Association of Endothelial and Vascular Smooth Muscle Dysfunction with Cardiovascular Risk Factors, Vascular Complications, and Subclinical Carotid Atherosclerosis in Type 2 Diabetic Patients

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Aim: Atherosclerosis and arteriosclerosis are mainly caused by the dysfunction of arterial components, namely, vascular endothelial cells, smooth muscle cells, and the extracellular matrix. Endothelial dysfunction is well established as a predictive surrogate marker of cardiovascular events; however, little is known regarding the clinical implications of vascular smooth muscle dysfunction for cardiovascular disease and microangiopathy. In the present study, we aimed to clarify the association of arterial dysfunction with micro-/macroangiopathy and conventional cardiovascular risk factors in 181 type 2 diabetic patients (T2DM; age ± SD, 64 ± 10 years; duration of diabetes, 12 ± 10 years).

Methods: Flow-mediated dilatation (FMD) and nitroglycerin-mediated dilatation (NMD) were assessed to evaluate endothelial dysfunction and vascular smooth muscle dysfunction, respectively, by using a novel ultrasound device, UNEXEF18G (Unex Co. Ltd., Japan).

Results: The FMD and NMD were 6.4 ± 3.9% and 13.4 ± 6.6%, respectively. No significant differences in FMD were noted between T2DM with and without micro- or macroangiopathy; however, NMD in T2DM patients with micro- and macroangiopathy was significantly lower than that in T2DM patients without angiopathy. NMD decreased with the progression of chronic kidney disease (CKD) stage (p = 0.005), but not FMD (p = 0.071). On multiple regression analysis, significant independent contributors to FMD were age, smoking, systolic blood pressure, glycosylated hemoglobin, and serum total cholesterol, while those for NMD were age, systolic blood pressure, and waist circumference.

Conclusion: The relationship of vascular complications and cardiovascular risk factors with NMD is different from that with FMD in type 2 diabetic patients.


Key words: FMD, NMD, IMT, Stiffness parameter β, CKD stage, Diabetic angiopathy

Introduction

Cardiovascular diseases (CVDs) are the main cause of death in type 2 diabetes mellitus (T2DM) patients. The early detection of atherosclerotic and/or arteriosclerotic changes in systemic arteries is essential for prevention of the development and/or progression of CVD. Endothelium-dependent flow-mediated dilatation (FMD) of the brachial artery, which is assessed using ultrasonography, has been established as an early surrogate marker for CVD⁴⁻⁶, while the clinical implications of endothelium-independent nitroglycerin-mediated dilatation (NMD) remain unclear. Although
the assessment of these markers using ultrasonography is non-invasive and cost-effective, the precision and reliability of the method are questionable, and the method is technically complex. Recently, a novel ultrasound device has been developed for the measurement of FMD and NMD, which enables a more precise evaluation of these parameters by using a technique that is simpler than the conventional method.

The surrogate marker intima-media thickness (IMT) of the common carotid artery (CCA) has also been well established as a surrogate marker for CVD. The IMT represents morphological changes of the arterial wall, such as plaque formation, stenosis, and/or wall thickening. Another surrogate marker, stiffness parameter $\beta$, which is not as well known as IMT, represents the functional, elastic changes of the local arterial wall. We have previously reported that stiffness parameter $\beta$ is associated with insulin resistance, the plasma levels of adiponectin, pioglitazone administration, and exercise in T2DM patients. Furthermore, the association of concurrent coronary artery disease with both IMT and stiffness parameter $\beta$ together was stronger than that with either of the parameters alone.

In general, FMD and/or NMD are believed to be impaired in the early stages of athero/arteriosclerotic changes, while morphological and functional changes of the tunica intima and tunica media, IMT, and stiffness parameter $\beta$ are believed to occur secondary to endothelial dysfunction. However, the results of the evaluation of the abovementioned surrogate markers may be heterogeneous even in the same patient at risk for CVD, as commonly observed in clinical settings. In fact, only a limited number of studies have investigated mutual relationships among these surrogate atherosclerosis markers in the same patient. Furthermore, little is known about whether the levels of these surrogate markers exacerbate simultaneously and whether the risk factors contributing to their increase are the same.

**Aim**

In the present study, we used a cross-sectional design to investigate 181 T2DM patients and to clarify the relationship between FMD/NMD and other cardiovascular markers, namely, IMT/stiffness parameter $\beta$, measured for the carotid artery; accordingly, we assessed the levels of these surrogate markers for CVD and explored the impacts of possible clinical risk factors and coexisting complications on these markers.

**Methods**

**Subjects**

Consecutive T2DM patients ($n=181$) who were admitted to our diabetes center at Osaka City University Hospital for participation in diabetes educational and/or complication-check programs were enrolled in this study. The diagnosis of T2DM was made on the basis of a history of diabetes or the Japan Diabetes Society criteria. Eighty-four of these patients received insulin; 13, sulfonylureas; 6, alpha-glucosidase inhibitors; 9, biguanides; 9, insulin secretagogues; 2, thiazolidinediones (pioglitazone); and 33, multidrug therapy. Seventy-four patients received 3-hydroxy-3-coenzyme A reductase inhibitors (statin), and 11 received fibrates as lipid-lowering therapy. As anti-hypertensive treatment, 74 patients received calcium channel blockers, and 72 received an angiotensin-converting enzyme inhibitor (ACEI) or an angiotensin II receptor blocker (ARB). Diabetic microangiopathy was diagnosed on the basis of the presence of nephropathy (advanced stage of microalbuminuria, ≥30 mg albumin/g creatinine), simple or more advanced stage retinopathy, and/or peripheral/autonomic neuropathy, as per the criteria defined by the Japan Diabetes Society. The stage of chronic kidney disease (CKD) was evaluated, and the estimated glomerular filtration (eGFR) was calculated as per the guidelines proposed by the Japanese Society of Nephrology. Diabetic macroangiopathy was clinically diagnosed by the coexistence of coronary artery disease, cerebrovascular disease, and peripheral artery disease and/or the administration of medications for their treatment.

All patients provided written informed consent to participate in this study. The study protocol was approved by the ethics review board of our institution.

**Vascular Measurement**

**Measurement of FMD and NMD using a Novel Ultrasound System**

We measured FMD and NMD according to the international guidelines and Japanese guidelines from Vascular Failure Workshop Group using a novel vascular ultrasound system equipped with an edge-tracking system for 2D imaging and pulsed Doppler flow velocimeter for automatic measurement (UNEXEF; Unex Co. Ltd., Nagoya, Japan). Regarding the reproducibility of the measurement of FMD using this system, its correlation coefficient between two examinations was reported to be 0.86 with a coefficient of variance (CV) of 11.2%.

In brief, the diameter of the brachial artery at rest was measured in
the cubital region, and subsequently, the cuff was inflated to 50 mm Hg above systolic blood pressure for 5 min and deflated. The diameter at the same point of the artery was monitored continuously, and the maximum dilatation from 45-60s after deflation was recorded. For the measurement of NMD, after a 15-min interval for vessel recovery, sublingual glyceril trinitrate (75 μg) was administered, and the maximum dilatation of the brachial artery at the same point was confirmed and measured by a plateau or no increase of artery diameter using real-time monitoring of artery diameter during at least 1 min after the initiation of maximum dilatation. In 34 subjects, NMD with nitroglycerin administration were not measured because of bradycardia, hypotension, or glaucoma. FMD and NMD were calculated as follows: FMD or NMD (%) = (maximum diameter − diameter at rest) × 100/diameter at rest.

### Measurement of IMT and Stiffness Parameter β by using an Ultrasonic Phase-Locked Echo-Tracking System

The IMT and stiffness parameter β of the CCA were measured by an ultrasonic phase-locked echo-tracking system, which was equipped with a high-resolution real-time 13-MHz linear scanner (Prosound SDD 6500; Aloka, Tokyo), as previously reported\(^\text{11, 14, 19}\). In brief, approximately 4 cm of the CCA was examined. The image was focused on the far wall of the artery. The IMT was measured in both carotid arteries at the site of the most advanced atherosclerotic lesion, i.e., at the site where the distance between the lumen-intimal interface and the media-adventitia interface of the far wall was the greatest. Stiffness parameter β was calculated from the blood pressure and the diameter of the artery as follows:

\[
\text{stiffness parameter } \beta = \ln \left( \frac{P_s/P_d}{D_d/(D_s-D_d)} \right)
\]

where Ps and Pd are systolic and diastolic blood pressures and Ds and Dd are systolic and diastolic inner diameters of the artery, respectively. The highest value of the carotid IMT and the greater of the stiffness parameter values determined for the CCA on both sides were defined as stiffness parameter β for each patient.

### Physical and Laboratory Findings

Blood pressure was determined by the conventional cuff method using a mercury sphygmomanometer after the subject had rested for at least 15 min. Blood was withdrawn after an overnight fast for analysis of the serum concentrations of glucose, total cholesterol. The glycosylated hemoglobin HbA1c (%) was estimated as an NGSP equivalent value (%) calculated by the formula HbA1c (%) = HbA1c (JDS; %) + 0.4%, considering the relational expression of HbA1c (%) measured by standard laboratory methods and the previous Japanese standard materials\(^\text{19}\).

### Statistical Analysis

Statistical analyses were performed using the Stat View 5 system (SAS Institute, Cary, NC). All results are expressed as the means±SD, unless otherwise indicated. Analysis of variance (ANOVA) with Scheffe’s test was used for comparisons among groups. Simple regression analyses and multiple regression analyses were performed to evaluate the relationships between principal cardiovascular risk factors and among the parameters of atherosclerosis (FMD, NMD, IMT, and stiffness parameter β). In multiple regression analyses, FMD/NMD and IMT/stiffness parameter β were set as dependent variables, and age, waist circumference, smoking status, duration of diabetes, systolic blood pressure, HbA1c level, serum total cholesterol level, serum creatinine level, and treatment of statin or ACEI/ARB were set as independent variables. A p value of 0.05 was considered significant.

### Results

Table 1 shows the clinical characteristics and the values of the surrogate markers for atherosclerosis, namely, FMD/NMD, IMT, and stiffness parameter β for all T2DM patients and their CKD stage. FMD was \(6.4 ± 3.9\%\) (range, \(0.7-18.9\%\)), and NMD was \(13.4 ± 6.6\%\) (range, \(0.5-28.9\%\)). The IMT of the carotid artery was \(1.14 ± 0.64\) mm (range, \(0.20-3.80\) mm), and stiffness parameter β was \(12.1 ± 4.8\) (range, \(4.5-35.4\)). No significant differences were noted in FMD between T2DM patients with and those without microangiopathy (\(6.3 ± 3.9\%\) vs. \(6.6 ± 3.8\%, \ p = 0.724\)). The NMD in T2DM patients with microangiopathy was significantly lower than that in those without (\(12.1 ± 6.3\%\) vs. \(15.7 ± 6.4\%, \ p = 0.001\)). Both IMT and stiffness parameter β in T2DM patients with microangiopathy were significantly higher than those in T2DM patients without microangiopathy (\(1.26 ± 0.67\) vs. \(0.91 ± 0.49\), \(p = 0.003\); \(12.7 ± 4.9\) vs. \(10.8 ± 4.6\, \ p = 0.011\), respectively). Similarly, with regard to the coexistence of cardiovascular complications, no significant difference was noted in FMD between T2DM patients with and those without macroangiopathy (\(5.8 ± 3.8\%\) vs. \(6.6 ± 3.9\, \ p = 0.225\)). The NMD in T2DM patients with macroangiopathy was significantly lower than in those without macroangiopathy (\(10.3 ± 5.7\%\) vs. \(14.4 ± 6.5\, \ p = 0.0009\)). Furthermore, we examined the impact of the stage of CKD, a strong determinant of atherosclerosis, on FMD and...
NMD. As shown in Fig.1, FMD tended to decrease with the progression of CKD, but the decrease was statistically insignificant (stage 1, 8.1 ± 4.7; stage 2, 6.3 ± 3.8; stage 3, 6.4 ± 3.4; stage 4/5, 5.0 ± 3.9; p = 0.071); however, NMD decreased significantly with the progression of CKD (stage 1, 15.8 ± 7.1; stage 2, 14.2 ± 6.3; stage 3, 12.5 ± 6.6; stage 4/5, 8.3 ± 4.3; p = 0.005). The decrease in NMD was greater than that in FMD for the progression of CKD.

Table 2 shows the correlation coefficients obtained by simple linear regression analysis between clinical risk factors and the surrogate markers FMD/NMD and IMT/stiffness parameter β. FMD was significantly correlated with age and systolic blood pressure, while NMD was correlated with age, duration of diabetes, systolic blood pressure, and serum creatinine level. NMD showed a closer association with these risk factors than FMD.

In all T2DM patients, FMD was negatively correlated with stiffness parameter β, but not with IMT (r = -0.221, p = 0.003; r = -0.031, p = 0.681, respectively), while NMD was negatively correlated with both stiffness parameter β and IMT (r = -0.193, p = 0.019; r = -0.243, p = 0.003, respectively), as shown in Fig.2. The association between FMD and NMD was weakly significant (r = 0.292, p = 0.0003).

To identify possible factors contributing to an increase in FMD/NMD and IMT/stiffness parameter β, multiple regression analyses were performed as described in the statistical analysis section (Table 3). In this model, the significant independent contributors to FMD were age, smoking, systolic blood pressure, HbA1c level, and serum total cholesterol and those to NMD were age, systolic blood pressure, and waist circumference.

Discussion

The present study investigated the association of clinical risk factors and concomitant diabetic micro- and macroangiopathy with FMD and NMD and compared this association with that between the former and subclinical carotid atherosclerosis, IMT, and stiffness parameter β in 181 T2DM patients. Our findings showed that FMD and NMD show different degrees of association with clinical risk factors and coexistent micro-/macroangiopathy, although they were associated with each other to some extent. Furthermore, they showed different associations with the surrogate markers of subclinical carotid atherosclerosis, namely, IMT and stiffness parameter β. These findings indicate that endothelial and smooth muscle dysfunction of the arterial wall do not worsen to the same degree during the development of atherosclerosis.

Table 1. Clinical characteristics and vascular surrogate markers, FMD/NMD, IMT, and stiffness parameter β in type 2 diabetic subjects according to CKD stage groups
and that FMD and NMD have different clinical implications as surrogate markers for CVDs.

The FMD of the brachial artery has been reported to be closely associated with endothelial function of the coronary artery\(^1,2\) and is recognized as an initial marker of atherosclerotic change. Evidence indicates that it can predict the development of CVD in high-risk individuals, such as those with peripheral artery diseases, coronary artery disease, and CKD\(^1,2,20\). On the other hand, other studies have reported that the evaluation of FMD has limited application in some clinical situations. FMD has been reported to be associated with the principal cardiovascular risk factors and the estimated 10-year risk of coronary heart disease only in low-risk populations\(^21\). In addition, it has been reported to be a predictor of future cardiovascular events but has shown very little prognostic accuracy for predicting traditional cardiovascular risk scores/factors in older adults\(^22\).

Unlike FMD, reports regarding the prognostic value of NMD are limited. The vasodilator response to exogenous nitric oxide was reported to be impaired in asymptomatic subjects with reduced FMD, which is consistent with smooth muscle dysfunction in adults at risk for atherosclerosis\(^23\). NMD in type 1 diabetes was negatively correlated with the HbA1c level, suggesting that chronic hyperglycemia may further deteriorate vascular smooth muscle cell dysfunc-
FMD/NMD and Type 2 Diabetes

FMD and NMD are closely related with those of previous studies. NMD exhibited a stronger association than FMD with carotid IMT, stiffness parameter $\beta$, and the coexistence of micro- and macroangiopathy. In particular, NMD decreased significantly with the progression of CKD. To date, the possible implications of NMD as a surrogate marker remain unclear. In many studies, NMD has been used as a control-like marker for comparison with FMD. NMD is affected or determined by vascular smooth muscle cell dysfunction and surrounding extracellular matrix in the medial layer of the arterial wall. The accelerating risk factors for atherosclerosis, such as hyperglycemia, dyslipidemia, hypertension, and uremia, may exert long-term cumulative effects on vascular smooth muscle cell and the surrounding matrix in the medial layer rather than on the endothelium. In fact, advanced glycation end-product (AGE) accumulation has been shown in the intima-media region of human and canine vascular tissues $^{28, 29}$ and is closely associated with arterial stiff-

![Figure 2](image)

**Fig. 2.** Association of flow-mediated dilatation (FMD) (A, B) and nitroglycerin-mediated dilatation (NMD) (C, D) with subclinical carotid atherosclerosis markers, intima-media thickness (IMT) (A, C) and stiffness parameter $\beta$ (B, D) in all type 2 diabetes patients.

FMD shows an inverse correlation with stiffness parameter $\beta$ (B: $n=181$, $r=-0.221$, $p=0.003$), but not with IMT (A: $n=181$, $r=-0.031$, $p=0.681$), while NMD shows an inverse correlation with both IMT (C: $n=147$, $r=-0.243$, $p=0.003$) and stiffness parameter $\beta$ (D: $n=147$, $r=-0.193$, $p=0.019$).

tion $^{24}$. Opinions regarding the interrelations between FMD and IMT are discrepant, although both are recognized as surrogate markers for CVDs. Ravikumar et al. showed that FMD correlated well with carotid IMT or the augmentation index, which is a marker of functional changes in the systemic arterial tree inagematched non-diabetic subjects, but not in diabetic subjects $^{25}$. Similarly, FMD was reported to show an inverse association with the index of small artery elasticity in both diabetic and healthy subjects, but not with IMT $^{26}$. Recently, Halcox et al. reported that FMD failed to show any significant association with carotid IMT at baseline but that it predicted annual progression of carotid IMT at the 6-year follow-up of 213 non-smoking middle-aged participants who did not initially have clinical CVD and diabetes $^{27}$. In our study, FMD did not show any significant association with IMT, but showed a close association with stiffness parameter $\beta$, which is a functional index of the artery. Thus, our findings regarding FMD are consistent with those of previous studies.

NMD exhibited a stronger association than FMD with carotid IMT, stiffness parameter $\beta$, and the coexistence of micro- and macroangiopathy. In particular, NMD decreased significantly with the progression of CKD. To date, the possible implications of NMD as a surrogate marker remain unclear. In many studies, NMD has been used as a control-like marker for comparison with FMD. NMD is affected or determined by vascular smooth muscle cell dysfunction and surrounding extracellular matrix in the medial layer of the arterial wall. The accelerating risk factors for atherosclerosis, such as hyperglycemia, dyslipidemia, hypertension, and uremia, may exert long-term cumulative effects on vascular smooth muscle cell and the surrounding matrix in the medial layer rather than on the endothelium. In fact, advanced glycation end-product (AGE) accumulation has been shown in the intima-media region of human and canine vascular tissues $^{28, 29}$ and is closely associated with arterial stiff-
of FMD and NMD. However, most diabetic patients encountered in clinical settings have received or are under treatment with multiple anti-atherosclerotic drugs; therefore, these clinical conditions should be considered when the relevance of these surrogate markers is evaluated. Further prospective studies are required to clarify the effects of these limitations on the results of our study.

**Conclusion**

We examined the association of FMD and NMD by assessment with a novel ultrasound system with cardiovascular risk factors, micro-/macroangiopathy, and surrogate markers, IMT and stiffness parameter $\beta$ of carotid artery in 181 patients with T2DM. The NMD in patients with micro- and macroangiopathy was significantly lower and decreased with the progression of the CKD stage, but FMD did not. Furthermore, independent determinant factors of NMD were found to be different from those of FMD on multiple regression analyses. These findings reveal that the association of NMD with cardiovascular risk factors and diabetic angiopathy is different from that of FMD in T2DM.

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**Table 3.** Standard coefficients of possible contributing factors for FMD/NMD and carotid atherosclerosis markers obtained on multiple regression analyses

<table>
<thead>
<tr>
<th></th>
<th>FMD (%)</th>
<th>NMD (%)</th>
<th>IMT (mm)</th>
<th>Stiffness parameter $\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>$-0.335^{***}$</td>
<td>$-0.373^{***}$</td>
<td>$0.262^{**}$</td>
<td>$0.356^{***}$</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>$0.149$</td>
<td>$0.061$</td>
<td>$0.008$</td>
<td>$-0.013$</td>
</tr>
<tr>
<td>Smoking (yes $= 1$)</td>
<td>$-0.167^*$</td>
<td>$0.022$</td>
<td>$0.131$</td>
<td>$0.115$</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>$0.097$</td>
<td>$-0.162^*$</td>
<td>$0.059$</td>
<td>$0.229^{**}$</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>$-0.188^*$</td>
<td>$-0.205^*$</td>
<td>$0.215^{**}$</td>
<td>$0.214^{**}$</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>$-0.161^*$</td>
<td>$-0.072$</td>
<td>$0.025$</td>
<td>$0.136$</td>
</tr>
<tr>
<td>Total-c (mg/dL)</td>
<td>$0.201^*$</td>
<td>$0.005$</td>
<td>$0.102$</td>
<td>$0.105$</td>
</tr>
<tr>
<td>sCre (mg/dL)</td>
<td>$-0.077$</td>
<td>$-0.166$</td>
<td>$0.115$</td>
<td>$0.087$</td>
</tr>
<tr>
<td>Statin (yes $= 1$)</td>
<td>$-0.059$</td>
<td>$0.020$</td>
<td>$0.201^{**}$</td>
<td>$0.123$</td>
</tr>
<tr>
<td>ACEI/ARB (yes $= 1$)</td>
<td>$0.100$</td>
<td>$0.045$</td>
<td>$0.092$</td>
<td>$-0.052$</td>
</tr>
</tbody>
</table>

$R^2$ 0.177 $^{***}$ 0.250 $^{***}$ 0.267 $^{***}$ 0.251 $^{***}$

*p < 0.05; **p < 0.01; ***p < 0.001

Abbreviations: SBP, systolic blood pressures; HbA1c, glycosylated hemoglobin; Total-c, total cholesterol; sCre, serum creatinine; Statin, 3-hydroxy-3-coenzyme A reductase inhibitors; ACEI/ARB, angiotensin-converting enzyme inhibitor or an angiotensin II receptor blocker.
Conflict of Interest

None declared.

References


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