

Association between Subclinical Atherosclerosis Markers and the Level of Accumulated Advanced Glycation End-Products in the Skin of Patients with Diabetes

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Aim: The level of accumulated advanced glycation end-products (AGEs) in the skin has been shown to predict the risk of complications in patients with diabetes mellitus (DM). Recently, the level of accumulated fluorescent AGEs in the skin has become measurable as skin autofluorescence (skin AF) using a non-invasive apparatus, autofluorescence reader. The purpose of this study was to evaluate the association between skin AF and the subclinical atherosclerosis markers, especially endothelial dysfunction, in patients with DM.

Methods: We enrolled 140 Japanese subjects with DM who attended Osaka University Hospital, and measured the skin level of AGEs by skin AF and three subclinical atherosclerosis markers: endothelial function by flow-mediated vasodilation, FMD; carotid intima-media thickness, IMT; and brachial-ankle pulse wave velocity, baPWV.

Results: FMD was significantly associated with skin AF ($r = -0.259$, $p = 0.002$). Furthermore, a stepwise multivariate regression analysis revealed that skin AF was an independent determinant of FMD ($\beta = -0.180$, $p = 0.038$). Although there were significant associations between skin AF and maximum carotid intima-media thickness (max-IMT) ($r = 0.298$, $p < 0.001$) as well as baPWV ($r = 0.284$, $p = 0.001$) in univariate analysis, skin AF was not an independent determinant of either carotid max-IMT or baPWV after adjustment for conventional cardiovascular risk factors. Receiver-operating characteristic curve analysis revealed that skin AF can identify the subjects whose FMD, max-IMT, and baPWV were completely within the normal range (C-statistics, 0.73; 95% confidence interval, 0.61–0.84; $p < 0.001$).

Conclusions: Skin AF was independently associated with FMD as an indicator of endothelial dysfunction, and can be utilized as a screening marker of atherosclerosis in Japanese patients with DM.

Key words: Skin autofluorescence, Advanced glycation end products, Endothelial dysfunction, Atherosclerosis, Diabetes

Introduction/Aim

Atherosclerosis, which is accelerated in patients with prolonged diabetes mellitus (DM), leads to the reduction of the quality of life and is one of the major causes of mortality. Subclinical atherosclerotic changes without symptoms precede the onset of arterial disease

such as cardiovascular disease (CVD) and peripheral arterial disease. The more commonly used subclinical atherosclerosis markers in the clinical setting are endothelial function assessed by flow-mediated vasodilation (FMD) of the brachial artery, arterial stiffness assessed by brachial-ankle pulse wave velocity (baPWV), and vascular structure assessed by the intima-media thick-

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Received: March 21, 2018 Accepted for publication: May 9, 2018

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ness (IMT) of the carotid artery. These are well established indices of relatively early-stage atherosclerosis, and the significant relation between these parameters and CVD has been demonstrated in many previous studies¹⁻³.

Advanced glycation end products (AGEs), a complex group of compounds produced from slowly occurring non-enzymatic glycation of proteins, represent the combined influence of various vascular risk factors including chronic hyperglycemia, and play important roles in the pathogenesis of diabetic vascular disease. Interestingly, the level of skin AGEs has been shown to predict the risk of future 10-year progression of diabetic complications in patients with type 1 DM (T1DM)⁴.

Recently, the level of accumulated fluorescent AGEs in the skin has become measurable non-invasively as skin autofluorescence (skin AF) using an autofluorescence reader. Skin AF intensity is correlated with accumulation of skin AGEs assessed by skin biopsy⁵ and has been shown to be associated with coronary artery calcification score used for the predictor of CVD⁶, diabetic complications and above mentioned indices of early-stage atherosclerosis such as carotid IMT and PWV⁷⁻⁹. With regard to endothelial dysfunction, however, such an association has not yet been validated in patients with DM, while Wang *et al.* found a strong association between skin AF and FMD in uremic patients on hemodialysis¹⁰.

Therefore, the primary purpose of this study was to evaluate the association between skin AF and endothelial function assessed by FMD in patients with DM. We also investigated the association between skin AF and IMT and baPWV and assessed the relationship between skin AF and these three subclinical atherosclerosis markers at the same time.

Methods

Subjects

The present investigation used the baseline data obtained from an ongoing prospective observational study to evaluate the association between skin AF and vascular complications. The main eligibility criteria of the original study were as follows: 1) a diagnosis of type 1 or 2 DM based on the criteria of the Japan Diabetes Society; and 2) age ≥ 20 but < 80 years. We aimed to recruit 447 subjects with and without DM for the comparison between groups. This prospective observational study started in May 2014 at the Osaka University Hospital in Japan, and 372 subjects were enrolled. For the present investigation, we selected all the diabetic subjects who met the following criteria: 1) being hospitalized for the treatment of diabetes, 2)

completed FMD, baPWV, and carotid ultrasonography examinations, 3) were not under dialysis treatment, 4) were not receiving nitrous acid treatment, and 5) were not current smokers.

The protocol was approved by the ethical review board at the Osaka University Hospital and the study was conducted in compliance with the Declaration of Helsinki and current legal regulations in Japan. Written informed consent for participation and publication was obtained from all the participants after a full explanation of the study was provided.

Measurement of Skin AF

Skin AF was measured using the AGE ReaderTM (DiagnOptics TechnologiesBV, Groningen, the Netherlands), a non-invasive desktop device. This device detects the characteristic fluorescence of some AGEs and was used to estimate the level of AGEs in the skin according to a previously reported protocol⁵. In short, the AGE ReaderTM uses an excitation light source with a peak excitation of 370 nm to illuminate a skin surface area of 4 cm². Subsequently, the emitted fluorescence light (within the wavelength range of 420–600 nm) and the reflected excitation light (within the wavelength range of 300–420 nm) from the skin are measured with a spectrometer. Skin AF is calculated in arbitrary units (AU) as the ratio between the emitted light and the reflected light. Skin AF was measured three times in series at a site on the ventral side of the forearm and the mean of those assessments was used as the representative value. The coefficient of variation between investigators for measurement of skin AF in the present study was 4.2%, demonstrating good reproducibility. In addition, intra-patient seasonal variance and the inter-day variance for measurement of skin AF were reported to be about 5%⁵.

Measurement of FMD, IMT, and baPWV

FMD of the brachial artery was measured in a quiet, temperature-controlled room on the morning after at least 12 hours of fasting. In order to minimize intra- and inter-investigator variation, all measurements were performed by a single expert investigator using the UNEX EF 38G (UNEX Corporation, Nagoya, Japan) in a previously reported manner^{11, 12}. To summarize, after measuring the vessel diameter of the brachial artery at rest, the forearm cuff was inflated to 50 mmHg above the systolic blood pressure and maintained at 50 mmHg for 5 minutes before deflation. After deflation, the change in vessel diameter was measured at the same point and the maximum value was recorded. FMD was calculated as follows: $\text{FMD} (\%) = (\text{maximum diameter} - \text{diameter at rest}) \times 100 / \text{diameter at rest}$. UNEX EF 38G determines the dia-

stolic diameters of the brachial artery semi-automatically using an instrument equipped with software for monitoring the brachial artery diameter, and thus, can measure FMD with high reproducibility. The coefficient of variation for measurement of FMD using this method was reported to be 10.1%¹².

Carotid IMT was evaluated using B-mode imaging ultrasonography with a 7.5-MHz probe. According to the guidelines of the Japan Society of Ultrasonics in Medicine, all scanning was performed by experienced physicians using the same method¹³. The thickest point (including plaque lesions) measured from the common carotid artery to the internal carotid artery bilaterally was defined as the max-IMT.

The measurement of baPWV was performed using the same volume-plethysmographic apparatus (BP-203RPEII formPWV/ABI, Nippon Colin, Japan, with subjects in the supine position after at least 5 minutes of rest. Oscillometric cuffs were wrapped on both ankles and upper arms, and simultaneously pressurized to the approximate value of the subject's diastolic pressure so that the pulse volume waveforms could be recorded using semiconductor pressure sensors. The distance between the sampling points of baPWV was calculated based on the height of the subject. The path length from the suprasternal notch to the ankle (La) was calculated as follows: $La = 0.8129 \times \text{height (cm)} + 12.328$. The path length from the suprasternal notch to the brachium (Lb) was calculated as follows: $Lb = 0.2195 \times \text{height} - 2.0734$. baPWV was calculated according to the following formula: $baPWV = (La - Lb) / T_{ba}$, where T_{ba} is the time interval between the wave front of the brachial waveform and that of the ankle waveform¹⁴. The higher value of baPWV that was recorded on the right side or left side was selected for this analysis.

Assessment of Clinical and Biochemical Characteristics

Clinical characteristics data such as duration of DM, current medication use, smoking history and biochemical data were collected. Fasting blood samples were collected and hemoglobin A1c (HbA1c), serum total, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol, triglycerides, and creatinine (Cr) levels were measured using standard laboratory protocols. The presence of hypertension and dyslipidemia was diagnosed by the primary doctors based on the criteria of the Japanese Society of Hypertension¹⁵ and the Japan Atherosclerosis Society¹⁶.

Statistical Analysis

Data are presented as the mean \pm standard deviation for continuous variables, and percentage for

dichotomous variables. The relationships among clinical characteristics, skin AF, and three subclinical atherosclerosis markers (FMD, max-IMT, or baPWV) were evaluated using the Pearson rank correlation coefficient. Forward and backward stepwise multivariate linear regression analyses to identify determinants of subclinical atherosclerosis markers were performed. In these analyses, variables with $p < 0.05$ in univariate analysis were entered into the model. The cutoff of max-IMT was set at 1.5 mm¹⁷ and that of baPWV was set at 1800 cm/sec^{18, 19} in accordance with previous reports. Since the specific standard value of FMD doesn't exist, we adopted 6.0% as the cutoff value in accordance with a previous report that investigated FMD as a predictor of coronary artery stenosis in Japanese subjects¹. We evaluated the association between skin AF and the number of atherosclerotic indices that showed abnormal values using the Jonckheere–Terpstra trend test. The ability of skin AF to detect the subjects whose FMD, max-IMT, and baPWV were all within the normal range, was evaluated by receiver-operating characteristic (ROC) analysis. A p value < 0.05 was considered statistically significant. These statistical analyses were performed using SPSS version 22 (SPSS Inc., Chicago, IL, USA).

Results

In the present study, a total of 140 subjects with DM were selected for analyses. Their clinical and biochemical characteristics are presented in **Table 1**. Skin AF values were significantly associated with gender (male, $r = 0.173$, $p = 0.041$), age ($r = 0.398$, $p < 0.001$), duration of DM ($r = 0.274$, $p = 0.001$), and estimated glomerular filtration (eGFR) ($r = -0.254$, $p = 0.002$). The subjects with hypertension ($r = 0.231$, $p = 0.006$) and dyslipidemia ($r = 0.233$, $p = 0.006$) showed higher level of skin AF. There was no statistically significant association between skin AF values and the other parameters (data not shown).

FMD was significantly associated with age ($r = -0.273$, $p = 0.001$), BMI ($r = -0.188$, $p = 0.026$), and skin AF ($r = -0.259$, $p = 0.002$) (**Fig. 1**, **Table 2**). The patients who were prescribed renin–angiotensin system (RAS) inhibitors ($r = -0.178$, $p = 0.036$) and statins ($r = -0.183$, $p = 0.031$) had lower levels of FMD. To evaluate whether skin AF is a determinant of FMD independent of other risk factors, we performed a stepwise multivariate regression analysis including age, BMI, and administration of RAS inhibitors and statins as explanatory variables. This analysis revealed that age, BMI, and skin AF were independent determinants of FMD (**Table 2**).

Max-IMT was significantly associated with age (r

Table 1. Clinical characteristics of study subjects

| | |
|------------------------------------|----------------|
| Type of diabetes (type 1/type 2) | 25/115 |
| Sex (male) | 65 (46.4%) |
| Age (years) | 59.3 ± 12.8 |
| BMI (kg/m ²) | 27.3 ± 5.5 |
| Duration of diabetes (years) | 13.5 ± 9.5 |
| Smoking (never) | 85 (60.7%) |
| FPG (mg/dL) | 151.2 ± 61.5 |
| HbA1c (%) | 8.9 ± 1.7 |
| LDL-cholesterol (mg/dL) | 112.1 ± 34.9 |
| HDL-cholesterol (mg/dL) | 51.3 ± 15.1 |
| Triglyceride (mg/dL) | 158.3 ± 104.4 |
| eGFR (mL/min/1.73 m ²) | 76.2 ± 23.9 |
| SBP (mmHg) | 123 ± 17.4 |
| DBP (mmHg) | 72.5 ± 12.6 |
| Hypertension | 88 (62.9%) |
| Dyslipidemia | 98 (70.0%) |
| FMD (%) | 6.2 ± 2.8 |
| max-IMT (mm) | 1.8 ± 0.8 |
| baPWV (cm/s) | 1698.2 ± 404.0 |
| Skin AF (AU) | 2.5 ± 0.5 |
| Medications | |
| OHA | 95 (67.9%) |
| Insulin | 61 (43.6%) |
| RAS inhibitor | 70 (50.0%) |
| Statin | 67 (47.9%) |
| Anti-platelet drugs | 32 (22.9%) |
| Complications | |
| Macroangiopathy | 24 (17.1%) |
| Microangiopathy | |
| Retinopathy | 46 (33.6%) |
| Nephropathy | 41 (29.3%) |
| Neuropathy | 62 (44.3%) |

Values are shown as mean ± standard deviation or number (percentage).

Abbreviations: BMI, body mass index; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; LDL-cholesterol, low density lipoprotein cholesterol; HDL-cholesterol, high density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; FMD, flow mediated dilation; max-IMT, maximum carotid intima-media thickness; baPWV, brachial-ankle pulse wave velocity; skin AF, skin autofluorescence; OHA, oral hypoglycemia agents; RAS inhibitor, renin-angiotensin system inhibitor.

The patients under dialysis were excluded from this study. Macroangiopathy included ischemic heart disease, cerebral vascular disease, and peripheral artery disease.

=0.416, $p < 0.001$), eGFR ($r = -0.212$, $p = 0.012$), SBP ($r = 0.258$, $p = 0.002$), hypertension ($r = 0.283$, $p = 0.001$), dyslipidemia ($r = 0.187$, $p = 0.027$), use of RAS inhibitors ($r = 0.287$, $p = 0.001$), use of statins ($r = 0.257$, $p = 0.002$), and skin AF ($r = 0.298$, $p < 0.001$). baPWV was significantly associated with age ($r = 0.499$, $p < 0.001$), duration of DM ($r = 0.325$, $p < 0.001$), BMI ($r = -0.187$, $p = 0.027$), eGFR ($r = -0.173$, $p = 0.041$), SBP ($r = 0.238$, $p = 0.005$), hyper-

tension ($r = 0.216$, $p = 0.001$), and skin AF ($r = 0.284$, $p = 0.001$). Although skin AF was significantly associated with both max-IMT and baPWV in univariate analysis (**Fig. 1**), it was not an independent determinant of either carotid max-IMT or baPWV after adjustment for conventional cardiovascular risk factors (**Table 2**).

Next, we identified the number of subclinical atherosclerosis tests (FMD, IMT, and baPWV) that

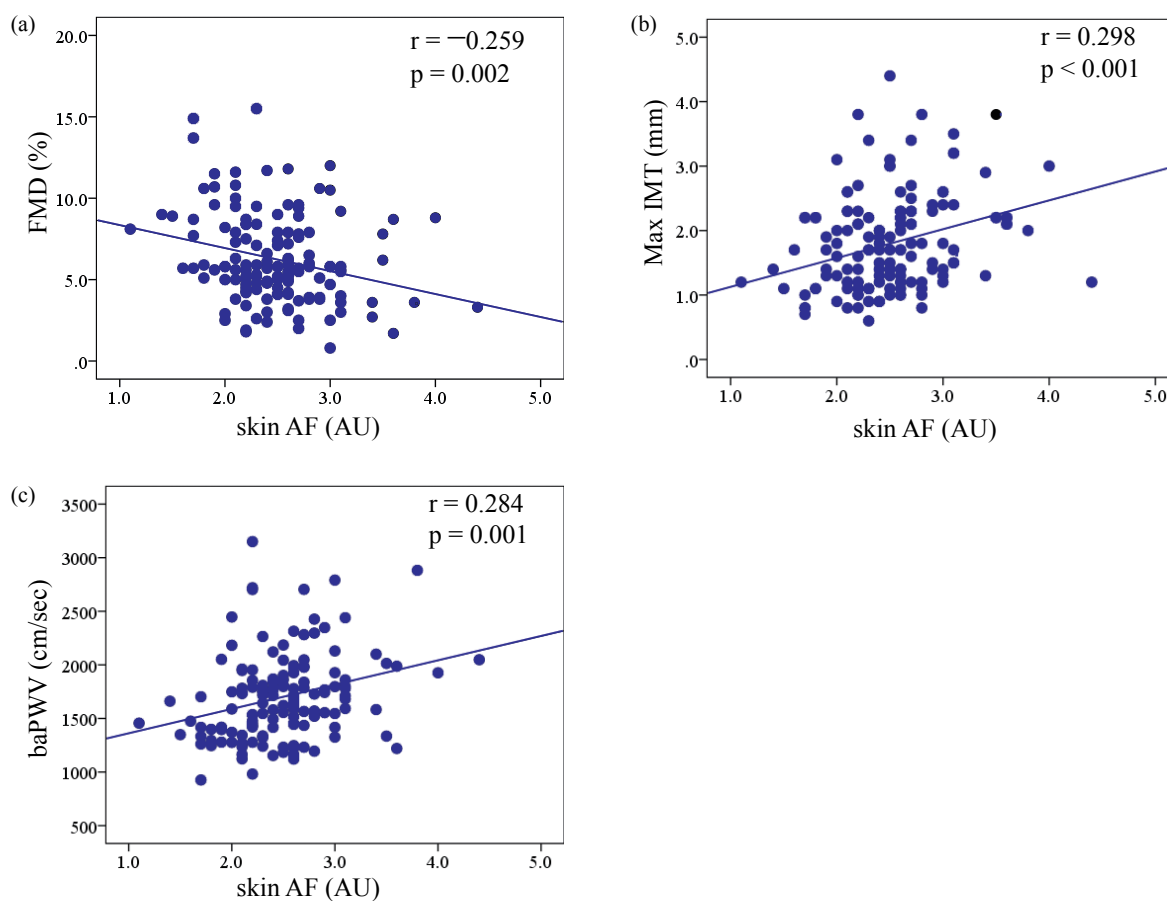


Fig. 1. Associations between skin AF and (a) FMD, (b) max-IMT, and (c) baPWV

Scatter plots for associations between skin AF and (a) FMD, (b) max-IMT, and (c) baPWV. Pearson correlation analysis was performed. The threshold of statistical significance was defined as $p < 0.05$.

Abbreviations: Skin AF, skin autofluorescence; FMD, flow mediated dilation; max-IMT, max intima-media thickness of the carotid artery; baPWV, brachial-ankle pulse wave velocity.

showed an abnormal range and evaluated their association with skin AF values. Interestingly, as the number of abnormal subclinical atherosclerosis parameters increased, skin AF showed higher values (p for trend < 0.001) (Fig. 2a). The ROC analysis for the absence of any subclinical atherosclerosis, that means all the subclinical atherosclerosis parameters were within the normal range, revealed that the area under the curve (AUC) for skin AF was 0.73 (95% confidence interval, 0.61–0.84, $p < 0.001$), and that the threshold of skin AF ≤ 1.9 AU was associated with a sensitivity of 36% and a specificity of 95%, respectively (Fig. 2b).

Discussion

The present study revealed that skin AF, a non-invasive and simple marker of the accumulation of AGEs in the skin, is an independent determinant of

brachial FMD, an indicator of endothelial dysfunction, in Japanese patients with DM. This finding is compatible with the idea that AGEs and their receptor system (RAGE) play an important role in the impairment of vascular function. Oxidative stress provoked by the generation of AGEs as well as AGE-RAGE interactions inhibit the endothelial nitric oxide synthase, thereby impairing endothelial function²⁰. The binding of AGEs to RAGE is also known to cause phenotypic changes in various cells such as endothelial cells and smooth muscle cells. In addition, AGEs, by cross-linking proteins and thereby changing their three-dimensional structures, impair vasodilation. Thus, AGEs are not only markers of “metabolic memory” in various metabolic disorders, but also have important pathogenic roles. This explains why skin AF can serve as a marker of endothelial dysfunction independent of other established risk factors.

Table 2. The associations between FMD/max-IMT/baPWV and skin AF

| | FMD | | | | max-IMT | | | | baPWV | | | |
|------------------------------------|------------|----------|--------------|----------|------------|----------|--------------|----------|------------|----------|--------------|----------|
| | Univariate | | Multivariate | | Univariate | | Multivariate | | Univariate | | Multivariate | |
| | <i>r</i> | <i>p</i> | β | <i>P</i> | <i>r</i> | <i>P</i> | β | <i>p</i> | <i>r</i> | <i>p</i> | β | <i>p</i> |
| Skin AF (AU) | -0.259 | 0.002 | -0.180 | 0.038 | 0.298 | <0.001 | NI | | 0.284 | 0.001 | NI | |
| Sex (male) | 0.000 | 0.999 | - | - | 0.153 | 0.072 | - | - | -0.051 | 0.546 | - | - |
| Age (years) | -0.273 | 0.001 | -0.232 | 0.008 | 0.416 | <0.001 | 0.339 | <0.001 | 0.499 | <0.001 | 0.402 | <0.001 |
| Duration of diabetes (years) | -0.167 | 0.050 | - | - | 0.107 | 0.210 | - | - | 0.325 | <0.001 | 0.177 | 0.018 |
| BMI (kg/m ²) | -0.188 | 0.026 | -0.229 | 0.005 | -0.148 | 0.082 | - | - | -0.187 | 0.027 | -0.174 | 0.020 |
| FPG (mg/dL) | 0.028 | 0.744 | - | - | 0.006 | 0.941 | - | - | -0.079 | 0.356 | - | - |
| HbA1c (%) | -0.111 | 0.191 | - | - | -0.073 | 0.392 | - | - | -0.046 | 0.590 | - | - |
| LDL-C (mg/dL) | 0.042 | 0.625 | - | - | -0.048 | 0.577 | - | - | -0.080 | 0.354 | - | - |
| HDL-C (mg/dL) | 0.015 | 0.865 | - | - | -0.007 | 0.931 | - | - | 0.138 | 0.104 | - | - |
| Log(TG) | -0.087 | 0.304 | - | - | 0.136 | 0.109 | - | - | -0.056 | 0.508 | - | - |
| eGFR (mL/min/1.73 m ²) | 0.156 | 0.066 | - | - | -0.212 | 0.012 | NI | | -0.173 | 0.041 | NI | |
| SBP (mmHg) | -0.155 | 0.068 | - | - | 0.258 | 0.002 | 0.256 | 0.001 | 0.238 | 0.005 | 0.247 | 0.001 |
| DBP (mmHg) | -0.041 | 0.630 | - | - | 0.116 | 0.174 | - | - | 0.007 | 0.934 | - | - |
| Hypertension | -0.150 | 0.077 | - | - | 0.283 | 0.001 | NI | | 0.216 | 0.010 | NI | |
| Dyslipidemia | -0.150 | 0.078 | - | - | 0.187 | 0.027 | NI | | 0.140 | 0.100 | - | - |
| RAS inhibitor | -0.178 | 0.036 | NI | | 0.287 | 0.001 | NI | | 0.090 | 0.290 | - | - |
| Statin | -0.183 | 0.031 | NI | | 0.257 | 0.002 | 0.179 | 0.026 | 0.123 | 0.147 | - | - |
| R ² | 0.134 | | | | 0.236 | | | | 0.327 | | | |

The threshold of statistical significance was defined as $p < 0.05$.

Stepwise multivariate regression analysis was performed to determine the predictor of FMD, max-IMT, or PWV. The significant variables in the Pearson correlation analysis were selected for multivariate analysis.

Abbreviations: Skin AF, skin autofluorescence; BMI, body mass index; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; LDL-cholesterol, low density lipoprotein cholesterol; HDL-cholesterol, high density lipoprotein cholesterol; TG, triglyceride; SBP, systolic blood pressure; DBP, diastolic blood pressure; RAS inhibitor, renin-angiotensin system inhibitor; max-IMT, maximum carotid intima-media thickness; baPWV, brachial-ankle pulse wave velocity.

The present study also confirmed that skin AF was associated with arterial thickening assessed by max-IMT and arterial stiffness assessed by baPWV, which was consistent with previous studies^{7-9, 21}. Considering the crucial roles of AGEs in the pathogenesis of atherosclerosis, it is reasonable that the index of AGEs accumulation in skin is associated with arterial thickening and/or stiffness.

Atherosclerosis is a multifactorial and systemic disease with a broad spectrum of morphologic and functional features and clinical presentations. Therefore, to assess the presence and extent of atherosclerosis in individuals, it would be desirable to perform multiple vascular tests. Indeed, to detect atherosclerotic changes in individuals as early as possible, combinations of multiple non-invasive vascular tests, including FMD, carotid IMT, and PWV, are often utilized. Interestingly, the present study showed that the value of skin AF ≤ 1.9 had strong screening power to identify individuals without subclinical atherosclerosis assessed by FMD, carotid IMT, and baPWV

(sensitivity of 36%, specificity of 95%). Since measurement of the skin AF value using the AGE ReaderTM is a very simple and non-invasive approach, it would be clinically worthwhile, from a time and convenience perspective, to use the skin AF value as a screening tool for atherosclerosis.

There are several limitations in this study. First, this is a cross-sectional survey with a relatively small number of subjects and the associations shown in this study were statistically significant but not very strong. Secondly, since the recruited subjects were patients who had been hospitalized for glycemic control or DM education, almost all the subjects were under poor glycemic control; the average of HbA1c was 8.9%. Therefore, it would be premature to generalize our findings to all individuals with DM. Thirdly, this study included premenopausal women. Since FMD is affected by the female menstrual cycle²², we cannot deny that the female menstrual cycle may have affected the results. Fourthly, this study included both T2DM and T1DM patients. When we divided the

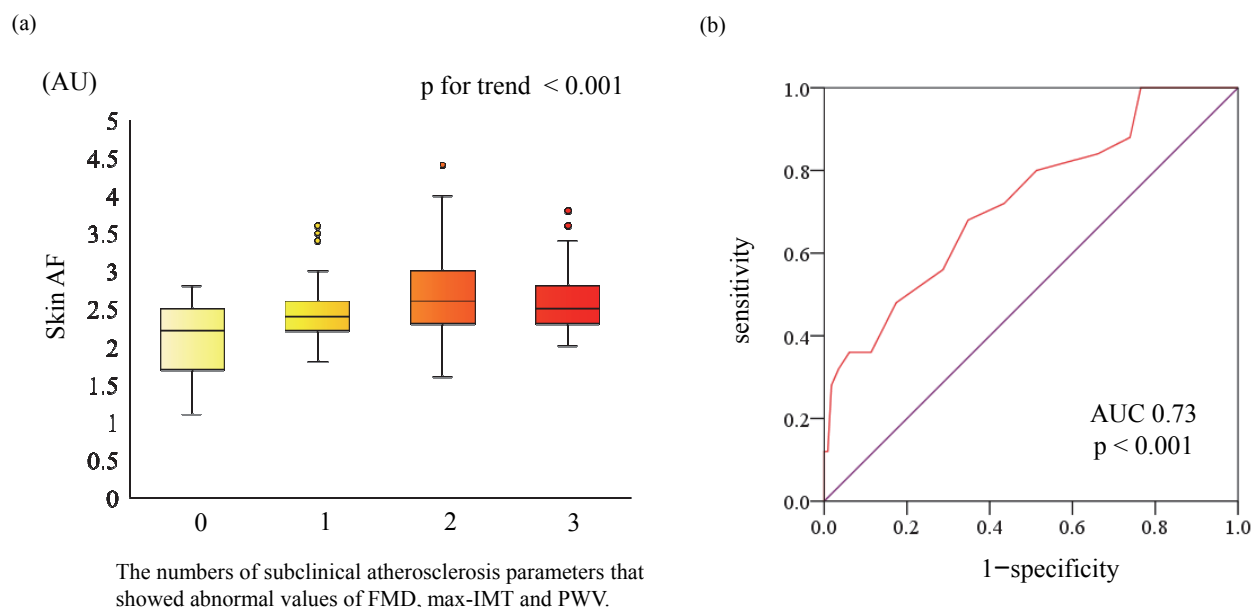


Fig. 2. Associations between skin AF and the presence of subclinical atherosclerosis

(a) Association between skin AF and the number of atherosclerotic indices that showed abnormal values using the Jonckheere–Terpstra trend test. As the number of subclinical atherosclerosis parameters that showed abnormal range was gaining, skin AF showed higher values (p for trend < 0.001). The cutoffs of FMD, max-IMT, and baPWV were set a 6.0%, 1.5 mm, and 1800 cm/sec, respectively. (b) The detection ability of skin AF for the absence of any atherosclerosis, which means that FMD, max-IMT, and baPWV were within the normal range, was evaluated by receiver-operating characteristic analysis. Skin AF \leq 1.9 AU predicted the absence of subclinical atherosclerosis change at a sensitivity of 36% and a specificity of 95% with an AUC of 0.73 (95% CI 0.61–0.84, $p < 0.001$).

study subjects into two groups (patients with T2DM and those with T1DM) and performed statistical analyses in these two groups separately, findings in the patients with T2DM were similar to those in the total study subject population: skin AF was significantly associated with markers of subclinical atherosclerosis ([Supplementary Table 1](#), [Supplementary Table 2](#), and [Supplementary Fig. 1](#)). Similarly, skin AF was significantly associated with max-IMT ($r = -0.408$, $p = 0.043$) and baPWV ($r = 0.727$, $p < 0.001$) in patients with T1DM. Although the coefficient of correlation value between skin AF and FMD in patients with T1DM was similar to that in patients with T2DM, it did not reach statistical significance in patients with T1DM ($r = -0.244$, $p = 0.239$). However, we could not draw any conclusions since analyses in patients with T1DM lack sufficient statistical power due to the small number of subjects. A larger scale study is necessary to explore whether skin AF was associated with these subclinical markers of atherosclerosis in patients with T1DM. Finally, it has been reported that some diabetes medications such as dipeptidyl peptidase-4 inhibitors and α -glucosidase inhibitors improved endothelial function^{23, 24}. Although multivariate regression analyses indicated that there was no signifi-

cant impact of type of DM medication on the association between skin AF and FMD (data not shown), we cannot rule out the influence of DM medications because of the cross-sectional nature of the present study. Future studies with longitudinal designs will be needed to address this point.

Notwithstanding these limitations, our study indicates an association between skin AF and endothelial function assessed by FMD as well as the other early-stage atherosclerotic indices like carotid-max IMT and baPWV. We also show that skin AF might serve as a screening marker of atherosclerosis in Japanese patients with DM.

Declarations

Ethics Approval and Consent to Participate

The study protocol was approved by the Research Ethics Committee of the Osaka University Graduate School of Medicine, and the study was conducted in accordance with the principles of the Helsinki Declaration. Written informed consent for participation and publication was obtained from all the participants after a full explanation of the study was provided.

Competing Interests

None.

Funding

This research was supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports, Culture and Technology [KAKENHI 16K09747].

Authors' Contributions

All authors contributed to the concept and design of this ongoing study. All authors read and approved the final manuscript. NK was the principal guarantor of this work, has full access to all the data, and takes the responsibility for the integrity of the data and accuracy of the data analysis.

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Supplementary Table 1. The associations between FMD/max-IMT/baPWV and skin AF among the subjects with T2DM ($n=115$)

| | FMD | | | | max-IMT | | | | baPWV | | | |
|------------------------------------|------------|----------|--------------|----------|------------|----------|--------------|----------|------------|----------|--------------|----------|
| | Univariate | | Multivariate | | Univariate | | Multivariate | | Univariate | | Multivariate | |
| | <i>r</i> | <i>p</i> | β | <i>p</i> | <i>r</i> | <i>p</i> | β | <i>p</i> | <i>r</i> | <i>p</i> | β | <i>p</i> |
| Skin AF (AU) | -0.231 | 0.013 | -0.220 | 0.019 | 0.255 | 0.006 | NI | | 0.198 | 0.034 | NI | |
| Sex (male) | -0.026 | 0.787 | - | - | 0.226 | 0.015 | -0.180 | 0.051 | -0.041 | 0.661 | - | - |
| Age (years) | -0.180 | 0.054 | - | - | 0.339 | <0.001 | 0.217 | 0.009 | 0.439 | <0.001 | 0.408 | <0.001 |
| Duration of diabetes (years) | -0.217 | 0.020 | NI | | 0.117 | 0.216 | - | - | 0.329 | <0.001 | NI | |
| BMI (kg/m ²) | -0.104 | 0.271 | - | - | -0.288 | 0.002 | -0.299 | 0.002 | -0.297 | 0.001 | NI | |
| FPG (mg/dL) | 0.022 | 0.818 | - | - | 0.045 | 0.631 | - | - | -0.050 | 0.596 | - | - |
| HbA1c (%) | -0.100 | 0.288 | - | - | -0.090 | 0.340 | - | - | -0.0079 | 0.401 | - | - |
| LDL-C (mg/dL) | 0.046 | 0.628 | - | - | -0.017 | 0.858 | - | - | 0.122 | 0.199 | - | - |
| HDL-C (mg/dL) | -0.100 | 0.289 | - | - | 0.114 | 0.224 | - | - | 0.267 | 0.004 | 0.197 | 0.021 |
| Log(TG) | 0.000 | 0.999 | - | - | 0.079 | 0.399 | - | - | -0.154 | 0.100 | - | - |
| eGFR (ml/min/1.73 m ²) | 0.150 | 0.111 | - | - | -0.146 | 0.121 | - | - | -0.114 | 0.225 | - | - |
| SBP (mmHg) | -0.045 | 0.630 | - | - | 0.220 | 0.018 | - | - | 0.177 | 0.059 | - | - |
| DBP (mmHg) | 0.080 | 0.394 | - | - | 0.069 | 0.461 | - | - | -0.068 | 0.472 | - | - |
| Hypertension | -0.146 | 0.120 | - | - | 0.247 | 0.008 | NI | | 0.182 | 0.051 | - | - |
| Dyslipidemia | -0.088 | 0.348 | - | - | 0.095 | 0.315 | - | - | 0.105 | 0.262 | - | - |
| RAS inhibitor | -0.133 | 0.155 | - | - | 0.239 | 0.010 | 0.273 | 0.003 | 0.038 | 0.688 | - | - |
| Statin | -0.169 | 0.072 | - | - | 0.197 | 0.035 | NI | | 0.079 | 0.402 | - | - |
| R ² | 0.040 | | | | 0.235 | | | | 0.230 | | | |

The threshold of statistical significance was defined as $p < 0.05$.

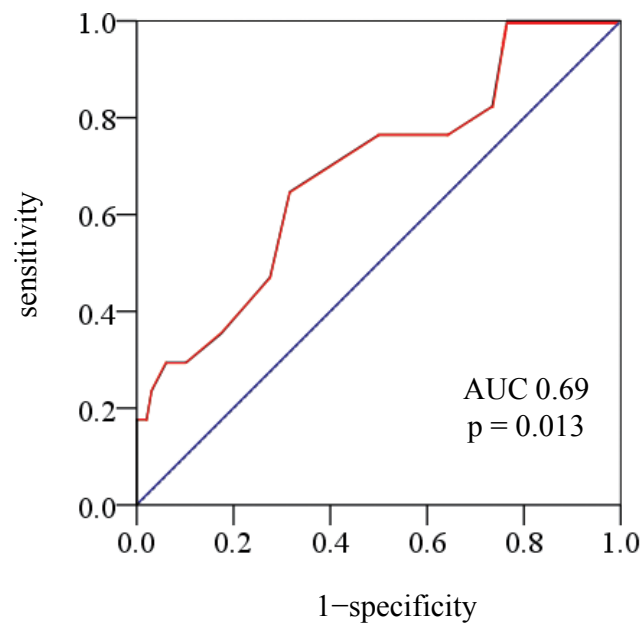
Stepwise multivariate regression analysis was performed to determine the predictor of FMD or max IMT or PWV. The significant variables in Pearson correlation analysis were selected for multivariate analysis.

Abbreviations: skin AF, skin autofluorescence; BMI, body mass index; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; LDL-cholesterol, low density lipoprotein cholesterol; HDL-cholesterol, high density lipoprotein cholesterol; TG, tryglyceride; SBP, systolic blood pressure; DBP, diastolic blood pressure; RAS inhibitor, renin-angiotensin system inhibitor; max IMT, max intima-media thickness of the carotid artery; baPWV, brachial-ankle pulse wave velocity.

Supplementary Table 2. Association between skin AF and the presence of subclinical atherosclerosis according to DM type

| | | The numbers of atherosclerosis indices that showed abnormal values | | | | <i>p</i> for trend |
|------------|-------------------|--------------------------------------------------------------------|------------------------|------------------------|------------------------|--------------------|
| | | 0 | 1 | 2 | 3 | |
| skinAF (U) | Total ($n=140$) | 2.13 ± 0.47 ($n=25$) | 2.46 ± 0.41 ($n=45$) | 2.63 ± 0.54 ($n=47$) | 2.64 ± 0.47 ($n=23$) | <0.001 |
| | T2DM ($n=115$) | 2.18 ± 0.51 ($n=17$) | 2.51 ± 0.41 ($n=35$) | 2.61 ± 0.55 ($n=40$) | 2.64 ± 0.47 ($n=23$) | 0.015 |
| | T1DM ($n=25$) | 2.01 ± 0.38 ($n=8$) | 2.30 ± 0.35 ($n=10$) | 2.74 ± 0.49 ($n=7$) | - | 0.002 |

Association between skin AF and the number of atherosclerotic indices that showed abnormal values using Jonckheere-Terpstra trend test. Regardless of DM type, as the number of subclinical atherosclerosis parameters that showed abnormal range was gaining, skin AF showed higher values (p for trend <0.001). The cutoffs of FMD, max-IMT, and baPWV were set 6.0%, 1.5 mm, and 1800 cm/sec, respectively.



Supplementary Fig. 1. The detection ability of skin AF for absence of any atherosclerosis among T2DM patients

The detection ability of skin AF for the absence of any atherosclerosis, which means that FMD, max-IMT, and baPWV were within the normal range, was evaluated by receiver-operating characteristic analysis among only T2DM patients. Skin AF ≤ 1.9 AU predicted the absence of subclinical atherosclerosis change at a sensitivity of 29% and a specificity of 94% with an AUC of 0.69 (95% CI 0.55–0.83, $p=0.013$). The cutoffs of FMD, max-IMT, and baPWV were set 6.0%, 1.5 mm, and 1800 cm/sec, respectively.