition of blood work. This facilitated the performance evaluation of the new SCOUT algorithm, which is more robust for dealing with dark skin and skin contamination. M/S VeraLight provided scores from their proprietary SCOUT algorithm.

ELISA Measurement

Estimation of Serum CML

Fasting blood samples were collected into serum separator tubes (SST) and allowed to clot for 30 minutes at room temperature. Samples were then centrifuged at 3000 rpm for 15 minutes to separate the serum portion and used for the assay. Human CML ELISA kit was purchased from Cusabio Biotech Co., Ltd (CSB-E12798h) and assay was performed according to the manufacturer’s protocol. In brief, 100 μL of standards, control, and samples were added to the wells and incubated for 2 hours at 37°C. Solution was aspirated from each well and 100 μL of conjugate solution was added and incubated for 1 hour at 37°C. Unbound antibodies were removed by washing three times using 200 μL of wash buffer and 100 μL of enzyme conjugate solution was added. Incubated for 1 hour at 37°C and later washed five times before the substrate solution (90 μL) was added. The plate was incubated in the dark at 37°C for 30 minutes. Read the plate at 450 nm after adding 50 μL of stop solution. CML levels were represented as pg/mL.

Estimation of Serum Levels of CEL

Fasting blood samples were collected into SST and allowed to clot for 30 minutes at room temperature. Samples were then centrifuged at 3000 rpm for 15 minutes to separate the serum portion and used for the assay. Human N (epsilon) (carboxyethyl) lysine ELISA kit was purchased from Cusabio Biotech Co., Ltd (CSB-E027210HU) and assay was performed according to the manufacturer’s protocol. In brief, 100 μL of standards, control, and samples were added to the respective wells and incubated at 37°C for 2 hours. Solution was aspirated from each well and 100 μL of conjugate solution was added and incubated at 37°C for 1 hour. Wash the plate three times using 200 μL of wash buffer to remove the unbound antibodies. After that add 100 μL of enzyme conjugate solution. Incubated for 1 hour at 37°C and later washed five times before the substrate solution (90 μL) was added. The plate was incubated for 30 minutes in the dark at 37°C. Optical density was measured at 450 nm after adding 50 μL of the stop solution. CEL levels are represented as nmol/mL. The intra- and interassay CVs were <8 and <10, respectively.

Serum AGE Measurement

Serum AGE measurement was determined as previously described by Sampathkumar et al.¹³ with minor modifications. Intrinsic AGE-specific fluorescence was measured spectrophuorometrically (multimode plate reader—PerkinElmer) by exciting the samples at 370 nm and emission readouts at 440 nm (Black Opaque 96-well Microplate, PerkinElmer). The concentrations of the AGE products were directly proportional to the fluorescence intensity, with each addition of serum sample the fluorescence intensity increased and fitted to a linear regression line. The slope of the regression line is called the AGI and is expressed in AU (100 units = 1 unit AGI). AGI values (representing serum AGE levels) are five-point linear regression estimates rather than the one-point derivation of fluorescent AGE levels reported in previous studies.

Statistical Analysis

Based on the primary outcome variable (skin AGEs) of our pilot study, the required minimum sample size was calculated as n = 40 in each group, considering the significance level set at 0.05 and statistical power of 0.80. Therefore, a higher number of samples (n ≥ 40) were considered for the study to ensure appropriate statistical power. One-way analysis of variance was used for the comparison between groups with p < 0.05 as the criterion for significance. Pearson correlation analysis was performed to determine the relationship between skin AGE and risk factors. All statistical analyses were performed using the Windows-based statistical package, SPSS (version 20.0, Chicago, IL, USA).
Association of POC-based Measurement of Skin AGEs with Serum AGEs

**Results**

The clinical and biochemical characteristics are shown in Table 1. Waist circumference, FPG, 2-hour post-plasma glucose, HbA1c, systolic and diastolic blood pressure, and serum triglycerides were significantly higher in individuals with AGT when compared with NGT.

Figure 2 shows the levels of serum AGEs and skin AGES in the two study groups. Skin AGES were significantly higher in the subjects with AGT when compared with NGT ($61.3 \pm 8.8$ AU; $p < 0.0001$) (Fig. 2A). The serum AGES were also significantly higher in subjects with AGT when compared with NGT ($3.5 \pm 1.0$ AU; $p < 0.0001$) (Fig. 2B).

Figure 3 shows the levels of CML (138 vs $89 \mu g/mL$) (Fig. 3A), CEL (2.4 vs $1.4 \mu mol/mL$) (Fig. 3B), and pentosidine (64 vs $48 \mu mol/mL$, $p < 0.0001$) (Fig. 3C) were significantly higher ($p < 0.001$ for all three) in the subjects with AGT when compared with NGT.

Table 2 shows the Pearson correlation analysis of the skin AGEs with serum AGEs and other risk factors. Skin AGEs showed a positive association with serum AGEs ($r = 0.344$, $p < 0.001$), CML ($r = 0.323$, $p < 0.001$), CEL ($r = 0.308$, $p < 0.001$), and pentosidine ($r = 0.251$, $p < 0.001$). They also showed a positive correlation with FPG ($p < 0.001$), 2-hour post-glucose ($p < 0.001$), HbA1c ($p < 0.001$), and BMI ($p < 0.05$). Multiple logistic regression analysis using AGT as dependent variable showed that skin AGE scores were significantly ($p < 0.001$) associated with AGT (odds ratio: 1.133, confidence intervals: 1.067–1.203) and this association persisted ($p < 0.038$) even after adjusting for age, waist circumference, BMI, FPG, and HbA1c.

Figure 4 illustrates the scatter plot showing the correlation of skin AGES with serum AGES (Fig. 4A), HbA1c (Fig. 4B), FPG (Fig. 4C) and 2-hour post-plasma glucose (Fig. 4D).

**Discussion**

Many studies have confirmed the accumulation of AGES and its role in the pathogenesis.
of diabetes and its complications.\textsuperscript{15} AGEs induce tissue dysfunction through receptor for advanced glycation end products (RAGE) that has been reported to be enhanced in diabetes mellitus.\textsuperscript{16,17} Conventional methods that has been reported to be enhanced in diabetes mellitus.\textsuperscript{16,17}

### Table 2: Pearson correlation analysis of skin AGEs with serum AGEs and other risk factors

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<th>Parameters</th>
<th>Skin AGEs</th>
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<td>BMI</td>
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<td>Serum AGEs</td>
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<td>0.308</td>
<td>&lt;0.01</td>
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<tr>
<td>Pentosidine</td>
<td>0.251</td>
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2h-PPG, 2-hour post-plasma glucose; AGE, Advanced glycation end product; BMI, Body mass index; CEL, Carboxymethyl lysine; CEL, Carboxymethyl lysine; FPG, Fasting plasma glucose; HbA1c, Glycated hemoglobin; HDL-C, High-density lipoprotein-cholesterol; LDL-C, Low-density lipoprotein-cholesterol; TC, Total cholesterol; TG, Triglyceride; bold indicate p value <0.05.

In our previous study, we reported the usefulness of the noninvasive POC device, SCOUT DS for assessment of skin fluorescence in type 2 diabetes where the sensitivity of SCOUT DS and HbA1c to detect AGT were 87 and 86%, respectively.\textsuperscript{14} The main advantages of the SCOUT machine over the FPG or HbA1c are that it does not need a blood draw; there is no need of any reagents or disposables, and the test result is available within minutes. In this study, we have validated the sensitivity further by showing its association with the levels of serum AGEs as well as other AGE-specific biomarkers viz., CML, CEL, and pentosidine. The study also shows a good correlation of skin AGEs fluorescence with HbA1C and blood glucose levels which, in turn, substantiates the use of the instrument for mass screening.

### Figs 4A to D: Scatter plot showing the correlation of skin AGEs with (A) Serum AGEs; (B) HbA1c; (C) Fasting plasma glucose; (D) 2-hour post-plasma glucose

AGEs formed after the molecular rearrangements of glycosylated proteins or lipids accumulate in the vessel wall where they may alter the cell structure and function thereby leading to micro- and macrovascular complications of diabetes.\textsuperscript{21} Hofmann et al.\textsuperscript{22} reported that skin autofluorescence (SAF) was correlated to the AGEs in the cardiac tissue in CAD patients thus positive to the correlation between arterial tissue AGEs and skin AGEs. Due to their low concentration in tissue proteins, specific detection of AGEs is cumbersome and requires preanalytical methods that may alter the sensitivity of the measurements.

A number of studies have shown the association of skin AGEs with HbA1c.\textsuperscript{23,24} In our study also we observed that skin AGEs had a significant correlation with HbA1c in diabetic subjects. Moreover, due to the short lifespan of red blood cells, skin fluorescence appears to be a good marker of past long-term tissue damage compared to the traditional HbA1c measure. In this study, we have demonstrated the association of skin AGEs with serum levels of AGEs.

Carboxymethyl lysine, CEL, and pentosidine represent the most prevalent AGEs in vivo and these are frequently used as AGE markers.\textsuperscript{25,26} Temma et al.\textsuperscript{27} and Yuan et al.\textsuperscript{28} have shown that the levels of skin AGEs in different patient groups and control subjects, measured by an AGE reader, are significantly correlated to levels of both nonfluorescent AGEs (e.g., CML and CEL) and fluorescent (e.g., pentosidine) products assessed in skin biopsies. Meerwaldt et al.\textsuperscript{22} first reported the validity of the AGE reader as a tool to measure AGEs. A study by Kida et al.\textsuperscript{30} showed that SAF correlated significantly with skin pentosidine but not with bone pentosidine content. Another study by Hu et al.\textsuperscript{31} reported that among diabetic and nondiabetic Chinese subjects with lower-limb amputation, the SAF was independently associated with different AGEs such as CML in skin and pentosidine in artery and nerve. In our study also the level of AGEs measured as both skin fluorescence and serum CML, CEL, and pentosidine were both consistently and significantly elevated in individuals with AGT when compared to those with NGT. Some studies have also confirmed that serum levels of non-CML AGEs are significantly associated with the severity of micro- and macrovascular complications of diabetes.\textsuperscript{32}

In conclusion, our study shows that skin AGEs measurement using a POC device may be useful in mass screening of diabetes in epidemiological surveys even in low-resource
settings, as there are literally no consumable costs. The correlation with serum AGEs and with biomarkers like CML, CEL, and pentosidine further support these findings.

**Ethical Standard**

The study was approved by the Ethical Committee of Madras Diabetes Research Foundation.

**Acknowledgment**

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**References**