Contents available at ScienceDirect

Diabetes Research and Clinical Practice

journal homepage: www.elsevier.com/locate/diabres

Serum cystatin C level is associated with carotid arterial wall elasticity in subjects with type 2 diabetes mellitus: A potential marker of early-stage atherosclerosis

Rei Kaneko [a], Shojiro Sawada [a,*], Ai Tokita [a], Rieko Honkura [a], Noriko Tamura [a], Shinjiro Kodama [a], Tomohito Izumi [a], Kei Takahashi [a], Kenji Uno [a], Junta Imai [a], Tetsuya Yamada [a], Yukiya Miyachi [b], Hideyuki Hasegawa [c], Hiroshi Kanai [d], Yasushi Ishigaki [e], Hideki Katagiri [a,*]

[a] Department of Metabolism and Diabetes, Tohoku University Graduate School of Medicine, Sendai, Japan
[b] Medical Systems Research and Development Center R&D Management Headquarters, FUJIFILM Corporation, Kaisei, Japan
[c] Graduate School of Science and Engineering, University of Toyama, Toyama, Japan
[d] Department of Electronic Engineering, Tohoku University Graduate School of Medicine, Sendai, Japan
[e] Department of Internal Medicine, Division of Diabetes and Endocrinology, Iwate Medical University Hospital, Morioka, Japan

ARTICLE INFO

Article history:
Received 1 August 2017
Received in revised form
29 January 2018
Accepted 1 February 2018
Available online 15 February 2018

Keywords:
Early-stage atherosclerosis
Cystatin C
Arterial wall elasticity
Type 2 diabetes mellitus

ABSTRACT

Aims: Detection of early-stage atherosclerosis in type 2 diabetes mellitus (T2DM) patients is important for preventing cardiovascular disease. A phased tracking method for evaluating arterial wall elasticity sensitively detects early-stage atherosclerosis. However, biochemical markers for early-stage atherosclerosis have yet to be established.

Methods: This cross-sectional study enrolled 180 T2DM patients, who were classified as not having atherosclerosis according to the carotid intima-media thickness (IMT) criteria. We measured serum cystatin C, the estimated glomerular filtration rate (eGFR) and urinary albumin-to-creatinine ratio (ACR), and analyzed the associations between these markers and arterial wall elasticity (Eθ), IMT and the cardio-ankle velocity index.

Results: Multiple linear regression analyses revealed that cystatin C was significantly associated with Eθ, while neither eGFR nor ACR showed an association. Furthermore, among the examined atherosclerotic markers, Eθ was most reliably associated with cystatin C. Additionally, the association between cystatin C and Eθ disappeared in the low elasticity subgroup, which included subjects in whom no atherosclerotic changes had yet been initiated.

Conclusions: In T2DM patients without apparent arterial wall thickening, cystatin C is strongly and independently associated with arterial wall elasticity, which reflects the degree of subclinical atherosclerosis. Thus, cystatin C is a potentially useful marker of early-stage atherosclerosis.

* Corresponding authors at: Department of Metabolism and Diabetes, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan.
E-mail addresses: ssawada@med.tohoku.ac.jp (S. Sawada), katagiri@med.tohoku.ac.jp (H. Katagiri).
https://doi.org/10.1016/j.diabres.2018.02.003
0168-8227/

1. Introduction

Type 2 diabetes mellitus (T2DM) is associated with atherosclerosis, which leads to cardiovascular disease and increased mortality [1]. However, it is difficult to detect atherosclerosis at its early stages. Arterial stenosis often develops without symptoms in T2DM patients [2], subsequently leading to cardiovascular events. Therefore, development of strategies for easily detecting early-stage atherosclerosis, such as identifying reliable biochemical markers, is particularly important in those with T2DM.

Measurement of carotid intima-media thickness (IMT) is widely used for atherosclerosis screening in clinical practice but this examination detects morphological changes of atherosclerosis. For detecting early-stage atherosclerosis before the appearance of such morphological changes, we developed a novel non-invasive ultrasonic phased tracking method, which evaluates deformity at multiple sites in arterial walls during one heartbeat [3–6]. Since the arterial wall deforms less easily at harder sites during a single heartbeat, this innovative phased tracking method has enabled us to quantitatively analyze arterial wall elasticity. The results obtained with this method correspond to pathological findings in the extracted human iliac artery [5]. In addition, we reported that phased tracking arterial wall elasticity, but not IMT, pulse wave velocity (PWV) or the plaque score, is associated with an increasing number of coronary risk factors such as diabetes, hypertension, hyperlipidemia and current smoking, in T2DM patients with IMT <1.1 mm [7], who were classified as not having atherosclerosis according to the IMT criteria [8]. The elasticity in the arterial wall is also associated with visceral fat mass in subjects with obesity [9] and an increasing number of coronary risk factors in T2DM patients who have not developed apparent cardiovascular disease [10]. Furthermore, arterial wall elasticity is reportedly increased in smokers, as compared with non-smokers [11], and in subjects with the metabolic syndrome, as compared with those without metabolic syndrome [12], among subjects with IMT <1.1 mm. Collectively, these findings indicate that arterial wall elasticity is useful for detecting early-stage atherosclerosis, i.e. that developing before IMT increases detectably. Therefore, in order to search for biochemical markers of early-stage atherosclerosis, we sought serum markers which have a strong association with arterial wall elasticity values.

In the present study, we focused on serum cystatin C. Cystatin C, a 13-kD endogenous cysteine proteinase inhibitor, is considered to be a more reliable marker for glomerular filtration rates than the estimated glomerular filtration rate (eGFR) [13]. Moreover, cystatin C was reported to be associated with plaque morphology using multi-detector computed tomography [14] and coronary angiography [15], even in patients without established chronic kidney disease. Furthermore, Wang et al. demonstrated cystatin C, but not eGFR, to be associated with the presence of cardiovascular disease in subjects with mild renal impairment [16]. Similar findings, indicating that cystatin C is superior to eGFR for predicting cardiovascular events, were also reported in subjects with diabetes [17] as well as those with [18] and without [19] cardiovascular disease. Thus, cystatin C is not simply regarded as a marker of impaired kidney function but also as one which reflects cardiovascular disease due to advanced atherosclerosis. However, the association of cystatin C and early-stage atherosclerosis remains unclear.

Herein, to assess whether the serum cystatin C level is linked to, and useful for detecting, early-stage atherosclerosis, we enrolled T2DM patients who had IMT of less than 1.1 mm, the cut-off value for a plaque lesion, and analyzed the associations of cystatin C with examinations for assessing atherosclerosis, such as Eθ (an indicator of arterial wall elasticity) as well as IMT and the cardio-ankle vascular index (CAVI). We also compared the utility of cystatin C as an early-stage atherosclerosis marker with those of other renal markers, such as eGFR and urinary albumin excretion.

2. Materials and methods

2.1. Study subjects

This was a cross-sectional study consisting of 180 subjects with T2DM. The subjects were recruited from patients with T2DM admitted to the Department of Metabolism and Diabetes, Tohoku University Hospital for glycemic control during the period from January 2012 to December 2015. T2DM was defined according to the World Health Organization (WHO) criteria, or treatment for diabetes mellitus. The exclusion criteria for the enrollment in this study were pregnancy, thyroid disease, tumor and severe inflammation, because these conditions are known to affect cystatin C levels. Subjects with atrial fibrillation were also excluded because the intima media complex (IMC) during one heart beat was used for calculating Eθ. Since this study aimed to evaluate renal markers in early-stage atherosclerosis, subjects with IMT ≥1.1 mm, were also excluded. The study protocol was approved by the Tohoku University Institutional Review Board, in accordance with the World Medical Association Declaration of Helsinki. Written informed consent was obtained from each patient.

We used the following criteria for cardiovascular risk factors. Hyperlipidemia was defined as total cholesterol ≥ 5.7 mmol/l (220 mg/dl) and/or triglycerides ≥1.7 mmol/l (150 mg/dl), based on the definition proposed by the Japan Atherosclerosis Society, or taking anti-hyperlipidemic drugs. The subjects whose systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg, based on the definition proposed by the Japanese Society of Hypertension, or taking anti-hypertensive drugs were defined as having hypertension. The subjects who currently smoked were classified as current smokers.

2.2. Measurement of Eθ

Eθ in the carotid artery was evaluated using the phased tracking method. The arteries were scanned with ultrasonic diagnostic equipment (FUJIFILM FAZONE M) and a linear type probe with a frequency range of 5–10 MHz (FUJIFILM FZT L10-5 probe) in the supine position. By B-mode ultrasound, the IMC was longitudinally scanned at two points on the far wall of the common carotid artery, 1 cm and 3 cm prox-

imal to the bifurcation. The portion of the IMC including plaques was excluded. The plaque was defined as a focal raised lesion, using an IMT cut-off value of 1.1 mm [8]. The quadrature demodulated signals of the IMC during 8 heartbeats were obtained with a high frame rate of 200 Hz. From these 8 heartbeats, we selected one heartbeat which yielded the most precise image. The sampling interval of the quadrature demodulated signal and the pitch of scan lines were 0.035 and 0.12 mm, respectively. The quadrature demodulated signals were saved as raw data to a personal computer. A single well-trained sonographer (N.T.), who was unaware of the clinical characteristics of the subjects, analyzed the raw data to introduce Eθ based on the principal described previously [20] (Supplementary Fig. 1). Multiple points were preset from the luminal surface to the adventitia along each beam. Then, the displacement of each point during one heartbeat was obtained by applying the phased tracking method [3–6]. Er, which is the elastic modulus in the radial direction of each of the sampled points, was calculated using the following equation: $Er = \Delta P/\varepsilon$, where $\varepsilon = \Delta h/h_0$, and Δh is the maximum decrease in the thickness of the arterial wall during one heartbeat. ΔP is the pulse pressure, defined as the difference between systolic and diastolic brachial blood pressure. The h_0 and r_0 variables are the initial thickness and radius of the vessel at end-diastole, respectively. Eθ, which is the elastic modulus in the circumferential direction was calculated using the following equation: $E\theta = (3/8) \times (2r_0/h_0 + 1) \times Er$. The intraobserver coefficient of variation for Eθ was 7.0%.

Due to the absence of normative data for Eθ, we referred to the Eθ values from the data of a healthy population from another cohort. The Sendai knowledge cluster initiative organized by the Ministry of Education, Culture, Sports, Science and Technology, Japan, performed population-based health care examinations including the determination of Eθ in the carotid artery. After excluding hypertension, hyperlipidemia, and diabetes mellitus, 156 cases were defined as healthy subjects. Their mean age was 47.7 ± 10.3 years and Eθ was 228.3 ± 72.0 kPa (unpublished data).

2.3. Measurement of IMT

IMT of the common carotid artery was measured employing the same scanning image, as that mentioned above for the measurement of Eθ. IMT was measured at a point on the far wall of the common carotid artery, in a 1 cm segment proximal to the bifurcation in a region free of plaque [21]. The mean value of the bilateral measurements was used for the analysis. The intraobserver coefficient of variation for IMT was 8.3%.

2.4. Measurement of CAVI

CAVI was measured using a pulse wave analytical instrument (VaSera; Fukuda Denshi, Tokyo, Japan) as described previously [22]. After a 10 min rest, cuffs were applied to the bilateral upper arms and ankles to detect each of the pulse waves. Electrocardiograph, phonocardiograph, and blood pressures were measured. CAVI was calculated using a conventional PWV measurement with the formula: $CAVI = a\{(2\rho/\Delta P) \times \ln(Ps/Pd)PWV^2\} + b$, where Ps is systolic blood pressure, Pd is diastolic blood pressure, PWV is pulse wave velocity, ΔP is Ps − Pd, ρ is blood density, and a and b are both constant.

2.5. Biochemical markers of renal function

The eGFR was calculated using the formula recommended by the Japanese Society of Nephrology [23], derived from the Modification of Diet in Renal Disease (MDRD) study group. The urinary albumin excretion is presented as the albumin-to-creatinine ratio (ACR; mg/g creatinine). Cystatin C was assayed using colloidal gold immunoassay (Nescaute GC cystatin C; Alfresa Pharma, Osaka, Japan).

2.6. Statistical analysis

Continuous variables were expressed as means ± S.D. or medians (inter-quantile range (IQR)). The difference between 2 groups was determined using the unpaired *t*-test for continuous variables and Fisher's exact test for non-continuous variables. Data were logarithmically transformed before statistical analysis, if they were not normally distributed. Pearson correlation was used for correlation analysis. Multiple linear regression analysis was performed to evaluate the independent parameters that were found to be significantly related to the objective variable. The independent parameters tested were age, male gender, systolic blood pressure, hyperlipidemia, HbA1c, BMI, duration of diabetes, treatment with RAS inhibitors, calcium channel blockers, other antihypertension agents, statins, fibrates, other antihyperlipidemic agents, insulin, GLP-1 receptor agonists and other anti-diabetic agents, eGFR, cystatin C and log ACR. Among these variables, no extreme collinearities were recognized. Receiver-operating characteristic (ROC) curve for cystatin C was generated to determine the optimal cut-off value for high elasticity of the arterial wall. We computed the sensitivity and specificity for high elasticity of the arterial wall using this cut-off value for cystatin C. As for sample size calculation, the correlation coefficient of cystatin C and Eθ was estimated 0.25 from our preliminary study. We calculated that 164 patients would be needed at a significance level $\alpha = 0.05$, with power of 90%. The number of required patients was thus set at 180. A level of $p < 0.05$ was considered to indicate a statistically significant association. Analyses were carried out with JMP 11.2 Pro statistical analysis software (SAS Institute, Cavy, NC, USA).

3. Results

3.1. Subject characteristics

All 180 diabetic subjects enrolled in this study had IMT of less than 1.1 mm, the cut-off value for detecting atherosclerosis with this examination. The clinical characteristics of these subjects are shown in Table 1. Overall, 56.7% of the subjects were male, mean age was 52.0 ± 15.6 years, the diabetes duration was 8.0 (3.0–15.0) years, BMI was 27.3 ± 6.2 kg/m^2, 64.4% of the subjects had hypertension, 70.0% had hyperlipidemia.

Table 1 – Subject characteristics.

	All subjects (n = 180)	Low Eθ (n = 90)	High Eθ (n = 90)	p
Male gender (%)	56.7	50.0	63.3	0.10
Age (years)	52.0 ± 15.6	48.0 ± 15.1	55.4 ± 15.3	<0.01
Duration of diabetes (years)	8.0 (3.0–15.0)	6.0 (2.0–13.0)	10.0 (4.0–20.0)	<0.01
Body mass index (kg/m^2)	27.3 ± 6.2	27.9 ± 6.0	26.7 ± 6.3	0.21
Systolic blood pressure (mmHg)	118.3 ± 15.7	114.8 ± 13.4	121.8 ± 17.0	<0.01
Diastolic blood pressure (mmHg)	73.5 ± 11.5	74.0 ± 9.8	72.9 ± 13.0	0.53
Fasting plasma glucose (mg/dl)	151.8 ± 54.1	157.0 ± 58.5	146.3 ± 48.9	0.17
HbA1c (%)	10.0 ± 2.0	10.3 ± 2.1	9.7 ± 1.9	<0.05
Total cholesterol (mg/dl)	182.8 ± 41.6	188.6 ± 41.8	177.0 ± 40.8	0.06
Triglyceride (mg/dl)	131.5 (93.0–175.3)	132.5 (94.8–193.3)	131.5 (90.5–160.3)	0.91
HDL cholesterol (mg/dl)	43.6 ± 12.6	43.7 ± 13.4	43.4 ± 11.8	0.89
LDL cholesterol (mg/dl)	108.5 ± 35.6	115.0 ± 34.7	102.3 ± 35.5	<0.05
eGFR (ml/min/1.73 m^2)	85.9 ± 29.5	92.0 ± 26.8	79.9 ± 30.9	<0.01
Cystatin C (mg/l)	0.94 ± 0.30	0.89 ± 0.21	1.00 ± 0.37	<0.05
ACR (mg/g creatinine)	15.6 (6.3–52.3)	15.7 (6.4–38.0)	12.3 (6.3–54.7)	0.97
Hypertension (%)	64.4	54.4	70.0	0.16
Hyperlipidemia (%)	70.0	68.9	71.1	0.87
Current smokers (%)	35.8	38.6	33.0	0.53
Eθ (kPa)	259.1 ± 82.1	197.0 ± 31.2	321.3 ± 69.2	<0.01
IMT (mm)	0.70 ± 0.16	0.69 ± 0.17	0.70 ± 0.15	0.81
CAVI	7.9 ± 1.6	7.5 ± 1.6	8.4 ± 1.6	<0.01
RAS inhibitors (%)	55.6	52.2	58.9	0.45
Calcium channel blockers (%)	33.3	23.3	43.3	<0.01
Other agents for hypertension (%)	16.1	8.9	23.3	<0.05
Statins (%)	45.6	41.1	50.0	0.29
Fibrates (%)	3.9	2.2	5.6	0.44
Other agents for hyperlipidemia (%)	12.2	8.9	15.6	0.25
Insulin (%)	57.2	52.2	62.2	0.23
GLP-1 receptor agonists (%)	7.2	5.6	8.9	0.57
Other agents for diabetes (%)	80.0	80.0	80.0	1.00

Data are presented as means ± SD and or medians (inter-quantile range (IQR)) for continuous variables and % for categorical variables. p value for the comparison between low and high Eθ group.

3.2. *Associations of Eθ with cardiovascular risk factors*

We first assessed the associations of Eθ with cardiovascular risk factors. Eθ was significantly associated with age ($r = 0.35$, $p < 0.01$), BMI ($r = -0.17$, $p = <0.05$), systolic blood pressure ($r = 0.35$, $p < 0.01$), HbA1c ($r = -0.19$, $p < 0.05$), eGFR ($r = -0.36$, $p < 0.01$) and serum cystatin C ($r = 0.42$, $p < 0.01$) (Table 2). We then focused on the associations of Eθ with renal markers such as eGFR, cystatin C and ACR levels. Eθ was significantly associated with eGFR ($r = -0.36$, $p < 0.01$) and cystatin C ($r = 0.42$, $p < 0.01$), but not with ACR ($r = 0.05$, $p = 0.48$) (Supplementary Fig. 2A–C). Next, the subjects were divided into two groups by eGFR = 60 mL/min/1.73 m^2, cystatin C = 0.95 mg/l and ACR = 30 mg/g creatinine. Subjects with eGFR <60 had significantly higher Eθ than those with eGFR ≥60, and subjects with cystatin C ≥0.95 had significantly higher Eθ than those with cystatin C <0.95. In contrast, subjects with ACR ≥30 had Eθ values similar to those with ACR <30 (Supplementary Fig. 3A–C).

Table 2 – Correlations of Eθ with cardiovascular risk factors in all subjects.

	r	p
Age (years)	0.35	<0.01
Body mass index (kg/m^2)	−0.17	<0.05
Systolic blood pressure (mmHg)	0.35	<0.01
Diastolic blood pressure (mmHg)	−0.02	0.79
Fasting blood glucose (mg/dl)	−0.12	0.10
HbA1c (%)	−0.19	<0.05
eGFR (ml/min/1.73 m^2)	−0.36	<0.01
Cystatin C (mg/l)	0.42	<0.01
log ACR (mg/g creatinine)	0.05	0.48
Hyperlipidemia	0.02	0.80

Next, to identify the independent variables affecting Eθ, we performed multiple regression analysis. Several clinical parameters, including age, gender, systolic blood pressure, hyperlipidemia, body mass index, HbA1c, duration of diabetes, use of medications, and each of the renal markers were included in the regression models. Cystatin C, eGFR and ACR were employed in models 1, 2 and 3, respectively. This analysis revealed that cystatin C ($\beta = 0.29$, $p < 0.01$), but neither eGFR ($\beta = -0.15$, $p = 0.09$) nor ACR ($\beta = -0.01$, $p = 0.88$), was independently associated with Eθ in T2DM patients with IMT <1.1 mm (Table 3). Thus, in T2DM subjects with IMT of less than 1.1 mm, serum cystatin C is strongly and independently associated with arterial wall elasticity which reflects the degree of atherosclerosis in its early stages.

3.3. *Associations of cystatin C with Eθ, IMT, and CAVI*

To determine which of the atherosclerosis examinations, Eθ, IMT or CAVI, is most strongly associated with cystatin C, we next analyzed the results employing a different approach.

Table 3 – Multiple regression analysis of Eθ associations with cardiovascular risk factors in all subjects.

	Model 1[a]		Model 2[b]		Model 3[c]	
	β	p	β	p	β	p
Age (years)	0.21	<0.05	0.20	<0.05	0.27	<0.01
Male gender	0.10	0.15	0.12	0.10	0.14	0.05
Systolic blood pressure (mmHg)	0.33	<0.01	0.33	<0.01	0.34	<0.01
Hyperlipidemia	0.01	0.95	0.02	0.85	0.00	0.97
Body mass index (kg/m^2)	−0.24	<0.01	−0.20	<0.05	−0.19	<0.05
HbA1c (%)	−0.12	0.09	−0.15	<0.05	−0.16	<0.05
Duration of diabetes (years)	−0.02	0.82	0.02	0.84	0.03	0.71
RAS inhibitors	−0.14	0.07	−0.14	0.09	−0.12	0.13
Calcium channel blockers	0.05	0.55	0.04	0.62	0.05	0.60
Other agents for hypertension	0.00	0.98	0.05	0.51	0.07	0.41
Statins	−0.02	0.79	−0.02	0.85	0.01	0.90
Fibrates	0.06	0.39	0.08	0.25	0.10	0.15
Other agents for hyperlipidemia	−0.05	0.48	−0.07	0.34	−0.06	0.40
Insulin	0.10	0.16	0.13	0.10	0.11	0.14
GLP-1 receptor agonists	0.22	<0.01	0.21	<0.01	0.21	<0.01
Other agents for diabetes	0.05	0.52	0.03	0.71	0.01	0.87
Cystatin C (mg/l)	0.29	<0.01	–	–	–	–
eGFR (ml/min/1.73 m^2)	–	–	−0.15	0.09	–	–
log ACR (mg/g creatinine)	–	–	–	–	−0.01	0.88

Multiple regression analysis was used to examine the relationships of Eθ (objective variable) with age, male gender, systolic blood pressure, hyperlipidemia, body mass index, HbA1c, duration of diabetes, RAS inhibitors, calcium channel blockers, other agents for hypertension, statins, fibrates, other agents for hyperlipidemia, insulin, GLP-1 receptor agonists, other agents for diabetes, and each renal marker (explanatory variables).

[a] $R^2 = 0.43$.

[b] $R^2 = 0.38$.

[c] $R^2 = 0.37$.

First, simple correlation analysis revealed that Eθ ($r = 0.42$, $p < 0.01$) and CAVI ($r = 0.26$, $p < 0.01$) were significantly associated with cystatin C, while IMT was not (Supplementary Fig. 4A–C). In addition, multiple regression analysis showed Eθ alone to be associated with cystatin C ($\beta = 0.29$, $p < 0.01$) independently of age, gender, systolic blood pressure, hyperlipidemia, body mass index, HbA1c, duration of diabetes, use of medications, while neither IMT ($\beta = -0.04$, $p = 0.66$) nor CAVI ($\beta = 0.05$, $p = 0.45$) showed association (Table 4). These results indicate that cystatin C is strongly associated with arterial wall elasticity, i.e. Eθ, which is the most sensitive examination of the three for early-stage atherosclerosis. Collectively, these observations suggest the serum cystatin C level to be a highly sensitive marker for early-stage atherosclerosis.

3.4. *Subgroup analysis based on low and high Eθ*

Finally, we investigated whether cystatin C is associated with early-stage atherosclerosis, i.e., whether the cystatin C association with Eθ values is less significant in subjects who have not yet developed atherosclerosis. All 180 diabetic subjects with IMT of less than 1.1 mm were divided into two groups using the median Eθ value (243 kPa) as the cut-off. In the low Eθ group, mean Eθ was 197.0 ± 31.2 kPa (Table 1). In comparison to the Eθ values of the aforementioned healthy population (228.3 ± 72.0 kPa), we assumed that there would be essentially no atherosclerosis, even in the early stage, in subjects in the low Eθ group. In contrast, subjects in the high Eθ group (321.3 ± 69.2 kPa) would be likely to have early-stage atherosclerosis not yet detectable as IMT change but with higher than average Eθ. Consistent with this assumption, age, duration of diabetes, systolic blood pressure, cystatin C and CAVI values were significantly higher in the high Eθ group (Table 1). Eθ showed a weak correlation with eGFR in the low Eθ group ($r = -0.21$, $p < 0.05$), while Eθ showed a modest correlation with eGFR in the high Eθ group ($r = -0.39$, $p < 0.01$) (Supplementary Fig. 2D, G). Interestingly, Eθ showed a strong correlation with cystatin C in the high Eθ group ($r = 0.56$, $p < 0.01$), while no significant difference was detected in the low Eθ group ($r = 0.05$, $p = 0.67$). (Supplementary Fig. 2E, H). The subjects with eGFR <60 had significantly higher Eθ than those with eGFR ≥60 in the high Eθ group (Supplementary Fig. 3G). Additionally, subjects with cystatin C ≥0.95 had significantly higher Eθ than those with cystatin C <0.95 in the high Eθ group (Supplementary Fig. 3H). However, there were no differences in Eθ between any two groups according to the renal markers in the low Eθ group (Supplementary Fig. 3D–F). In addition, multiple regression analysis demonstrated cystatin C to be associated with Eθ in the high Eθ group independently of age, gender, systolic blood pressure, hyperlipidemia, body mass index and HbA1c, duration of diabetes and use of medications ($\beta = 0.51$, $p < 0.01$), while no significant association was observed in the low Eθ group (Table 5). This subgroup analysis showed the association between cystatin C and Eθ to be even stronger in the high Eθ group than in the low Eθ group. Thus, cystatin C was not associated with arterial wall elasticity in subjects in whom no atherosclerotic changes had yet been initiated (IMT <1.1 mm and Eθ <243 kPa). In contrast, cystatin C very sensitively reflects the degree of atherosclerotic change in the early stage, as indicated by increased arterial elasticity (IMT <1.1 mm and Eθ ≥243 kPa). Finally, the ROC curve for cystatin C was generated to deter-

Table 4 – Multiple regression analysis of Eθ, IMT and CAVI associations with cardiovascular risk factors.

	Eθ[a]		IMT[b]		CAVI[c]	
	β	p	β	p	β	p
Age (years)	0.21	<0.05	0.56	<0.01	0.54	<0.01
Male gender	0.10	0.15	0.15	<0.05	0.19	<0.01
Systolic blood pressure (mmHg)	0.33	<0.01	0.19	<0.05	0.02	0.76
Hyperlipidemia	0.01	0.95	0.11	0.27	0.02	0.83
Body mass index (kg/m^2)	−0.24	<0.01	0.05	0.59	−0.19	<0.01
HbA1c (%)	−0.12	0.09	0.15	<0.05	−0.06	0.29
Duration of diabetes (years)	−0.02	0.82	−0.07	0.40	0.18	<0.05
RAS inhibitors	−0.14	0.07	0.07	0.39	0.10	0.15
Calcium channel blockers	0.05	0.55	−0.08	0.38	−0.08	0.27
Other agents for hypertension	0.00	0.98	−0.06	0.43	−0.14	<0.05
Statins	−0.02	0.79	0.02	0.85	0.08	0.29
Fibrates	0.06	0.39	0.03	0.64	0.03	0.58
Other agents for hyperlipidemia	−0.05	0.48	−0.03	0.70	0.03	0.62
Insulin	0.10	0.16	0.02	0.83	−0.15	<0.05
GLP-1 receptor agonists	0.22	<0.01	0.03	0.66	−0.04	0.54
Other agents for diabetes	0.05	0.52	0.05	0.54	−0.17	<0.01
Cystatin C (mg/l)	0.29	<0.01	−0.04	0.66	0.05	0.45

Multiple regression analysis was used to examine the relationships of Eθ, IMT, and CAVI (objective variables) with age, male gender, systolic blood pressure, hyperlipidemia, body mass index, HbA1c, duration of diabetes, RAS inhibitors, calcium channel blockers, other agents for hypertension, statins, fibrates, other agents for hyperlipidemia, insulin, GLP-1 receptor agonists, other agents for diabetes, and cystatin C (explanatory variables).

[a] $R^2 = 0.43$.

[b] $R^2 = 0.33$.

[c] $R^2 = 0.58$.

Table 5 – Multiple regression analysis of Eθ associations with cardiovascular risk factors, in low and high Eθ subgroups.

	Low Eθ (n = 90)[a]		High Eθ (n = 90)[b]	
	β	p	β	p
Age (years)	0.29	<0.05	0.07	0.58
Male gender	0.10	0.36	−0.04	0.70
Systolic blood pressure (mmHg)	0.25	<0.05	0.37	<0.01
Hyperlipidemia	0.21	0.14	0.07	0.63
Body mass index (kg/m^2)	−0.29	<0.05	−0.29	<0.05
HbA1c (%)	−0.03	0.79	−0.17	0.10
Duration of diabetes (years)	−0.26	0.07	−0.03	0.76
RAS inhibitors	0.16	0.23	−0.05	0.66
Calcium channel blockers	−0.09	0.50	−0.04	0.73
Other agents for hypertension	−0.04	0.73	−0.02	0.86
Statins	−0.21	0.15	−0.08	0.58
Fibrates	0.14	0.17	0.00	1.00
Other agents for hyperlipidemia	−0.33	<0.01	−0.11	0.24
Insulin	0.18	0.15	0.08	0.45
GLP-1 receptor agonists	0.45	<0.01	0.15	0.10
Other agents for diabetes	0.07	0.57	−0.09	0.40
Cystatin C (mg/l)	0.03	0.84	0.51	<0.01

Multiple regression analysis was used to examine the relationships of Eθ (objective variable) with age, male gender, systolic blood pressure, hyperlipidemia, body mass index, HbA1c, duration of diabetes, RAS inhibitors, calcium channel blockers, other agents for hypertension, statins, fibrates, other agents for hyperlipidemia, insulin, GLP-1 receptor agonists, other agents for diabetes, and cystatin C (explanatory variables).

[a] $R^2 = 0.39$.

[b] $R^2 = 0.55$.

mine the optimal cut-off for high elasticity of the arterial wall (Supplementary Fig. 5). The area under the curve was 0.59, and the sensitivity and specificity were 0.66 and 0.49, respectively, at cystatin C = 0.86 mg/l.

4. Discussion

In the present study, to examine whether the serum cystatin C level is linked to early-stage atherosclerosis, we analyzed

the associations between renal markers and atherosclerosis markers in T2DM patients with IMT of less than 1.1 mm, who are classified as not having atherosclerosis according to the IMT criteria [8]. In these subjects, multiple regression analyses revealed cystatin C to be strongly and independently associated with Eθ, while neither eGFR nor ACR showed a significant association. In addition, Eθ was the more strongly associated with cystatin C than either IMT or CAVI. These findings demonstrate that serum cystatin C levels reflect the degrees of atherosclerosis before arterial wall thickening becomes detectable.

Furthermore, the association between cystatin C and Eθ was more evident in the high Eθ subgroup. In contrast, the significance of this association disappeared in the low Eθ subgroup. Eθ values in the low Eθ subgroup were significantly smaller than those in a healthy population. Considering that all of the enrolled subjects had IMT of less than 1.1 mm, the subjects in the low Eθ subgroup presumably had normal arterial structure and stiffness. Therefore, these findings indicate that the association between cystatin C and Eθ manifests after atherosclerotic changes have been initiated. Thus, the serum cystatin C level is likely to be useful as a marker for screening early-stage atherosclerosis, i.e. that after increased elasticity has been initiated but before arterial wall thickening becomes apparent. Moreover, in our study, the optimal cut-off value of cystatin C for predicting high arterial wall elasticity was 0.86 mg/l. Thus, in clinical practice, considerable attention should be paid to diabetic patients with cystatin C >0.86 mg/l as potentially having early stage-atherosclerosis, even if thickened carotid intima-media has not been detected.

T2DM is well known to be associated with atherosclerosis, which leads to cardiovascular disease and increasing mortality rates [1]. In patients with T2DM especially, arterial stenosis often develops without symptoms [2]. According to a meta-analysis of prospective studies, 26.1% of asymptomatic T2DM patients have silent myocardial ischemia based on screening by myocardial perfusion scintigraphy [24]. Therefore, to reduce the risk of future cardiovascular disease in patients with T2DM, detection of subclinical atherosclerosis is particularly important. Conventional angiography, computed tomography and magnetic resonance angiography are often used to diagnose atherosclerosis in clinical practice. However, these modalities are useful for detecting advanced, rather than early-stage, atherosclerosis. Our phased tracking method, which evaluates the deformities of multiple sites in arterial walls during one heartbeat, is useful for detecting early-stage atherosclerosis [3–6]. Employing the results obtained with the phased tracking method, the elastic modulus in the radial direction (Er) and that in the circumferential direction (Eθ) are quantitatively calculated. We and other groups previously reported these elastic modulus values to be more reliable and useful for detecting early-stage atherosclerosis than examining carotid IMT, PWV and the plaque score of the carotid artery, all of which are widely used for atherosclerosis screening in clinical practice [7,9–12]. When considering Er and Eθ, the latter appears to be a more reliable marker for arterial wall elasticity, since Eθ is less influenced by the thickness of the arterial wall [3]. Therefore, we measured the elastic modulus, Eθ, in the present study. We obtained results indicating the serum cystatin C level to be strongly associated with Eθ in T2DM patients with IMT <1.1 mm. This association was independent of age, gender, systolic blood pressure, hyperlipidemia, body mass index, HbA1c, duration of diabetes, and use of medications. Thus, the phased tracking method enabled us to show that the serum cystatin C level is associated with atherosclerosis development at an early stage, i.e. before arterial wall thickening becomes apparent.

Carotid arterial IMT is a well-established surrogate marker for cardiovascular disease. Measuring IMT with ultrasonography is non-invasive, reproducible and easy to perform, and, therefore, is widely used in clinical practice. A number of studies have shown that IMT predicts future myocardial infarction [25,26] and cerebrovascular disease [26,27]. However, employing this procedure, it is not possible to make a diagnosis of atherosclerosis until the appearance of arterial wall thickening. Therefore, IMT measurement is not optimal for sensitively detecting early-stage atherosclerosis. Indeed, the significance of carotid IMT in addition to conventional coronary risk factors for predicting future cardiovascular events remains a matter of debate [28,29]. In the present study, we enrolled T2DM patients with IMT of less than 1.1 mm, who are classified as not having atherosclerosis according to the IMT criteria [8]. Within this IMT range, there was no significant association between cystatin C and IMT.

Cystatin C may provide a more sensitive and accurate estimation of renal function than eGFR, since cystatin C is less influenced by age and muscle mass [13]. We obtained data indicating the serum cystatin C level to be strongly associated with Eθ, a sensitive marker of early-stage atherosclerosis, while neither eGFR nor ACR was associated with Eθ, In addition, Eθ was more strongly associated with cystatin C than was either IMT or CAVI. Furthermore, multiple regression analysis revealed a strong relationship between cystatin C and Eθ, especially in the subgroup with relatively high arterial wall elasticity. One possible explanation of these results is that renal impairment is associated with atherosclerosis development starting in the very early stages of both disorders. On the other hand, it is also possible that cystatin C might itself be directly involved in the development of atherosclerosis [30]. Cystatin C is an endogenous inhibitor of cysteine protease. Atherosclerosis is an inflammatory disease characterized by remodeling of the extracellular matrix of the arterial walls and cysteine protease induces degradation of the extracellular matrix. The imbalance between cysteine protease and its inhibitor, cystatin C, may result in increased degradation of extracellular matrix and migration of monocytes/macrophages into the intima, thus leading to the development of atherosclerosis. Tissue cystatin C levels are reportedly reduced in atherosclerotic plaques [31]. In contrast, inflammatory cytokines stimulate cells outside of the vascular walls to secrete cystatin C into the circulation. Thus, serum cystatin C is considered to be compensatorily upregulated in subjects who have developed atherosclerosis [32]. Although further studies are needed to examine whether this mechanism also accounts for serum cystatin C levels rising in states of subclinical atherosclerosis, as found in this study, the serum cystatin C level is likely to reflect the degree

of the atherosclerotic states via mechanisms both dependent on, and independent of, glomerular filtration rates.

This study has limitations. First, the possible causal relationship between cystatin C and Eθ could not be clarified due to the cross-sectional study design. Second, although we adjusted for well-known confounders such as age, gender, blood pressure, hyperlipidemia, body mass index, HbA1c, duration of diabetes, and use of medications, residual confounding, due to factors such as postmenopausal and inflammatory status, may have occurred. Third, our patients were recruited in a hospital setting after admission for poor glycemic control, such that recruitment bias may exist. In fact, the average HbA1c of our patients was 10.0%, which is extremely high. Moreover, because blood was drawn one day after the admission, fasting plasma glucose may have improved owing to dietary treatment while hospitalized. Also, fasting plasma glucose may not necessarily reflect the conditions of glycemic control. Fourth, there is little evidence indicating that carotid arterial wall elasticity determined by the phased tracking method is a good surrogate marker of cardiovascular diseases, because long-term outcomes have not been observed. In addition, although we and another group reported the significance of carotid arterial wall elasticity [3–7,9–12], further accumulation of evidence is needed to establish that Eθ is a good surrogate marker of early stage-atherosclerosis. Fifth, all of the subjects had T2DM and the lack of age- and gender-matched healthy controls limits the generalizability of our findings. Further studies, especially larger, population-based prospective studies, are needed to overcome these limitations.

In conclusion, our results indicate that the serum cystatin C level is associated with arterial wall elasticity, a sensitive examination for early-stage atherosclerosis, in subjects classified as not having atherosclerosis as defined by IMT criteria. Thus, the serum cystatin C level may reflect the degrees of atherosclerotic changes in the early stages of this disorder. Measuring the serum cystatin C level appears to be useful for detecting subclinical atherosclerosis in subjects with T2DM.

Funding sources

This work was supported by Grants-in-Aid for Scientific Research (16 K09773 and 15 K09416) to S. Sawada and Y. Ishigaki, respectively, from Japan Society for the Promotion of Science – Japan.

Conflicts of interests

The authors declare that they have no competing interests.

Author contributions

R.K. conceived the hypothesis, performed the experiments, and analyzed the data. S.S. A.T, R.H. and Y.I performed the experiments. S.K., T.I., K.T., K.U., J.I. and T.Y contributed to discussions and recruitment of patients. Y.M., H.H. and H.K. provided technical advice on the ultrasonic equipment. N.T. provided ultrasonic analysis of arterial wall elasticity. H.K. directed the project, and prepared the manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.diabres.2018.02.003.

REFERENCES

[1] Kannel WB, McGee DL. Diabetes and cardiovascular disease. The Framingham study. Jama 1979;241(19):2035–8.
[2] Nesto RW, Phillips RT. Asymptomatic myocardial ischemia in diabetic patients. Am J Med 1986;80(4c):40–7.
[3] Hasegawa H, Kanai H, Hoshimiya N, Koiwa Y. Evaluating the regional elastic modulus of a cylindrical shell with nonuniform wall thickness. J Med Ultrason (2001) 2004;31(2):81–90.
[4] Hasegawa H, Kanai H, Ichiki M, Tezuka F. Tissue structure of arterial wall revealed with elasticity imaging. J Med Ultrason (2001) 2007;34(1):73–4.
[5] Kanai H, Hasegawa H, Ichiki M, Tezuka F, Koiwa Y. Elasticity imaging of atheroma with transcutaneous ultrasound: preliminary study. Circulation 2003;107(24):3018–21.
[6] Kanai H, Koiwa Y, Zhang J. Real-time measurements of local myocardium motion and arterial wall thickening. IEEE Trans Ultrason Ferroelectr Freq Control 1999;46(5):1229–41.
[7] Okimoto H, Ishigaki Y, Koiwa Y, Hinokio Y, Ogihara T, Suzuki S, et al. A novel method for evaluating human carotid artery elasticity: possible detection of early stage atherosclerosis in subjects with type 2 diabetes. Atherosclerosis 2008;196(1):391–7.
[8] Katakami N, Kaneto H, Shimomura I. Carotid ultrasonography: A potent tool for better clinical practice in diagnosis of atherosclerosis in diabetic patients. J Diab Investig 2014;5(1):3–13.
[9] Tokita A, Ishigaki Y, Okimoto H, Hasegawa H, Koiwa Y, Kato M, et al. Carotid arterial elasticity is a sensitive atherosclerosis value reflecting visceral fat accumulation in obese subjects. Atherosclerosis 2009;206(1):168–72.
[10] Miyamoto M, Kotani K, Okada K, Ando A, Hasegawa H, Kanai H, et al. Arterial wall elasticity measured using the phased tracking method and atherosclerotic risk factors in patients with type 2 diabetes. J Atheroscler Thrombosis 2013;20(8):678–87.
[11] Yamagishi T, Kato M, Koiwa Y, Hasegawa H, Kanai H. Usefulness of measurement of carotid arterial wall elasticity distribution in detection of early-stage atherosclerotic lesions caused by cigarette smoking. J Med Ultrason (2001) 2006;33(4):203–10.
[12] Yamagishi T, Kato M, Koiwa Y, Hasegawa H, Kanai H. Impact of lifestyle-related diseases on carotid arterial wall elasticity as evaluated by an ultrasonic phased-tracking method in Japanese subjects. J Atheroscler Thrombosis 2009;16(6):782–91.
[13] Inker LA, Schmid CH, Tighiouart H, Eckfeldt JH, Feldman HI, Greene T, et al. Estimating glomerular filtration rate from serum creatinine and cystatin C. New Engl J Med 2012;367(1):20–9.
[14] Imai A, Komatsu S, Ohara T, Kamata T, Yoshida J, Miyaji K, et al. Serum cystatin C is associated with early stage coronary atherosclerotic plaque morphology on multidetector computed tomography. Atherosclerosis 2011;218(2):350–5.

[15] Batra A, Kapoor A, Sharma RK, Agrawal N, Sinha A, Kumar S, et al. Association of plasma cystatin C levels with angiographically documented coronary artery disease in patients of Indian origin. J Cardiol 2012;59(2):182–9.
[16] Wang J, Sim AS, Wang XL, Salonikas C, Moriatis M, Naidoo D, et al. Relations between markers of renal function, coronary risk factors and the occurrence and severity of coronary artery disease. Atherosclerosis 2008;197(2):853–9.
[17] Schottker B, Herder C, Muller H, Brenner H, Rothenbacher D. Clinical utility of creatinine- and cystatin C-based definition of renal function for risk prediction of primary cardiovascular events in patients with diabetes. Diabetes Care 2012;35(4):879–86.
[18] Koenig W, Twardella D, Brenner H, Rothenbacher D. Plasma concentrations of cystatin C in patients with coronary heart disease and risk for secondary cardiovascular events: more than simply a marker of glomerular filtration rate. Clin Chem 2005;51(2):321–7.
[19] Luc G, Bard JM, Lesueur C, Arveiler D, Evans A, Amouyel P, et al. Plasma cystatin-C and development of coronary heart disease: The PRIME Study. Atherosclerosis 2006;185(2):375–80.
[20] Yukiya M, Hideyuki H, Hiroshi K. Automated detection of arterial wall boundaries based on correlation between adjacent receive scan lines for elasticity imaging. Jpn J Appl Phys 2015;54(7S1):07HF18.
[21] Sidhu PS, Desai SR. A simple and reproducible method for assessing intimal-medial thickness of the common carotid artery. Brit J Radiol 1997;70:85–9.
[22] Shirai K, Utino J, Otsuka K, Takata M. A novel blood pressure-independent arterial wall stiffness parameter; cardio-ankle vascular index (CAVI). J Atheroscler Thrombosis 2006;13 (2):101–7.
[23] Matsuo S, Imai E, Horio M, Yasuda Y, Tomita K, Nitta K, et al. Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis: Off J National Kidney Found 2009;53(6):982–92.
[24] Zhang L, Li H, Zhang S, Jaacks LM, Li Y, Ji L. Silent myocardial ischemia detected by single photon emission computed tomography (SPECT) and risk of cardiac events among asymptomatic patients with type 2 diabetes: a meta-analysis of prospective studies. J Diabetes Complications 2014;28 (3):413–8.
[25] Bots ML, Hoes AW, Koudstaal PJ, Hofman A, Grobbee DE. Common carotid intima-media thickness and risk of stroke and myocardial infarction: the Rotterdam Study. Circulation 1997;96(5):1432–7.
[26] O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson Jr SK. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. New Engl J Med 1999;340(1):14–22.
[27] Touboul PJ, Elbaz A, Koller C, Lucas C, Adrai V, Chedru F, et al. Common carotid artery intima-media thickness and brain infarction: the Etude du Profil Genetique de l'Infarctus Cerebral (GENIC) case-control study. The GENIC Investigators. Circulation 2000;102(3):313–8.
[28] del Sol AI, Moons KG, Hollander M, Hofman A, Koudstaal PJ, Grobbee DE, et al. Is carotid intima-media thickness useful in cardiovascular disease risk assessment? The Rotterdam Study. Stroke J Cerebral Circ 2001;32(7):1532–8.
[29] Nambi V, Chambless L, Folsom AR, He M, Hu Y, Mosley T, et al. Carotid intima-media thickness and presence or absence of plaque improves prediction of coronary heart disease risk: the ARIC (Atherosclerosis Risk In Communities) study. J Am Coll Cardiol 2010;55(15):1600–7.
[30] Chapman HA, Riese RJ, Shi GP. Emerging roles for cysteine proteases in human biology. Annu Rev Physiol 1997;59:63–88.
[31] Shi GP, Sukhova GK, Grubb A, Ducharme A, Rhode LH, Lee RT, et al. Cystatin C deficiency in human atherosclerosis and aortic aneurysms. J Clin Investig 1999;104(9):1191–7.
[32] Salgado JV, Souza FL, Salgado BJ. How to understand the association between cystatin C levels and cardiovascular disease: Imbalance, counterbalance, or consequence? J Cardiol 2013;62(6):331–5.