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Impaired peripheral circulation in lower-leg arteries caused by higher arterial stiffness and greater vascular resistance associates with nephropathy in type 2 diabetic patients with normal ankle–brachial indices

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ABSTRACT

Diabetic nephropathy is a major cause of lower-limb amputation. We enrolled 250 type 2 diabetic patients without apparent occlusive peripheral arterial disease (ankle–brachial indices >0.9) and 40 age-matched nondiabetic subjects consecutively admitted to our hospital. Flow volume and resistive index (RI), an index of vascular resistance, at the popliteal artery were evaluated using gated two-dimensional cine-mode phase-contrast magnetic resonance imaging. Brachial–ankle pulse wave velocity (baPWV) was measured as an index of arterial distensibility. Flow volume was negatively correlated with both baPWV ($p = 0.0009$) and RI ($p < 0.0001$) among the patients. When the patients were grouped into four subgroups with or without albuminuria and renal insufficiency according to the levels of urinary albumin excretion rate ($\geq$20 or <20 μg/min) and estimated glomerular filtration rate (eGFR) (<60 or $\geq$60 ml/min/1.73 m^2), albuminuric patients with renal insufficiency ($n = 30$) showed the lowest flow volume ($p = 0.0078$) and the highest baPWV ($p = 0.0006$) and RI ($p = 0.0274$) among the groups. Simple linear regression analyses demonstrated that eGFR correlated positively with flow volume ($p = 0.0020$) and negatively with baPWV ($p = 0.0258$) and RI ($p = 0.0029$) in patients with albuminuria ($n = 92$), but not with normoalbuminuria ($n = 158$). Impaired peripheral circulation in lower-leg arteries associates with nephropathy in diabetic patients even though they have normal ankle–brachial indices.

1. Introduction

Diabetic nephropathy is the leading cause of lower-limb amputation [1], end-stage renal failure and death from cardiovascular disease (CVD) [2]. Elevated urinary albumin excretion [3] and decrease in glomerular filtration rate [4] are powerful markers of increased cardiovascular morbidity and mortality for diabetic patients. Therefore, current diabetes guidelines recommend screening for elevated urinary albumin excretion as the earliest clinical evidence of nephropathy [5] in

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addition to screening for decline in estimated glomerular filtration rate (eGFR) calculated by the Modification of Diet in Renal Disease (MDRD) formula to detect chronic kidney disease attributed to diabetes [6]. Duplex Doppler sonography has demonstrated that alterations in glomerular capillaries and tubulointerstitial compartments of the kidney result in increase of intrarenal vascular resistance [7]. Type 2 diabetic patients with nephropathy are known to have elevated intrarenal vascular resistance compared with other types of renal disease [8]. Furthermore, among diabetic patients with nephropathy, increased vascular resistance in the intrarenal arteries associates with the severity of systemic atherosclerosis assessed by brachial–ankle pulse wave velocity (baPWV) as a marker of large artery stiffness [8], or intima-media thickness (IMT) in the carotid or femoral artery [9]. In the lower-leg arteries, diabetic patients show two types of insufficient arterial blood flow associated with the vessel wall properties. Firstly, diabetic condition is likely to increase atherosclerotic plaque formation in the vessel wall and promote peripheral artery occlusive disease (PAOD) in the lower extremities, resulting in reduced blood flow to lower limbs during exercise or at rest [10]. To help identify high-risk patients with PAOD, the ankle–brachial index (ABI) is generally used [11]. Secondly, arterial distensibility and vascular resistance reduce blood supply in the lower-leg arteries even though the individual has no apparent PAOD [12]. Gradual accumulation of advanced glycation end-products (AGEs) [13], increased IMT [14], and radiologically detectable calcified deposits in the vessel walls [15] are seen at different stages of the atherosclerotic process and are considered to be responsible for the pathogenesis of vascular rigidity. Endothelial dysfunction develops during the atherosclerotic process and is associated with reduction in vasodilator capacity and increase in peripheral vascular resistance [16]. Although it is desirable to ameliorate insufficient arterial blood flow before the onset of lower-limb ischemia, the role of diabetic nephropathy in peripheral circulation in lower-leg arteries has not been fully elucidated.

The aim of the present study was to clarify the association of impaired peripheral circulation in lower-leg arteries with nephropathy in type 2 diabetic patients with normal ABI by using a new technique of two-dimensional cine-mode phase contrast magnetic resonance imaging (2D-cine-PC MRI).

2. Materials and methods

We enrolled 250 type 2 diabetic patients and 40 age-matched nondiabetic subjects ranging in age from 50 to 75 years who had been consecutively admitted to our hospital. All patients were admitted for strict glycemic control or assessment of diabetic complications, and no patients had history of cerebrovascular disease, coronary arterial disease, and/or PAOD. Patients were considered to have cerebrovascular disease if they had a history of sudden focal neurological deficit. Coronary arterial disease was diagnosed if the patients had a history of myocardial infarction or showed abnormal electrocardiographic findings. PAOD was diagnosed if the patients had an abnormal ABI [11]. Patients who had foot edema caused by heart failure, liver cirrhosis or severe nephropathy (serum creatinine >2 mg/dl), malignant neoplasm, alcohol abuse, acute illness, urinary tract infections or hematuria were excluded from the study. Presence of pyuria or hematuria was diagnosed by microscopic examination and counting of the number of white blood cells or red blood cells per high-power field in the last voided urine of a 24-h collection. Every patient with hypertension (>140/90 mmHg) received antihypertensive treatment to reduce the risk of CVD events. Although administration of renin–angiotensin system inhibitors, including angiotensin converting enzyme inhibitor (ACEI) [17] and angiotensin II receptor blocker (ARB) [18], can decrease the urinary albumin excretion and slow the decline in glomerular filtration rate among type 2 diabetic patients, those medications were used for the management of raised blood pressure. The study was approved by the ethics committee of our institution, and informed consent was obtained from all patients before the examinations.

An automatic device (BP-203RPE; Colin, Komaki, Japan) was used to measure ABI and baPWV, as an index of the elastic properties of large arteries [19]. Because the PWV from the heart to the brachial artery in diabetic patients is similar to that in control subjects, baPWV is regarded as a quantitative measure of arterial stiffness from the heart to the ankle. A trained ophthalmologist carried out fundus ophthalmoscopies and identified diabetic patients as either without retinopathy, having simple retinopathy or having proliferative retinopathy. Patients with diabetes were screened for distal symmetric polyneuropathy using a 128-Hz tuning fork applied to the bony prominence at the dorsalis surface of both great toes, just proximal to the nail bed [20]. When the tuning fork was placed on the foot for 10 s, if the patients required >10 s to detect the vibration, vibration perception was regarded as compromised. Each patient was also identified by smoking habit as being a current smoker or non-smoker. Nonsmokers were defined as not having tobacco consumption for at least the previous 3 years. Urinary albumin excretion rate (AER) was measured in 24-h urine samples. Diabetic patients were classified as having normoalbuminuria ($n = 158$), microalbuminuria ($n = 56$), or overt proteinuria ($n = 36$) when the AER was <20, 20–200, or >200 μg/min, respectively. The Japanese ethnic factor for the MDRD equation has been reported to be 0.881 [21]. Therefore, the eGFR is calculated by the MDRD formula as follows: eGFR (ml/min/1.73 m^2) = $0.881 \times 186.3 \times Age^{-0.203} \times SCr^{-1.154}$ (if female × 0.742), where SCr is serum creatinine (mg/dl). With or without renal insufficiency was defined as eGFR of <60 or ≥60 ml/min/1.73 m^2 [22].

An MRI scanner operating at 1.5 T (Signa Horizon-LX; GE Medical Systems, Milwaukee, WI) was used for the following experimental protocols as previously described [23]. All patients were at rest in the supine position during examinations, which were done in a temperature-controlled room at 25 °C. To set up the individual flow analysis, the popliteal artery was depicted by gated 2D time-of-flight magnetic resonance angiography. A single slice was oriented perpendicular to the flow direction, and flow data were obtained using 2D-cine-PC MRI with 80-cm/s velocity encoding triggered by peripheral gating. Flow data were analyzed on an Advantage Windows version 4.2 workstation (GE Medical Systems, Milwaukee, WI) to determine the direction and velocity through the cardiac cycle. The instantaneous flow volume

at 16 equally spaced time points through the cardiac cycle was calculated from the individual velocity images by integrating the velocity across the area of the vessel. A resistive index (RI), which allows quantitative analysis of the waveform and associates with arterial resistance to blood flow, has been defined as (A – B)/A, where A is the systolic peak velocity and B is the end-diastolic velocity [24].

Statistical evaluation was carried out on SPSS software version 11.0 for Windows (SPSS, Chicago, IL). Comparisons between the diabetic patients and their control group were done using the unpaired Student's t-test. A multiple comparison of significant differences among the four groups was carried out by one-way ANOVA followed by Scheffe's F-test. The chi-squared test for 2-by-2 or Bonferroni test for 2-by-4 contingency table was used to compare the frequencies between two or among four groups. Values were expressed as the means ± S.D. We considered p-values <0.05 to be statistically significant.

3. Results

3.1. *All subjects*

Clinical characteristics and vascular parameters in all subjects are shown in Table 1. There were no significant differences between the groups for prevalence of male sex, age, body mass index (BMI), total cholesterol (TC), prevalence of smoking status, and eGFR. However, compared with nondiabetic subjects, diabetic patients had higher fasting plasma glucose (FPG) ($p < 0.0001$), hemoglobin A1c (HbA1c) ($p < 0.0001$), triglycerides (TGs) ($p = 0.0261$), and systolic blood pressure (sBP) ($p = 0.0230$) and lower HDL cholesterol (HDL-C) ($p = 0.0024$), and diastolic blood pressure (dBP) ($p = 0.0202$). Although ABI, heart rate and systolic and early diastolic flow volumes were similar between the groups, diabetic patients had lower total ($p = 0.0005$) and late diastolic ($p < 0.0001$) flow volumes and higher baPWV ($p < 0.0001$) and RI ($p < 0.0001$) than those in the nondiabetic subjects, indicating that arterial stiffness and vascular resistance are possible risk factors for reduced blood flow in diabetic patients. To clarify the associations among those vascular parameters, simple linear regression analyses were performed. Total flow volume, baPWV, and RI were negatively (total flow volume vs. baPWV, $r = -0.209$, $p = 0.0009$; total flow volume vs. RI, $r = -0.645$, $p < 0.0001$) or positively (baPWV vs. RI, $r = 0.176$, $p = 0.0053$) correlated with each other, suggesting that coexistence of arterial stiffness and vascular resistance acts as a risk factor for impaired peripheral circulation in lower-leg arteries in diabetic patients even though they have a normal ABI.

3.2. *Peripheral circulation and nephropathy*

Simple linear regression analysis demonstrated that the entire group of diabetic patients ($n = 250$) did not show a significant

Table 1 – Clinical characteristics and vascular parameters in all subjects

	Nondiabetic subjects	Diabetic patients	p-Value
Number	40	250	
Male sex (%)	21 (52.5)	144 (57.6)	0.6652
Age (years)	59.8 ± 6.5	61.5 ± 6.6	0.1243
BMI (kg/m^2)	22.6 ± 1.9	23.6 ± 3.5	0.0821
Duration of diabetes (years)	–	12.3 ± 8.4	–
Treatment (D/OHD/I)	–	16/86/148	–
FPG (mmol/l)	5.35 ± 0.39	6.97 ± 1.61	<0.0001
HbA1c (%)	4.7 ± 0.4	8.2 ± 1.3	<0.0001
TC (mmol/l)	4.92 ± 0.57	4.85 ± 0.80	0.5898
HDL-C (mmol/l)	1.43 ± 0.42	1.24 ± 0.35	0.0024
TGs (mmol/l)	1.14 ± 0.31	1.40 ± 0.75	0.0261
Blood pressure (mmHg)			
Systolic	122 ± 7	128 ± 17	0.0230
Diastolic	75 ± 8	71 ± 10	0.0202
ACEI and/or ARB (%)	–	87 (34.8)	–
Smokers (%)	15 (37.5)	132 (52.8)	0.1038
Retinopathy (%)	–	107 (42.8)	–
Albuminuria (%)	–	92 (36.8)	–
Neuropathy (%)	–	110 (44.0)	–
eGFR (ml/min/1.73 m^2)	71.7 ± 7.2	72.9 ± 17.2	0.7401
ABI	1.12 ± 0.09	1.11 ± 0.09	0.4977
baPWV (cm/s)	1290 ± 113	1758 ± 359	<0.0001
Heart rate (bpm)	71 ± 9	72 ± 11	0.6298
Flow volume (ml/min)			
Total	90.6 ± 19.3	75.6 ± 26.0	0.0005
Systolic	84.5 ± 15.8	81.2 ± 19.7	0.3203
Early diastolic	−10.8 ± 7.7	−13.4 ± 9.3	0.0963
Late diastolic	17.0 ± 6.9	7.8 ± 9.1	<0.0001
Resistive index	0.969 ± 0.026	1.017 ± 0.045	<0.0001

Data are expressed as n (%) or means ± S.D. D, diet; OHD, oral hypoglycemic drugs; I, insulin.

Table 2 – Diabetic patients grouped into four subgroups with or without albuminuria and renal insufficiency according to the levels of urinary albumin excretion rate of ≥20 or <20 μg/min and estimated glomerular filtration rate of <60 or ≥60 ml/min/1.73 m²

	Normoalbuminuria		Albuminuria	
	Without renal insufficiency	With renal insufficiency	Without renal insufficiency	With renal insufficiency
eGFR (ml/min/1.73 m²)	79.3 ± 12.9	51.6 ± 6.9	78.7 ± 12.7	47.2 ± 9.8
Overt proteinuria (%)	–	–	13 (21.0)	23 (76.7)
Number	136	22	62	30
Male sex (%)	75 (55.1)	9 (40.9)	40 (64.5)	20 (66.7)
Age (years)	61.1 ± 6.7	64.0 ± 5.5	60.2 ± 6.1	64.6 ± 6.5[g]
BMI (kg/m²)	23.1 ± 3.2	24.5 ± 4.5	24.2 ± 3.5	24.0 ± 3.7
Duration of diabetes (years)	10.4 ± 7.6	11.3 ± 6.5	13.5 ± 8.5	19.1 ± 9.2[c,e,g]
Treatment (D/OHD/I)	7/52/77	2/5/15	5/21/36	2/8/20
FPG (mmol/l)	6.86 ± 1.55	7.16 ± 1.29	7.16 ± 1.83	6.99 ± 1.67
HbA1c (%)	8.3 ± 1.4	8.4 ± 1.0	8.2 ± 1.2	8.1 ± 1.4
TC (mmol/l)	4.80 ± 0.75	4.94 ± 0.70	4.90 ± 0.81	4.95 ± 1.02
HDL-C (mmol/l)	1.29 ± 0.34	1.31 ± 0.49	1.16 ± 0.32	1.14 ± 0.32
TGs (mmol/l)	1.26 ± 0.71	1.54 ± 1.07	1.57 ± 0.63	1.61 ± 0.73
Blood pressure (mmHg)				
Systolic	123 ± 14	124 ± 15	134 ± 19[c]	143 ± 17[c,f]
Diastolic	71 ± 9	69 ± 9	72 ± 10	75 ± 11
ACEI and/or ARB (%)	32 (23.5)	6 (27.3)	28 (45.2)[a]	21 (70.0)[b]
Smokers (%)	65 (47.8)	11 (50.0)	38 (61.3)	18 (60.0)
Retinopathy (%)	41 (30.1)	6 (27.3)	36 (58.1)[b]	24 (80.0)[b]
Neuropathy (%)	52 (38.2)	8 (36.4)	35 (56.5)	15 (50.0)
ABI	1.11 ± 0.08	1.09 ± 0.08	1.12 ± 0.08	1.11 ± 0.11
baPWV (cm/s)	1668 ± 330	1714 ± 407	1873 ± 367[b]	1961 ± 289[c]
Heart rate (bpm)	71 ± 12	71 ± 10	73 ± 10	75 ± 11
Flow volume (ml/min)				
Total	75.8 ± 25.7	84.3 ± 16.5	79.5 ± 27.1	59.8 ± 25.0[a,e,h]
Systolic	80.0 ± 18.9	86.2 ± 14.4	87.3 ± 20.8	70.6 ± 19.6[d,h]
Early diastolic	−13.1 ± 9.6	−10.2 ± 6.3	−15.2 ± 9.7	−13.7 ± 8.5
Late diastolic	8.9 ± 9.3	8.3 ± 6.5	7.4 ± 9.6	3.0 ± 8.0[a]
Resistive index	1.012 ± 0.046	1.011 ± 0.037	1.020 ± 0.042	1.039 ± 0.040[a]

Data are expressed as *n* (%) or means ± S.D. D, diet; OHD, oral hypoglycemic drugs; I, insulin. [a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$ vs. normoalbuminuric patients without renal insufficiency; [d]$p < 0.05$, [e]$p < 0.01$, [f]$p < 0.001$ vs. normoalbuminuric patients with renal insufficiency; [g]$p < 0.05$, [h]$p < 0.01$ vs. albuminuric patients without renal insufficiency.

correlation between total flow volume and eGFR. Therefore, to clarify the role of nephropathy in impaired blood flow in lower-leg arteries, diabetic patients were classified into four subgroups with or without albuminuria and renal insufficiency. Although they showed normoalbuminuria, 22 of the 250 (8.8%) patients had renal insufficiency. Clinical characteristics and vascular parameters in those subgroups are shown in Table 2. There were no significant differences among the groups for frequency of male gender, BMI, FPG, HbA1c, TC, HDL-C, TGs, frequency of smoking habit and neuropathy. However, albuminuric patients with renal insufficiency showed the oldest age ($p = 0.0249$), longest duration of diabetes ($p < 0.0001$), and highest sBP ($p < 0.0001$), frequency of patients taking ACEI and/or ARB ($p < 0.01$), and frequency of retinopathy ($p < 0.01$) among the groups. There were no significant differences in ABI among the groups. Normoalbuminuric patients with and without renal insufficiency had similar baPWV, whereas albuminuric patients with renal insufficiency showed the highest baPWV ($p = 0.0006$) among the groups, indicating that elevated urinary albumin excretion is a possible risk factor for arterial stiffness. Waveform analysis at the popliteal artery using gated 2D-cine-PC MRI is shown in Fig. 1. Normoalbuminuric patients without renal insufficiency showed a typically triphasic waveform, which could be clearly separated into systolic, early diastolic and late diastolic phases during the cardiac cycle (Fig. 1A). Waveforms in the normoalbuminuric patients with renal insufficiency (Fig. 1B) and albuminuric patients without renal insufficiency (Fig. 1C) were similar to those in the normoalbuminuric patients without renal insufficiency, whereas albuminuric patients with renal insufficiency (Fig. 1D) showed reduced blood flow and abnormal flow reversal in late diastole, suggesting the presence of higher arterial stiffness and greater vascular resistance in lower-leg arteries. There were no significant differences in heart rate and early diastolic flow reversal among the groups. Although normoalbuminuric patients with renal insufficiency and albuminuric patients without renal insufficiency had similar flow parameters, albuminuric patients with renal insufficiency demonstrated the lowest total ($p = 0.0078$), systolic ($p = 0.0019$) and late diastolic ($p = 0.0147$) flow volumes and the greatest RI ($p = 0.0274$) among the groups, suggesting that parallel development of albuminuria and renal dysfunction is a possible risk factor for vascular resistance and impaired blood

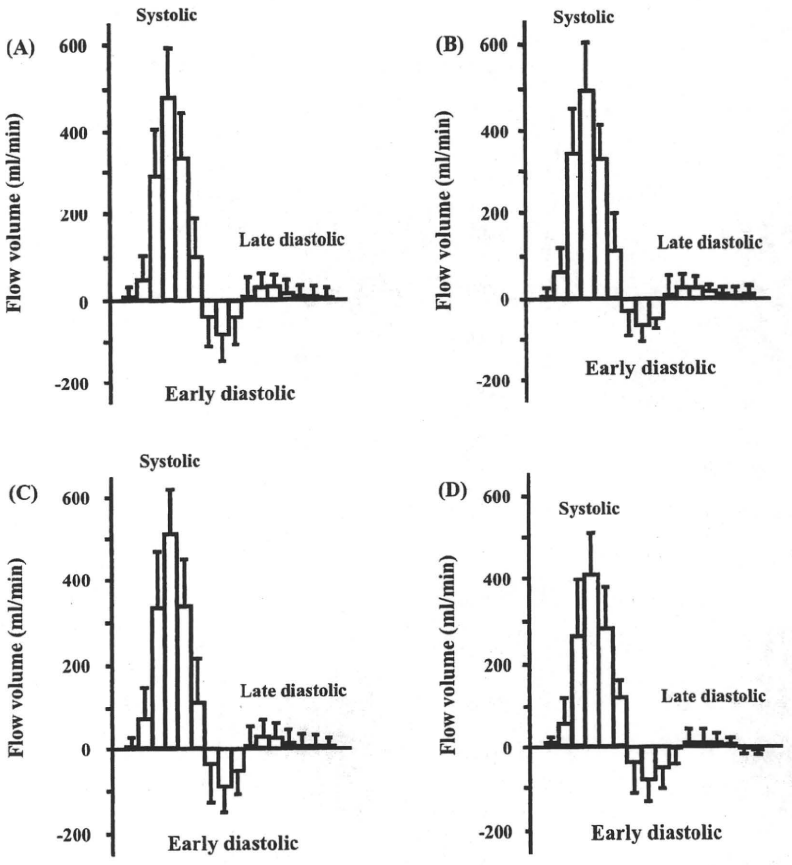

Fig. 1 – Waveform analysis at the popliteal artery in type 2 diabetic patients with normal ankle–brachial indices (ABI > 0.9) grouped into four subgroups with or without albuminuria and renal insufficiency according to the levels of urinary albumin excretion rate (AER) of ≥20 or <20 μg/min and estimated glomerular filtration rate (eGFR) of <60 or ≥60 ml/min/1.73 m^2. Data are expressed as means ± S.D. (A) Normoalbuminuric patients without renal insufficiency; (B) normoalbuminuric patients with renal insufficiency; (C) albuminuric patients without renal insufficiency; (D) albuminuric patients with renal insufficiency.

flow. To clarify the role of elevated urinary albumin excretion in peripheral circulation in lower-leg arteries, patients were classified into two subgroups with normoalbuminuria (n = 158) or albuminuria (n = 92). Simple linear regression analyses, as shown in Fig. 2, revealed that among the patients with albuminuria eGFR was correlated positively with total flow volume (r = 0.319, p = 0.0020) (Fig. 2A) and negatively with baPWV (r = −0.232, p = 0.0258) (Fig. 2B) and RI (r = −0.308, p = 0.0029) (Fig. 2C). However, there were no significant correlations between eGFR and those vascular parameters in patients with normoalbuminuria (Fig. 2D–F).

4. Discussion

4.1. *Peripheral circulation*

In the present study, waveforms at the popliteal artery in the normoalbuminuric patients with renal insufficiency and albuminuric patients without renal insufficiency were similar to those in the normoalbuminuric patients without renal insufficiency, whereas the albuminuric patients with renal insufficiency showed reduced blood flow and abnormal flow reversal in late diastole. These results suggest that albuminuric patients with renal insufficiency have higher arterial stiffness and greater vascular resistance in lower-leg arteries. There are important differences among elastic and muscular arteries and arterioles. All are impaired in diabetic patients [16,25]. Large arteries, including the aorta and its major branches, have elastic properties of the vessel wall and act as carrying vessels and blood supply reservoirs [26]. When there is a decrease in arterial elasticity, less blood can be stored in these arteries, resulting in a decrease in diastolic forward flow. The medium- and small-caliber arteries and arterioles, which have functional smooth muscles in the vessel wall, act as resistance vessels regulating blood flow to the capillaries [26]. Endothelial dysfunction and reduced lumen diameter in small vessels can increase peripheral vascular resistance [16], resulting in an abnormal flow reversal in late diastole. These vascular abnormalities can coexist in the same individual [27]. Our data demonstrated that blood flow, arterial stiffness, and vascular resistance were negatively or positively correlated with each other, suggesting that coexistence of arterial stiffness and vascular resistance acts as a risk factor for

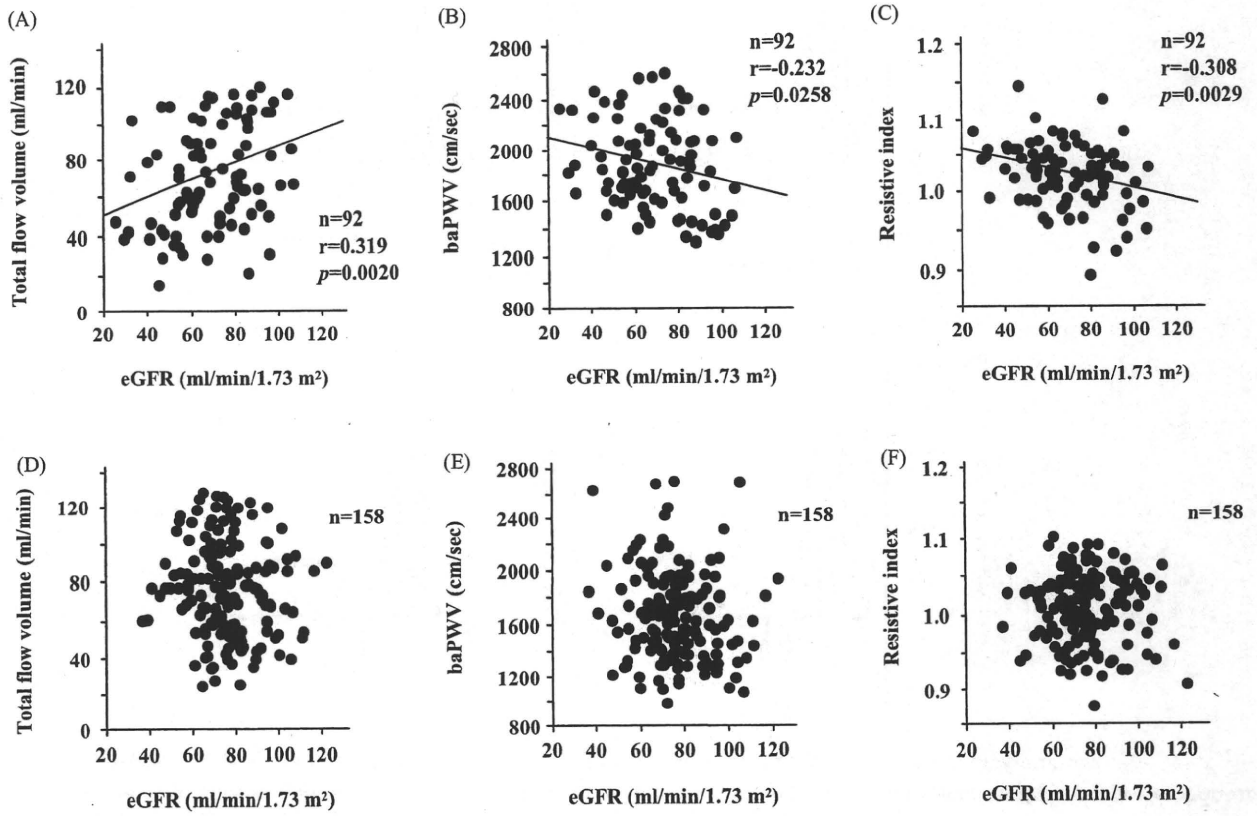

Fig. 2 – Simple linear regression analyses between estimated glomerular filtration rate (eGFR) and total flow volume, brachial–ankle pulse wave velocity (baPWV), or resistive index in lower-leg arteries in albuminuric (n = 92) (A–C) or normoalbuminuric (n = 158) (D–F) type 2 diabetic patients with normal ankle–brachial indices (ABI > 0.9).

impaired peripheral circulation in lower-leg arteries in diabetic patients even though they have a normal ABI.

4.2. *Nephropathy*

As we reported previously, when the diagnostic criterion for critical lower-limb ischemia in diabetic patients of transcutaneous oxygen tension of <50 mmHg at the dorsum of the foot was used [28], 16 of 60 (26.7%) diabetic patients had ischemic lower limbs although they had no apparent PAOD [23]. Diabetic nephropathy is a major cause of lower-limb amputation associated with peripheral vascular disorders [1]. It is desirable to ameliorate insufficient arterial blood flow before the onset of lower-limb ischemia. Endothelium actively regulates vascular tone and permeability and balance between coagulation and fibrinolysis [29]. Therefore, elevated urinary albumin excretion is regarded as a renal expression of systemic endothelial damage being extended to the whole arterial system [29]. In our present study, when the diabetic patients were classified into four subgroups with or without albuminuria and renal insufficiency, albuminuric patients with renal insufficiency showed the highest arterial stiffness, greatest vascular resistance, and lowest blood flow in lower-leg arteries among the groups. These results suggest that parallel development of albuminuria and renal dysfunction is a possible risk factor for arterial stiffness, vascular resistance, and impaired blood flow in lower-leg arteries. Our data revealed that renal function was correlated positively with blood flow and negatively with arterial stiffness and vascular resistance in lower-leg arteries among the patients with albuminuria, but not in those with normoalbuminuria. Alterations in capillaries, glomeruli and tubulointerstitial compartments of the kidney can increase renal vascular resistance assessed by duplex Doppler sonography [7], and those abnormalities associate with the severity of systemic atherosclerosis [8,9]. Therefore, although we did not measure intrarenal vascular resistance in the present study, we surmise that increased vascular resistance at the popliteal artery may associate with the severity of diabetic nephropathy.

Nephropathy in patients with type 2 diabetes is more heterogeneous than that in type 1 diabetes. Type 2 diabetic patients can develop renal impairment in the absence of increased albuminuria, and those conditions are initially developed during the prediabetic state secondary to age, hypertension and other factors [30]. In the United States, 30% of newly diagnosed type 2 diabetic patients showed renal insufficiency although they did not have retinopathy and albuminuria [31]. Our study revealed that 8.8% of our patients had normoalbuminuric renal insufficiency. It has been reported that risk for progression of renal failure or death in diabetic patients with normoalbuminuric renal insufficiency is lower than in those with albuminuric renal insufficiency [32]. In the present study, arterial stiffness, vascular resistance

and blood flow were similar between the normoalbuminuric patients with and without renal insufficiency. These results indicate that diabetic patients with normoalbuminuric renal insufficiency may have a lower risk of systemic arterial damage.

Among the study limitations of the present research, we note, firstly, that administration of ACEI [17] and ARB [18] can decrease urinary albumin excretion and slow the decline in the GFR among type 2 diabetic patients. Therefore, we cannot rule out the possibility that those medications may alter the natural history of diabetic nephropathy and peripheral circulation in lower-leg arteries. Secondly, our data were obtained in a Japanese population, and therefore it remains to be established whether these results can be generalized to other ethnicities. Additionally, we used cross-sectional study design. Further prospective study is necessary to clarify the impact of nephropathy on insufficient blood flow in lower-leg arteries among diabetic patients.

In conclusion, we have demonstrated that impaired blood flow in lower-leg arteries caused by higher arterial stiffness and greater vascular resistance associates with eGFR in albuminuric type 2 diabetic patients with normal ABI. Our findings are the first to clarify that diabetic nephropathy may increase the risk of lower-limb ischemia even though the patient has no apparent PAOD.

Conflict of interest

The authors state that they have no conflict of interest.

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Coronary artery calcification, arterial stiffness and renal insufficiency associate with serum levels of tumor necrosis factor-alpha in Japanese type 2 diabetic patients

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ABSTRACT

Although the kidneys are the major source of proinflammatory cytokines, association of tumor necrosis factor-alpha (TNF-α) with severity of atherosclerosis or kidney function in diabetic patients is unclear. Two hundred type 2 diabetic patients and 30 age-matched nondiabetic subjects consecutively admitted to our hospital were enrolled. The Agatston coronary artery calcium score (CACS), a quantitative marker of coronary atherosclerosis, was obtained using multidetector-row computed tomography. Arterial stiffness was assessed by brachial-ankle pulse wave velocity (baPWV). Diabetic patients had higher log(CACS + 1) ($p = 0.0089$), baPWV ($p = 0.0293$), frequency of elevated urinary albumin excretion (UAE) ($p < 0.0001$) and TNF-α ($p = 0.0029$) and similar estimated glomerular filtration rate (eGFR) compared to nondiabetic subjects. When diabetic patients were grouped into four subgroups with or without elevated UAE and renal insufficiency (UAE of ≥30 or <30 mg/24 h and eGFR of <60 or ≥60 ml/min per 1.73 m^2), patients with micro- and macroalbuminuric renal insufficiency showed the highest log(CACS + 1) ($p < 0.0001$), baPWV ($p = 0.0068$) and TNF-α ($p < 0.0001$) of these groups. Log(CACS + 1) ($p = 0.0008$) and baPWV ($p = 0.0006$) positively and eGFR ($p < 0.0001$) negatively correlated with TNF-α in diabetic patients. We find that coronary artery calcification, arterial stiffness, and renal insufficiency associate with circulating levels of TNF-α in type 2 diabetic patients.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in type 2 diabetic patients. Elevated urinary albumin excretion (UAE) [1,2] and a decrease in glomerular filtration rate [3,4] both contribute to the development of end-stage renal disease and CVD events in diabetic patients. Current diabetes guidelines recommend screening for elevated UEA as the earliest clinical evidence of nephropathy [5] as well as screening for a decline in estimated glomerular filtration rate (eGFR) as calculated by the modification of diet in renal disease (MDRD) formula to detect chronic kidney disease attributed to diabetes [6]. Vascular inflammation contributes to the pathogenesis of both micro- and macrovascular complications of diabetes through the actions of proinflammatory cytokines [7,8]. Tumor necrosis factor-alpha (TNF-α)

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can be produced by adipose tissues [9], kidneys [10] and atherosclerotic lesions in the arterial walls [11]. In the kidneys, advanced glycation end-products (AGEs) [12], angiotensin II [13] and oxidized LDL [14] can stimulate TNF-α synthesis from the renal cells and initiate local effects of renal damage. The activities of this cytokine are not limited to the renal injury; intra-arterial TNF-α administration can cause vascular inflammation and impair endothelial function [15]. Circulating levels of TNF-α are elevated in patients with metabolic syndrome [16], regarded as a prediabetic state, and in obese [17] or nephropathic [18] patients with type 2 diabetes. Such patients are therefore considered at high risk of CVD events.

Diabetic nephropathy is a major contributor to the development of atherosclerosis including vascular calcification [19] and arterial stiffness [20]. Vascular calcification is a manifestation of atherosclerosis that begins early in the atherosclerotic process [21]. A number of studies have documented the usefulness of electron-beam computed tomography (EBCT) [22] and multidetector-row computed tomography (MDCT) [23] for detection and evaluation of the extent of calcium deposits in the coronary arteries among asymptomatic patients before the onset of life-threatening cardiac events. Autopsy [22] and angiographic studies [24] of the coronary arteries reveal that the amount of calcification correlates with the burden of atherosclerosis. Diabetic patients show twofold prevalence of extensive coronary artery calcification compared with nondiabetic subjects [25]. Aortic stiffness is greater in diabetic patients than in nondiabetic subjects [26]. Large artery stiffness leads to increased systolic blood pressure and ventricular mass and hampers coronary filling in diastole [27], leading to increased CVD risk. Although coronary artery calcification and large artery stiffness are powerful predictors of CVD events [24,28], the association of TNF-α with these risk factors has not been fully elucidated in diabetic patients.

In the present study, we investigated the association of circulating levels of TNF-α with the severity of atherosclerosis or renal insufficiency in type 2 diabetic patients.

2. Materials and methods

Two hundred type 2 diabetic patients and 30 age-matched nondiabetic subjects ranging in age from 50 to 69 years who had been consecutively admitted to our hospital between November 2004 and October 2007 were recruited for the study. All patients were admitted for strict glycemic control or assessment of diabetic complications; no patients had clinical history of cerebrovascular disease, coronary arterial disease, or peripheral artery occlusive disease. Patients who abused alcohol or showed liver cirrhosis, severe nephropathy (serum creatinine > 176.8 μmol/l), malignant neoplasm, acute illness or urinary tract infections were excluded from the study. Patients were considered to have cerebrovascular disease if they had a history of sudden focal neurological deficit. Coronary arterial disease was diagnosed if the patients had a history of myocardial infarction or showed abnormal electrocardiographic findings. Peripheral arterial occlusive disease was diagnosed if the patient had an abnormal ankle-brachial index (ABI) of <0.9 at rest [29]. Presence of pyuria or hematuria was diagnosed by microscopic examination and counting of the number of white blood cells or red blood cells per high-power field in the last voided urine of a 24-h collection. Although administration of angiotensin converting enzyme inhibitor (ACEI) and angiotensin II receptor blocker (ARB) can act to prevent angiotensin II actions, decrease UAE, slow the decline in the glomerular filtration rate and reduce circulating levels of TNF-α due to anti-inflammatory effects [30–32], all patients with hypertension (>140/90 mmHg) received antihypertensive agents for the management of high blood pressure. Although hydroxymethylglutaryl coenzyme A reductase inhibitor (statins) can reduce serum levels of TNF-α [33], the medication was used for the treatment of dyslipidemia in patients. Nondiabetic subjects were admitted for hormonal evaluation of adrenal mass and subjects with normal function were included in the study. The study was approved by the ethics committee of our institution, and informed consent was obtained from all subjects before the examinations, which were done during their stay in the hospital.

Blood samples were drawn before breakfast in the morning after a 12-h overnight fast. Blood pressure was measured by a mercury sphygmomanometer with the patient in the sitting position after 5 min of rest. Three readings separated by 2 min were taken and the average was used for the analysis. An automatic device (BP-203RPE; Colin, Komaki, Japan) was used to measure ABI and brachial-ankle pulse wave velocity (baPWV), as an index of stiffness in elastic and muscular arteries [34]. A trained ophthalmologist carried out fundus ophthalmoscopies and defined diabetic patients as either without retinopathy, having simple retinopathy or proliferative retinopathy. Diabetic patients were classified by the measurement of UAE in 24-h urine collection as having normoalbuminuria, microalbuminuria, or macroalbuminuria when at least two of three specimens were at diagnostic thresholds of less than 30, 30–300, or greater than 300 mg/24 h, respectively [5]. The Japanese ethnic factor for the MDRD equation has been reported to be 0.881 [35]. Therefore, eGFR was calculated by the MDRD formula as follows: eGFR (ml/min per 1.73 m^2) = $0.881 \times 186.3 \times age^{-0.203} \times SCr^{-1.154}$ (if female × 0.742), where SCr is serum creatinine (mg/dl). Renal insufficiency was defined as eGFR of <60 ml/min per 1.73 m^2 [36]. Patients with diabetes were screened for distal symmetric polyneuropathy using a 128-Hz tuning fork applied to the bony prominence at the dorsalis surface of both great toes, just proximal to the nail bed [37]. When the tuning fork was placed on the foot for 10 s, if the patients required >10 s to detect the vibration, vibration perception was regarded as compromised. Each subject was also classified as a current smoker or nonsmoker. Nonsmokers were defined as not using tobacco for at least the previous 3 years. Serum TNF-α concentrations were measured by enzyme immunoassay kit (Quantikine HS Human TNF-α kit, R&D Systems, Minneapolis, MN).

Twenty-four contiguous slices of 2.5-mm thickness of the proximal coronary arteries were obtained during a single breath hold using a sixteen multidetector-row computed tomography (16-MDCT) scanner (LightSpeed Ultrafast 16, GE Healthcare, Milwaukee, WI). These scans are electrocardiographically triggered at 70% of the R–R interval, near the end of diastole and before atrial contraction, to minimize the effect of

cardiac motion. Coronary calcium score was analyzed on an Advantage Windows version 4.2 workstation (GE Healthcare, Milwaukee, WI) with scoring software (Smartscore version 3.5). The Agatston coronary artery calcium score (CACS), including both intimal and medial calcification in left main, left anterior descending, circumflex, and right coronary arteries, were obtained as a quantitative marker of calcium burden in the coronary artery [38]. From comparison between 16-MDCT and EBCT, 16-MDCT can detect coronary calcification and may be an alternative to EBCT [23]. The tests were almost equivalent in coronary artery calcium scoring: 16-MDCT score = 7.7 + 1.015 × EBCT score ($r^2 = 0.955$).

Statistical evaluation was carried out on SPSS software version 11.0 for Windows (SPSS Inc., Chicago, IL). Normality of distribution of each variable was assessed with the Kolmogorov–Smirnov test. Comparison between the two groups was done using the unpaired Student's *t*-test. A multiple comparison of significant differences among the four groups was carried out by one-way analysis of variance followed by Scheffe's *F*-test. The χ^2-test for 2 × 2 or Bonferroni test for 2 × 4 contingency tables was used to compare the frequencies between two groups or among four groups. Pearson's correlation coefficient was applied to assess the relation between normally distributed variables. Since distribution of CACS was highly skewed, common log-transformed CACS [log(CACS + 1)] was used for linear regression analysis. Stepwise multiple regression analyses were performed to evaluate the association of log(CACS + 1) or baPWV with 12 possible risk factors for atherosclerosis, four factors for microangiopathy and TNF-α in diabetic patients. The *F*-value was set at 4.0 at each step. Values are expressed as the means ± S.D. *p* values <0.05 were considered to be statistically significant.

3. Results

3.1. All subjects

Clinical characteristics of all subjects are summarized in Table 1. There were no significant differences between the groups for prevalence of male gender, age, body mass index (BMI), total cholesterol (TC), frequency of smoking habit and ABI. However, compared with nondiabetic subjects, diabetic patients had higher fasting plasma glucose (FPG) ($p < 0.0001$), hemoglobin A1c (HbA1c) ($p < 0.0001$), triglycerides (TGs) ($p = 0.0203$) and systolic blood pressure (sBP) ($p = 0.0186$) and lower HDL cholesterol (HDL-C) ($p = 0.0011$) and diastolic blood pressure (dBP) ($p = 0.0432$). Diabetic patients had higher frequency of elevated UAE ($p < 0.0001$), baPWV ($p = 0.0293$), log(CACS + 1) ($p = 0.0089$) and TNF-α ($p = 0.0029$) than in the nondiabetic subjects, whereas eGFR and serum levels of calcium (Ca), inorganic phosphate (IP) and alkaline phosphatase (ALP) were similar in the two groups. To clarify the associations among severity of atherosclerosis and kidney function in diabetic patients,

Table 1 – Clinical characteristics in diabetic patients and age-matched nondiabetic subjects

	Nondiabetic subjects	Diabetic patients	*p*-Value
Number	30	200	–
Male gender (%)	13 (43.3)	112 (56.0)	0.2703
Age (year)	58.9 ± 5.4	60.3 ± 5.8	0.2040
BMI (kg/m^2)	23.4 ± 3.5	24.2 ± 3.6	0.2709
Duration of diabetes (year)	–	11.5 ± 8.2	–
Treatment (D/OHD/I)	–	16/85/99	–
FPG (mmol/l)	5.50 ± 0.62	7.96 ± 2.39	<0.0001
HbA1c (%)	5.2 ± 0.4	8.4 ± 1.7	<0.0001
TC (mmol/l)	5.39 ± 0.68	5.29 ± 0.90	0.5632
HDL-C (mmol/l)	1.55 ± 0.43	1.31 ± 0.37	0.0011
TGs (mmol/l)	1.25 ± 0.53	1.60 ± 0.79	0.0203
Statins (%)	–	52 (26.0)	–
Blood pressure (mmHg)			
Systolic	129 ± 17	136 ± 15	0.0186
Diastolic	81 ± 11	77 ± 10	0.0432
ACEI and/or ARB (%)	–	70 (35.0)	–
Smokers (%)	6 (20.0)	60 (30.0)	0.3614
Retinopathy (%)	–	92 (46.0)	–
Micro- and macroalbuminuria (%)	0 (0)	82 (41.0)	<0.0001
eGFR (ml/min per 1.73 m^2)	71.4 ± 7.8	70.3 ± 14.4	0.6776
Neuropathy (%)	–	91 (45.5)	–
ABI	1.15 ± 0.07	1.15 ± 0.07	0.7132
Brachial-ankle PWV (cm/s)	1479 ± 223	1585 ± 250	0.0293
Log(CACS + 1)	0.80 ± 0.98	1.36 ± 1.09	0.0089
Ca (mmol/l)	2.29 ± 0.08	2.27 ± 0.10	0.1343
IP (mmol/l)	1.14 ± 0.15	1.12 ± 0.16	0.4419
ALP (IU/l)	230 ± 65	235 ± 63	0.6802
TNF-α (pg/ml)	1.05 ± 0.40	1.35 ± 0.51	0.0029

Data are expressed as *n* (%) or mean ± S.D. D; diet, OHD; oral hypoglycemic drugs, I; insulin.

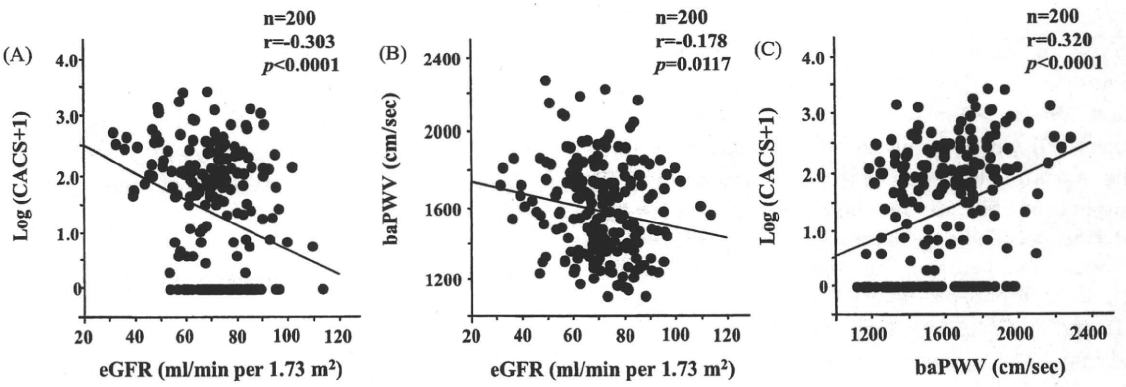

Fig. 1 – Simple linear regression analyses among estimated glomerular filtration rate (eGFR), coronary artery calcium score (CACS) and brachial-ankle pulse wave velocity (baPWV) in type 2 diabetic patients. (A) eGFR vs. log(CACS + 1); (B) eGFR vs. baPWV; (C) baPWV vs. log(CACS + 1).

simple linear regression analyses, as shown in Fig. 1, were performed. Log(CACS + 1), baPWV and eGFR were negatively [eGFR vs. log(CACS + 1), $p < 0.0001$; eGFR vs. baPWV, $p = 0.0117$] or positively [baPWV vs. log(CACS + 1), $p < 0.0001$] correlated with each other.

3.2. *Nephropathy*

To clarify the association of kidney disease with the severity of atherosclerosis, patients were classified into four subgroups with or without elevated UEA and renal insufficiency. The

Table 2 – Diabetic patients classified into four subgroups with or without elevated urinary albumin excretion (UAE) and renal insufficiency according to the levels of UAE of ≥30 or <30 mg/24 h and estimated glomerular filtration rate (eGFR) of <60 or ≥60 ml/min per 1.73 m²

	Normoalbuminuria		Micro- and macroalbuminuria	
	Without renal insufficiency	With renal insufficiency	Without renal insufficiency	With renal insufficiency
eGFR (ml/min per 1.73 m²)	75.3 ± 9.8	54.2 ± 6.3	76.7 ± 11.2	47.6 ± 8.0
Number	100	18	57	25
Male gender (%)	52 (52.0)	10 (55.6)	32 (56.1)	18 (72.0)
Age (year)	59.7 ± 5.8	63.3 ± 5.1	59.6 ± 5.4	62.6 ± 6.5
BMI (kg/m²)	24.1 ± 3.8	23.6 ± 3.3	24.4 ± 3.6	24.7 ± 3.4
Duration of diabetes (years)	10.3 ± 7.9	11.1 ± 7.9	12.0 ± 8.0	15.9 ± 8.7[a]
Treatment (diet/OHD/insulin)	10/49/41	1/9/8	3/20/34	2/7/16
FPG (mmol/l)	7.96 ± 2.66	7.47 ± 1.91	7.98 ± 2.18	8.30 ± 2.03
HbA1c (%)	8.3 ± 1.8	8.5 ± 1.4	8.6 ± 1.6	8.0 ± 1.6
TC (mmol/l)	5.18 ± 0.86	5.63 ± 0.84	5.24 ± 0.95	5.61 ± 0.94
HDL-C (mmol/l)	1.35 ± 0.42	1.37 ± 0.40	1.29 ± 0.27	1.15 ± 0.29
TGs (mmol/l)	1.45 ± 0.79	1.81 ± 1.09	1.60 ± 0.69	2.06 ± 0.56[b]
Statins (%)	24 (24.0)	6 (33.3)	14 (24.6)	8 (32.0)
Blood pressure (mmHg)				
Systolic	133 ± 15	131 ± 11	140 ± 16[a]	142 ± 12[a]
Diastolic	76 ± 10	74 ± 11	79 ± 9	77 ± 9
ACEI and/or ARB (%)	24 (24.0)	6 (33.3)	25 (43.9)	15 (60.0)[b]
Smokers (%)	32 (32.0)	3 (16.7)	18 (31.6)	7 (28.0)
Retinopathy (%)	28 (28.0)	7 (38.9)	38 (66.7)[b]	19 (76.0)[b]
Neuropathy (%)	34 (34.0)	10 (55.6)	30 (52.6)	17 (68.0)[a]
ABI	1.15 ± 0.07	1.14 ± 0.05	1.14 ± 0.07	1.14 ± 0.08
Brachial-ankle PWV (cm/s)	1520 ± 236	1611 ± 245	1637 ± 244[a]	1712 ± 261[b]
Log(CACS + 1)	1.03 ± 0.99	1.61 ± 1.18	1.51 ± 1.13	2.15 ± 0.82[c]
Ca (mmol/l)	2.27 ± 0.11	2.26 ± 0.08	2.27 ± 0.09	2.23 ± 0.11
IP (mmol/l)	1.12 ± 0.17	1.13 ± 0.16	1.10 ± 0.16	1.12 ± 0.14
ALP (IU/l)	235 ± 60	218 ± 70	240 ± 68	241 ± 57

Data are expressed as *n* (%) or means ± S.D. D, diet; OHD, oral hypoglycemic drugs; I, insulin.
[a] $p < 0.05$ vs. normoalbuminuric patients without renal insufficiency.
[b] $p < 0.01$ vs. normoalbuminuric patients without renal insufficiency.
[c] $p < 0.001$ vs. normoalbuminuric patients without renal insufficiency.

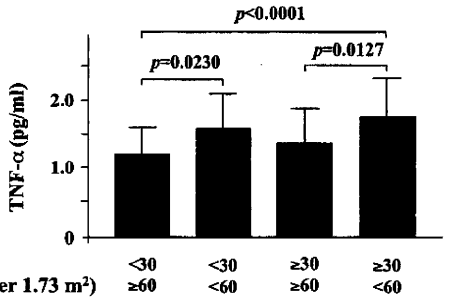

Fig. 2 – Serum levels of tumor necrosis factor-alpha (TNF-α) in patients with type 2 diabetes grouped into four subgroups with or without elevated urinary albumin excretion (UAE) and renal insufficiency according to the levels of UAE of ≥30 or <30 mg/24 h and estimated glomerular filtration rate (eGFR) of <60 or ≥60 ml/min per 1.73 m².

clinical characteristics are shown in Table 2. There were no significant differences among these groups for prevalence of male gender, age, BMI, FPG, HbA1c, TC, HDL-C, frequency of patients taking statins, dBP, prevalence of smoking habit, ABI, Ca, IP and ALP. Patients with micro- and macroalbuminuric renal insufficiency had the longest duration of diabetes ($p = 0.0225$), highest TGs ($p = 0.0061$), sBP ($p = 0.0364$), frequency of patients taking ACEI and/or ARB ($p < 0.01$), retinopathy ($p < 0.01$) and neuropathy ($p < 0.05$). Furthermore, these patients showed the highest baPWV ($p = 0.0068$) and log(CACS + 1) ($p < 0.0001$) among the groups. Although 18 of 200 (9%) patients showed renal insufficiency, they had normoalbuminuria and similar log(CACS + 1) and baPWV compared to normoalbuminuric patients without renal insufficiency.

3.3. TNF-α

Serum concentrations of TNF-α in each group are shown in Fig. 2. Both normoalbuminuric ($p = 0.0230$) and micro- and macroalbuminuric ($p < 0.0001$) patients with renal insufficiency had higher TNF-α than in normoalbuminuric patients without renal insufficiency. To clarify the association of the severity of atherosclerosis or kidney function with serum levels of TNF-α, simple linear regression analyses were performed as shown in Fig. 3. Log(CACS + 1) ($p = 0.0008$) (Fig. 3A) and baPWV ($p = 0.0006$) (Fig. 3B) positively and eGFR ($p < 0.0001$) (Fig. 3C) negatively correlated with TNF-α among these patients.

3.4. *Risk factors*

Stepwise multiple regression analyses were performed to examine the association of log(CACS + 1) or baPWV with 12 possible risk factors for atherosclerosis (age, male sex, BMI, duration of diabetes, FBS, HbA1c, sBP, dBP, TC, HDL-C, TGs and smoking habit), four factors for microangiopathy (retinopathy, micro- and macroalbuminuria, eGFR and neuropathy) and TNF-α. Age ($\beta = 0.034$; $F = 7.193$), smoking habit ($\beta = 0.410$; $F = 6.791$), retinopathy ($\beta = 0.575$; $F = 15.670$), eGFR ($\beta = -0.014$; $F = 6.768$) and TNF-α ($\beta = 0.323$; $F = 4.985$) for log(CACS + 1) ($r^2 = 0.214$, $p < 0.0001$), and age ($\beta = 10.791$; $F = 18.338$), sBP ($\beta = 6.593$; $F = 38.377$), retinopathy ($\beta = 79.888$; $F = 6.620$) and TNF-α ($\beta = 67.305$; $F = 5.111$) for baPWV ($r^2 = 0.326$, $p < 0.0001$) were identified as significant independent variables, respectively.

4. Discussion

In this study, we have demonstrated for the first time that severity of coronary artery calcification, arterial stiffness and renal insufficiency associate with circulating levels of TNF-α in type 2 diabetic patients. Our findings support the notion that TNF-α is a key molecule in the development of micro- and macrovascular complications and that use of anti-inflammatory agents may therefore act to reduce CVD events in diabetic patients. However, 9% of our patients with normoalbuminuric renal insufficiency showed characteristically distinct atherosclerotic features from those of patients with micro- and macroalbuminuric renal insufficiency, even though both groups had higher circulating levels of TNF-α than in normoalbuminuric patients without renal insufficiency.

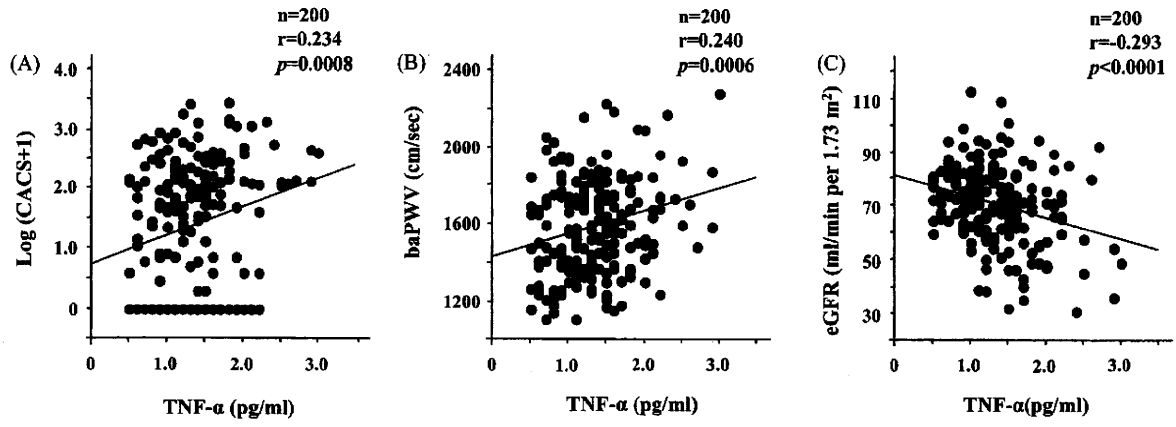

Fig. 3 – Simple linear regression analyses between serum levels of tumor necrosis factor-alpha (TNF-α) with coronary artery calcium score (CACS) (A), brachial-ankle pulse wave velocity (baPWV) (B) or estimated glomerular filtration rate (eGFR) (C) in type 2 diabetic patients.

Our data reveal that when diabetic patients are subgrouped with or without elevated UAE and renal insufficiency, patients with micro- and macroalbuminuric renal insufficiency show the highest coronary artery calcification, arterial stiffness and serum levels of TNF-α among the groups. Although these factors except for TNF-α have been reported previously, our study demonstrates that age [39], smoking habit [40], retinopathy [41], kidney function [19] and TNF-α are independent variables for coronary artery calcification, and age [42], hypertension [43], retinopathy [44] and TNF-α are independent variables for arterial stiffness, respectively. Kidney is a major source of TNF-α synthesis [10], but there are several extrarenal sources of this molecule, including adipose tissue [9] and atherosclerotic lesions [11]. These results indicate that TNF-α is a predictor of both coronary artery calcification and arterial stiffness and is independent of reduced kidney function for coronary artery calcification in type 2 diabetic patients.

Although we did not investigate the primary source of TNF-α, a previous report noted that renal production of this cytokine is elevated in patients with type 2 diabetes [45]. In the kidneys, TNF-α is produced mainly by infiltrating macrophages [46], as well as by intrinsic renal cells including endothelial, mesangial, glomerular and tubular epithelial cells [10]. Various atherogenic factors such as AGEs [12], angiotensin II [13] and oxidized LDL [14] can stimulate TNF-α synthesis from these cells and initiate local effects of renal damage. Endothelial cells in the intimal layer actively regulate vascular tone and permeability and the balance between coagulation and fibrolysis [47]. Thus, the increased permeability for albumin through the vascular wall in the kidneys reflects systemic endothelial injury that leads to increased risk of CVD events.

Nephropathy in patients with type 2 diabetes is more heterogeneous than in type 1 diabetic patients. Type 2 diabetic patients often develop renal dysfunction in the absence of increased albuminuria, which conditions initially develop during the prediabetic state secondary to age, hypertension and other factors [48]. In the United States, 30% of newly diagnosed type 2 diabetic patients were found to exhibit renal insufficiency without retinopathy and albuminuria [49]. A longitudinal study demonstrates that risk for progression of renal failure or death in diabetic patients with normoalbuminuric renal insufficiency is lower than in patients with albuminuric renal insufficiency [50]. In the present study, 9% of the diabetic patients had renal insufficiency without elevated UAE. Although these patients had higher serum levels of TNF-α compared to normoalbuminuric patients without renal insufficiency, the severity of atherosclerosis was similar in the two groups. This inconsistency between abnormal TNF-α production and the severity of atherosclerosis in patients with normoalbuminuric renal insufficiency has not been clarified.

Cytotoxic activities of inflammatory cytokines are not limited to renal damage. Intra-arterial TNF-α administration causes vascular inflammation and endothelial dysfunction [15], which is an early stage of the atherosclerotic process. Diabetic patients have a greater amount of coronary calcium deposition than in nondiabetic subjects [25] and excessive coronary calcification associates with insulin resistance [51]. Coronary artery calcium is present only in atherosclerotic lesions and can be quantified safely with ultrafast computed tomography. The resulting calcium score represents the presence and extent of atherosclerosis and is useful in predicting CVD events [24]. Vascular calcification is an active and regulated process related to various inflammatory cells and cytokines. Macrophages can contribute to the development of vascular calcification through TNF-α production, as a regulator of bone formation [52]. TNF-α changes the phenotype of vascular smooth muscle cells and induces osteoblastic differentiation [53]. Diabetic patients have a higher aortic stiffness than in nondiabetic subjects [26]. Pulse wave velocity, which depends on arterial radius and wall thickness [54], is widely used to assess arterial distensibility. Functional or structural alterations of vessel wall properties can alter arterial compliance and increase blood pressure. TNF-α can impair endothelial function and lead to functional stiffness in the vessel wall due to reduced nitric oxide bioavailability [55]. Vascular smooth muscle cell migration into the intima followed by proliferation and matrix deposition is a central feature of early atherosclerosis [56]. Thus, both an increase in intima-media thickness [57] and vascular calcification [52,53] caused by excessive TNF-α production in the diabetic state are involved in the pathogenesis of structural rigidity of the vessel wall.

Because the present study is a cross-sectional design, further prospective study is required to clarify the role of abnormal TNF-α secretion in the development of micro- and macrovascular complications in patients with type 2 diabetes. In addition, our data were obtained in a Japanese population, and it remains to be established whether these results can be generalized. Furthermore, the inconsistency between abnormal TNF-α production and the severity of atherosclerosis in patients with normoalbuminuric renal insufficiency has not been clarified. Finally, because the administration of ACEI, ARB and statins can affect UAE, glomerular filtration rate and circulating levels of TNF-α, the influences of these medications on our data are unclear.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Consumption of vegetables alters morning urinary 6-sulfatoxymelatonin concentration

Abstract: Melatonin, which is contained in certain vegetables, may have an influence on circulatory melatonin concentrations. This study examined the effects of the consumption of vegetables on 6-sulfatoxymelatonin concentrations in morning urine. Ninety-four healthy women aged 24–55 were recruited through a city public health center in Japan. The women randomly allocated to the intervention group were requested to consume high amounts of six selected vegetables, with a target of 350 g/day for 65 days, while those in the control group were asked to avoid the same six vegetables during the same period. First-void morning urine was collected before and at the end of the intervention period, and creatinine-adjusted 6-sulfatoxymelatonin concentrations were measured. At the end of the intervention period, daily mean intake of melatonin from the six vegetables was 1288.0 ng in the intervention group and 5.3 ng in the control group. In the intervention group, the mean concentration of 6-sulfatoxymelatonin changed from 48.1 [95% confidence interval (CI): 40.4–57.2] ng/mg creatinine to 49.6 (95% CI: 42.8–57.3) ng/mg creatinine across the intervention period. In the control group, the mean concentration of 6-sulfatoxymelatonin changed from 55.5 (95% CI: 48.7–63.2) ng/mg creatinine to 50.8 (95% CI: 44.0–58.7) ng/mg creatinine across the intervention period. A comparison of the two groups with regard to the changes in the 6-sulfatoxymelatonin concentrations across the intervention period showed a significant difference ($P = 0.03$). The results indicate that increased consumption of vegetables raises circulatory melatonin concentrations.

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Key words: 6-sulfatoxymelatonin, intervention study, Japanese, vegetables, women

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Introduction

Human melatonin has been studied with regard to its beneficial role in protecting against cancers [1–3]. Several in vitro studies demonstrated the oncostatic effect of melatonin on different tumor cells, such as cells from breast cancer, colon cancer, melanoma, ovarian carcinoma, prostate cancer, neuroblastoma, pituitary tumor, larynx carcinoma, oral carcinoma, bladder carcinoma and erythroleukemia [4–6]. Studies in animals reported the anti-carcinogenesis effect of orally ingested melatonin on tumors in mammary and liver [7, 8]. Antioxidant effects of melatonin and its metabolites such as N^1-acetyl-5-methoxykynuramine (AMK) and N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) were also examined in laboratory studies [9–13]; free radicals play an important role in cancer cell proliferation, and may influence on age-related diseases [14].

Compared with those, the epidemiologic studies of melatonin in relation to the risk of cancer are still scarce. Thus far, two epidemiologic studies were conducted prospectively to assess the association of melatonin concentrations with breast cancer risk; one study found a lower risk of breast cancer with higher melatonin concentrations measured in the first morning urine [15], and the other study found no evidence that melatonin concentrations in 24-hr urine samples associated with the risk for breast cancer [16]. Melatonin exists in edible plants and vegetables [17–19]. Beneficial effects of vegetable intake in protecting against cancer and cardiovascular disease have been suggested by epidemiologic evidence [20, 21]. Although several mechanisms for these beneficial effects have been already hypothesized and studied [21, 22], melatonin contained in the vegetables might be another possible agent to support the positive effects. In our previous cross-sectional study among Japanese females, urinary melatonin concentrations increased significantly with increased vegetable intake in the regular diet [23]. We hypothesized that the consumed vegetables may be one of the sources of melatonin in humans; no previous study had examined this hypothesis.

The aim of the present randomized-controlled study was to examine the effects of the consumption of vegetables containing high amounts of melatonin, on 6-sulfatoxymelatonin concentrations in first-void morning urine as a reflection of the nocturnal melatonin concentrations [24–26]. Hattori et al. [17] investigated the presence of melatonin in vegetables and identified that certain vegetables commonly consumed in Japan contained certain amounts of melatonin; however, the melatonin contents of all commercially available vegetables have not yet been

identified. Hence, our focus is on the effects of specific vegetables already identified to be containing known amounts of melatonin.

Materials and methods

Study participants

Study subjects were recruited through a city public health center in Gifu prefecture, Japan. Female volunteers who had no hypertension, who did not use medications for a sleep disorders, depression, or mental disorders, who were not pregnant, and who were not currently engaged in night-shift work were invited to participate in this study. Ninety-eight healthy women aged 24–55 who agreed to participate and completed the informed consent form were registered. The subjects were stratified into three age groups: 24–35 yr, 36–45 yr, and 46 yr and older. They were randomly assigned to either the intervention group or the control group. Forty-nine subjects were assigned to the intervention group, in which the subjects were provided with and requested to consume selected vegetables, and 49 subjects were assigned to the control group, in which the subjects were requested to avoid these vegetables. Before the intervention started, one subject withdrew from the intervention group. During the intervention period, two subjects in the intervention group and one subject in the control group dropped out of the study. Overall, 46 subjects in the intervention group and 48 subjects in the control group completed the study.

Data collection and intervention

Before entering the intervention period, all the subjects were asked to fill out a self-administered questionnaire to provide general information about their demographic characteristics and other information such as parity status, past disease history, medication use within the previous 6 months, and sleeping habits. Physical activity levels were measured before and at the end of the intervention period by using a validated questionnaire, and these data were translated into metabolic equivalents per week [27, 28]. A validated food frequency questionnaire was administered to estimate the nutrient intake in the regular diet [29]. All subjects were required to fill out a 3-day dietary record, which was performed and included detailed instructions, before and at the end of the intervention period. In the record, the subjects were asked to provide the names and quantities of all the foods, beverages, and snacks which they consumed. Other than following the requirements of the study, the subjects were advised to maintain their regular diet and lifestyle.

The intervention period was from October 4, 2005 through December 7, 2005, a period of 65 days. For intervention agents, we selected the following six vegetables which, according to Hattori et al. [17], are relatively high in melatonin of the vegetables whose melatonin concentrations are available, and are commonly consumed in Japan: sweet corn, *goya* (bitter gourd), *kaiware* (Japanese radish sprout), *shungiku* (garland chrysanthemum), *shimeji* mushroom, and *shiitake* mushroom. The subjects who were assigned to the intervention group were provided these vegetables in 10 weekly deliveries to their residences throughout the intervention period. The exact contents of each delivery were based on the availability of the vegetables in the local market, but each package contained a sufficient amount of all six vegetables to provide a total of 350 g/day. The subjects in the intervention group were given a target of consuming 350 g of the delivered vegetables per day throughout the study period. Using a preformatted journal, the subjects reported the contents of the deliveries and the amounts consumed. In the same journal, the subjects recorded their nightly bedtimes. On the other hand, the subjects assigned to the control group were required to avoid the above six vegetables during the intervention period. When consumption of one of the six vegetables was inevitable, they were to record the consumed amount. They also recorded their nightly bedtimes. The subjects in both groups were required to avoid the following food items and products as they have been reported to contain large amounts of melatonin [17]: barley, *awa* (foxtail millet), *hie* (Japanese barnyard millet), brown rice, *gokokumai* (rice with five kinds of grains), banana, *aojiru* (green juice), and kale.

First-void morning urine was collected from all the subjects on 2 consecutive days starting 2 days before the intervention started and the last 2 consecutive days of the intervention period. The subjects were requested to go to bed around midnight or earlier on the nights before urine collection. The times that the urine was collected were recorded by the subjects. The urine samples were immediately frozen and stored at −80°C until being assayed. Urinary 6-sulfatoxymelatonin was measured radioimmunologically with kits purchased from IBL Laboratories (Hamburg, Germany). The sensitivity was 1.0 ng/mL, and the interassay coefficient of variation was 11.3%. To adjust for a variation in the dilution of urine, 6-sulfatoxymelatonin concentrations were expressed as urine 6-sulfatoxymelatonin/urine creatinine [26, 30].

Data analysis

For the analysis of 6-sulfatoxymelatonin concentrations, we used the mean of the concentrations during each 2-day period. The 6-sulfatoxymelatonin concentrations and nutrient intake were logarithmically transformed for statistical analysis to approximately normalize their distributions. Geometric means and 95% confidence interval (CI) of 6-sulfatoxymelatonin concentrations were calculated. To compare the difference in the concentrations of urinary 6-sulfatoxymelatonin before and at the end of the intervention, a paired t-test was conducted separately for each group. To compare the two groups in terms of the change in the 6-sulfatoxymelatonin concentrations across the intervention period, a two sample t-test was conducted. All P-values are two sided.

To assess the difference in the consumption of the six vegetables as well as other food items and nutrients across the intervention period, the information from the 3-day dietary record was analyzed. One dietitian interpreted all the records and estimated the intake of each nutrient, and

then the other dietitian confirmed the estimations. Based on the 3-day record, the intake of melatonin from the six vegetables was estimated by multiplying the consumed amounts in grams by the following melatonin concentrations in the vegetables: 1366 pg/g tissue for sweet corn, 13,353 pg/g tissue for bitter gourd, 657 pg/g tissue for Japanese radish sprout, 417 pg/g for garland chrysanthemum, 300 pg/g for *shimeji* mushroom and 387 pg/g for *shiitake* mushroom (partially reported by Hattori et al.) [17]. The intake of nutrients other than melatonin was calculated according to the Standard Tables of Food Composition in Japan, 5th edition, published by the Science and Technology Agency of Japan. The total amounts, in gram units, of the six vegetables consumed by the subjects throughout the study period were estimated from the journal entries. The melatonin intake was then estimated using the method described above. This study was approved by the institutional review board at Gifu University Graduate School of Medicine. All the statistical analyses were performed with SAS (SAS Institute Inc., Gary, NC, USA).

Results

The baseline characteristics of the subjects by study group are summarized in Table 1. The change in the daily mean intake of the six vegetables before and at the end of the intervention period was obtained by using the data from the 3-day food records (Table 2). It was estimated that the subjects in both groups consumed similar amounts of the six vegetables before the intervention period. During the intervention period, the daily mean intake of the six vegetables in grams was almost 150 times higher among the subjects in the intervention group than among those in the control group. In line with the intake of the six vegetables, the estimated intake of melatonin sourcing from these vegetables increased in the intervention group and decreased in the control group. Similarly, it was estimated from the journals kept by the subject during the intervention period that the daily mean intake of melatonin from the six vegetables for the entire period was 1231 ng among the subjects in the intervention group whereas the equivalent value was 1.2 ng among the subjects in the control group. Among the subjects in the intervention group, sweet corn was the most consumed agent in grams [mean consumption for the entire period: 5263 g, standard error of mean (SEM): 204 g], and the bitter gourd was the second-most consumed agent (mean consumption for the entire period: 5104 g, SEM: 266 g). The total mean amount of sweet corn consumed by the control subjects during the intervention period was 10 g (SEM: 2 g), and that of bitter gourd was 3 g (SEM: 2 g). The estimated intake of other nutrients is also presented in Table 2. Among the subjects in the intervention group, the intake of crude fiber, vitamin A, vitamin C, and vitamin E increased significantly at the end of the intervention period. Between the groups, the difference in change for each of the above nutrients across the intervention period was significant. In both groups, caffeine intake increased significantly at the end of the intervention period.

The primary measures of this study, the concentrations of 6-sulfatoxymelatonin in first-void morning urine before and at the end of the intervention period, are shown in Table 3. At the baseline, the concentrations of urinary 6-sulfatoxymelatonin were higher among the subjects in the control group (55.5, 95% CI: 48.7, 63.2 ng/mg creatinine) than among the subjects in the intervention group (48.1, 95% CI: 40.4, 57.2 ng/mg creatinine), although the difference was not statistically significant. At the end of the intervention period, the mean concentration of 6-sulfatoxymelatonin significantly decreased by 8.5% ($P = 0.03$) in the control group, whereas the concentration increased slightly and non-significantly by 3.1% in the intervention group. A comparison of the net change in baseline-end values of 6-sulfatoxymelatonin between the two groups showed a significant difference across the intervention period ($P = 0.03$).

Table 1. Baseline characteristics of women in the melatonin intervention and control groups

	Melatonin-contained vegetables supplemented group (n = 46)	Control group (n = 48)
Age, years [mean ± standard error of mean (SEM)]	38.6 ± 0.9	40.3 ± 1.0
Height, cm (mean ± SEM)	157.5 ± 1.0	156.2 ± 0.6
Weight, kg (mean ± SEM)	51.7 ± 0.9	50.4 ± 0.9
Body mass index, kg/m^2 (mean ± SEM)	20.9 ± 0.4	20.7 ± 0.3
Married, no. (%)	41 (89.1)	44 (91.7)
Parous, no. (%)	36 (78.3)	45 (93.8)
Smoking status		
Current smoker, no. (%)	0 (0.0)	2 (4.2)
Past smoker, no. (%)	3 (6.5)	5 (10.4)
Ever used aspirin within 6 months, no. (%)	4 (8.7)	2 (4.2)
Reported 0 alcohol use in regular diet, no. (%)	9 (19.6)	20 (41.7)
Average bedtime (mean ± SEM)		
Weekday	23:19 ± 0:07	23:16 ± 0:08
Weekend	23:32 ± 0:08	23:36 ± 0:09
Average wake time (mean ± SEM)		
Weekday	6:06 ± 0:04	5:50 ± 0:05
Weekend	7:08 ± 0:09	6:54 ± 0:08