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Reliability of measurement of endothelial function across multiple institutions and establishment of reference values in Japanese

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ABSTRACT

Aims: For the standardization of flow-mediated vasodilatation (FMD) assessment as a clinical tool, validation of its reliability across multiple institutions and the establishment of normal/reference values based on reliable data from multiple institutions are needed.

Methods and results: In Study 1, assessment of FMD (scan recording and analysis) using an ultrasonographic semi-automatic measuring system (sFMD) was conducted at 18 participating institutions (sFMD-INST) (n = 981). All of the brachial arterial scans were also analyzed at a core laboratory (sFMD-COLB). After 111 subjects with inadequate sFMD recordings were excluded (n = 880), the correlation between the sFMD-INST and sFMD-COLB improved from R = 0.725 to R = 0.838 (p < 0.001). In Study 2, based on good-quality sFMD data obtained from 6660 subjects without cardiovascular disease (CVD) and 729 subjects with CVD from 27 institutions, reference values of sFMD are proposed by the Framingham risk score (FRS)-based risk categories and according to gender and age. The receiver-operating characteristic curve analysis revealed a significant power of sFMD values in reference ranges to discriminate between subjects with and without CVD (e.g., area under curve = 0.64 in the FRS-low risk group).

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Conclusions: When the analysis was limited to cases with clear sFMD recordings, the reliability of the sFMD assessment (scan and its analysis) conducted in individual institutions appeared to be acceptable. Reference sFMD values (lower cuff occlusion) for the Japanese population are proposed based on reliable data derived from multiple institutions, and the reference values may identify patients without advanced vascular damage.

1. Introduction

Although assessment of flow-mediated vasodilatation of the brachial artery (FMD) has potential as a useful clinical tool in the clinical management of patients with cardiovascular (CV) disease and/or its risk factors [1–5], highly skilled sonographers are required for the accurate assessment of FMD (brachial artery scan recording and analysis) [6–8]. Therefore, in most multi-center studies conducted to date, the brachial artery scans obtained in each participating institution have been analyzed at a core laboratory (COLB) [4,5,7,8]. For the standardization of FMD assessment as a clinical tool, validation of the FMD assessment method across multiple institutions and the establishment of normal/reference FMD values based on reliable data obtained from multiple institutions fulfilling the criteria for reliability of the assessment method are needed.

Recently, an ultrasound instrument dedicated to FMD assessment, equipped with both an on-line computer-assisted semi-automatic analysis software to measure the FMD and accessories for fixing the measured arm and ultrasound probe, has been introduced for commercial use [9–12]. A major advantage of this device is that it allows automatic direct A-mode signal analysis of changes of the vessel diameter during the FMD assessment procedure in real time under stereotaxic guidance based on 2-dimensional B-mode images for maintenance of the appropriate position for the vessel interfaces recording [9–12]. Thus, this device might be helpful for the standardization of FMD assessment via facilitating the assessment at individual institutions and improving the quality of analysis of the FMD scan records at each institution.

The FMD-J study was a prospective multicenter study conducted to examine the usefulness of FMD assessment using a semi-automatic device at individual institutions in the management of patients at risk for CV disease [9]. The present study was conducted as an extension of the FMD-J study to examine the following for the standardization of FMD assessment; Study 1) To validate the reliability of FMD assessment (scan and its analysis) using the aforementioned semi-automatic device at individual participant institutions as compared to analysis of the brachial artery scans at a COLB; Study 2) if the reliability was found to be acceptable, the FMD data obtained in the FMD-J study and in the previously reported Flow-mediated Dilatation Japan Registry study (FDR study) [11,12], in which the FMD assessment was conducted using the same protocol as that in the FMD-J study, would be collected to obtain the reference values of FMD. In addition, a receiver-operating-characteristic curve (ROC) analysis was carried out to evaluate the discriminative power of FMD in the reference ranges for the presence of CV disease (i.e., to discriminate between subjects with and without CV disease).

2. Methods

The study protocols conform to the principles of the Declaration of Helsinki, and have been approved by the Ethics Committee of Tokyo Medical University (The core center of FMD-J study) (No. 2456) and also by the Ethics Committees of other each of the participating institutions. The written informed consent was obtained from all of the study participants before participation in the FMD-J study or the FDR study.

2.1. Study cohorts

The present studies were conducted in subjects derived from study cohorts of the FMD-J study (2 study cohorts for Study 1 and 4 study cohorts for Study 2) (Fig. 1). The FMD-J study included 3 study arms (Study A, Study B, and Study C) (Fig. 1).

The details of the study protocols and the subjects are described in Supplement file 1 and Reference 9. In the FDR study, all of the participants underwent annual health check-ups and assessment of FMD at one of 4 institutions (clinics/hospitals) affiliated to the following 3 institutions participating in the FMD-J study {Tokyo Medical University (COLB for the FMD-J study), National Defense Medical College, and Hiroshima University}. Some parts of the data from the FDR study have already been reported elsewhere [11,12]. The protocol for the assessment of FMD was the same in the FDR study as that in the FMD-J study, and the aforementioned semi-automatic device was used for the FMD assessment in all the subjects of the FDR study. In Study C (an arm of FMD-J study) and the FDR study, the FMD was measured at the time of the annual health check-up in the subjects, and the presence/absence of CV disease in the subjects (heart disease and/or cerebrovascular disease: the details were not described) was confirmed by a questionnaire.

2.2. Study design

2.2.1. Study 1: examination of the reliability of assessment of FMD at individual participant institutions

Some of the study subjects in Study A and Study B (arms of FMD-J study) (from 18 participating institutions) participated in Study 1 (Fig. 1). The results of assessment of the quality of the brachial artery scans and of the data analyses conducted at each institution were registered on the WEB. Each participant institution (18 institutions) was instructed to send the USB devices containing the brachial artery scans obtained during the assessment of FMD without the results of the data analysis to the COLB located in Tokyo Medical University, and the recordings were individually analyzed by a well-experienced reader at the COLB (FMD-COLB) without any information concerning the FMD data assessment at the participant institution. This well-experienced reader (T.S.) had the experience of conducting this analysis in more than 500 cases (as at the end of December 2010). Then, the results of the FMD assessment at each of the institutions registered on the WEB, and the FMD-COLB were compared (Study 1-1, Study 1-2, and 1-3).

2.2.2. Study 2: establishment of reference values of FMD and evaluation of the discriminative power of FMD for the presence of CV disease

In addition to the subjects from Study B, subjects without CV disease from Study C and the FDR study [11,12] were enrolled for
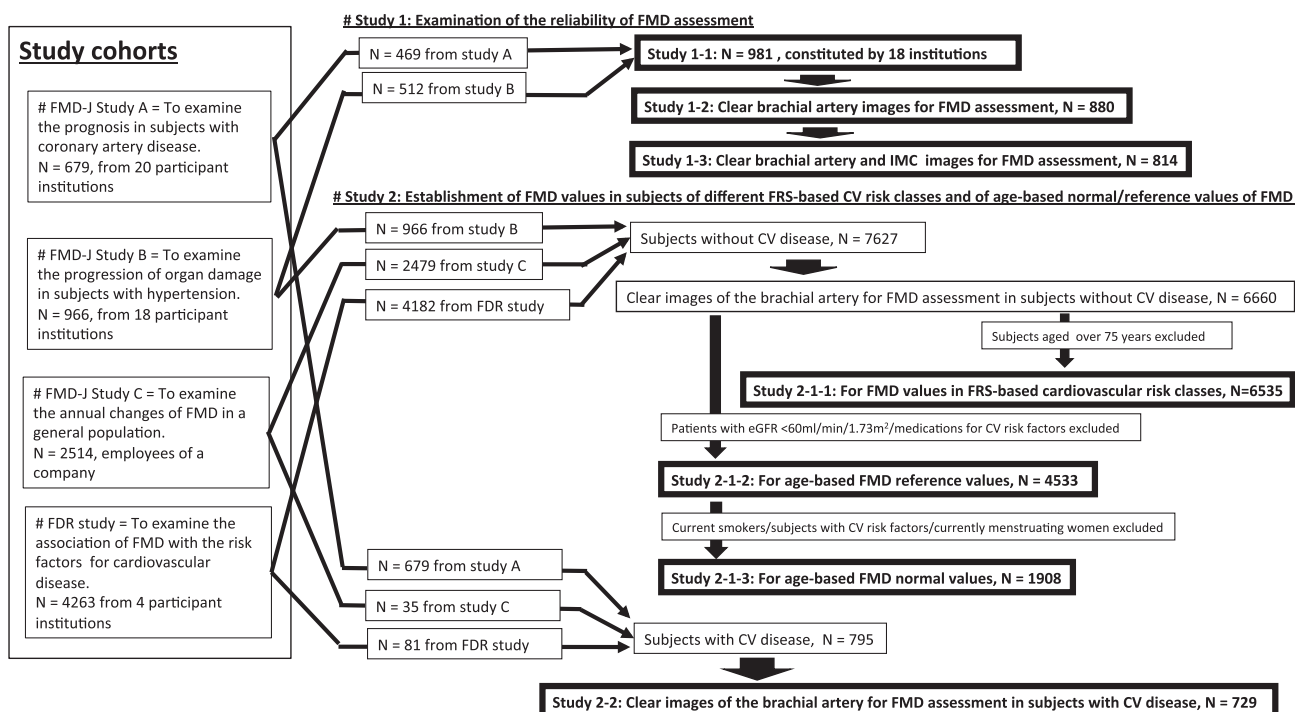
Study cohorts

FMD-J Study A = To examine the prognosis in subjects with coronary artery disease. N = 679, from 20 participant institutions

FMD-J Study B = To examine the progression of organ damage in subjects with hypertension. N = 966, from 18 participant institutions

FMD-J Study C = To examine the annual changes of FMD in a general population. N = 2514, employees of a company

FDR study = To examine the association of FMD with the risk factors for cardiovascular disease. N = 4263 from 4 participant institutions

Study 1: Examination of the reliability of FMD assessment

N = 469 from study A

N = 512 from study B

Study 1-1: N = 981 , constituted by 18 institutions

Study 1-2: Clear brachial artery images for FMD assessment, N = 880

Study 1-3: Clear brachial artery and IMC images for FMD assessment, N = 814

Study 2: Establishment of FMD values in subjects of different FRS-based CV risk classes and of age-based normal/reference values of FMD

N = 966 from study B

N = 2479 from study C

N = 4182 from FDR study

Subjects without CV disease, N = 7627

Clear images of the brachial artery for FMD assessment in subjects without CV disease, N = 6660

Subjects aged over 75 years excluded

Study 2-1-1: For FMD values in FRS-based cardiovascular risk classes, N=6535

Patients with eGFR <60ml/min/1.73m² /medications for CV risk factors excluded

Study 2-1-2: For age-based FMD reference values, N = 4533

Current smokers/subjects with CV risk factors/currently menstruating women excluded

Study 2-1-3: For age-based FMD normal values, N = 1908

N = 679 from study A

N = 35 from study C

N = 81 from FDR study

Subjects with CV disease, N = 795

Study 2-2: Clear images of the brachial artery for FMD assessment in subjects with CV disease, N = 729

Fig. 1. Flow-chart of the study subject selections. Abbreviations FMD = flow-mediated vasodilatation of brachial artery; FRS = Framingham risk score; eGFR = estimated glomerular filtration rate; CV risk = risk factors for cardiovascular disease; CV disease = cardiovascular disease; IMC = intima−media complex.

the studies 2-1-1, 2-1-2, and 2-1-3 (Fig. 1), and reference FMD values were obtained. A ROC analysis was carried out to evaluate the discriminative power of FMD for subjects with and without CV disease (Study 2-2).

2.2.2.1. Study 2-1-1: FMD values in the Framingham Risk Score (FRS)-based CV risk classes (Fig. 1). The values of FMD were examined in subjects without CV disease classified into different CV risk categories based on the FRS. Then, they were divided by the gender and the FRS-based CV risk category (high-risk; 10-year CAD risk $\geq$20%/intermediate-risk; 10-year CAD risk 10−19%/low-risk; 10-year CAD risk <10%) [13].

2.2.2.2. Study 2-1-2: age-based reference values (Fig. 1). The reference values were examined in subjects who did not have a history of CV disease, values of the serum creatinine-derived estimated glomerular filtration rate (eGFR) of <60 ml/min/1.73 m² [14], and were not receiving medications for CV risk factors. Then, the subjects were divided by the gender and age (divided in decades).

2.2.2.3. Study 2-1-3: age-based normal values (Fig. 1). The normal values were examined in subjects for establishing age-based reference values, after excluding subjects with risk factors for CV disease (current smoker, BMI $\geq$ 27.5, SBP/DBP $\geq$ 140/90 mm Hg, TC $\geq$ 6.2 mmol/L, HbA1c $\geq$ 6.5%, and/or medication for hypertension, dyslipidemia, and/or diabetes mellitus). In addition, women who were menstruating at the time of the FMD assessment were also excluded. Then, the subjects were divided by the gender and age (classified in decades).

2.2.2.4. Study 2-2: FMD values in subjects with CV disease (Fig. 1). In addition to subjects from Study A, subjects with CV disease from Study C and the FDR study were also enrolled, and the FRSs were calculated in these subjects.

2.3. FMD procedures, brachial artery scan, assessment of the quality of the scans, and their analysis

In the FMD-J study, two ultrasound instruments equipped with an on-line computer-assisted semi-automatic analysis software were available to measure the FMD: EF (Unex Co. Ltd, Nagoya, Japan and e-TRACKING (ALOKA Co. Ltd, Tokyo, Japan) [9]. Except for the case of 10 subjects in Study A, however, the Unex equipment was used for the assessment of FMD in all the study subjects, because it is an even more dedicated equipment to assess FMD than e-TRACKING.

In regard to the procedure for the FMD assessment, an occlusion cuff is wrapped around the forearm with the proximal edge of the cuff at the elbow, and cuff is inflated to a compression pressure of 50 mm Hg over the systolic blood pressure value for 5 min. The details of these procedures and the device used to measure the values of FMD are described in supplemental file 2. In this system, the diastolic diameter of the brachial artery is determined semi-automatically and the changes of the diameter are tracked automatically [9].

Inadequate scans were assessed to check which of the following two criteria for inadequate scans were met; exclusion criterion 1 = vessel interfaces in the longitudinal B-mode images not clear and/or the images inadequate due to patient movement; exclusion criterion 2 = exclusion criteria 1 plus unclear signal of the IMC in the A-mode images. In cases where the IMC was clear in the A-mode images, the diameter was automatically determined from the peak of the IMC signal (Fig. 2A), and such cases were considered as not falling under exclusion criteria 2. In cases without a clear signal of the IMC, the diameter was manually determined by the rising point of the media-adventitia signals (Fig. 2B), and such cases were considered as not falling under exclusion criteria 1. FMD was calculated as {Diameter of the brachial artery at the baseline (DIAbase) - diameter of the brachial artery at maximum dilatation (DIAmax)} x 100/DIAbase. In addition, because DIAbase is an
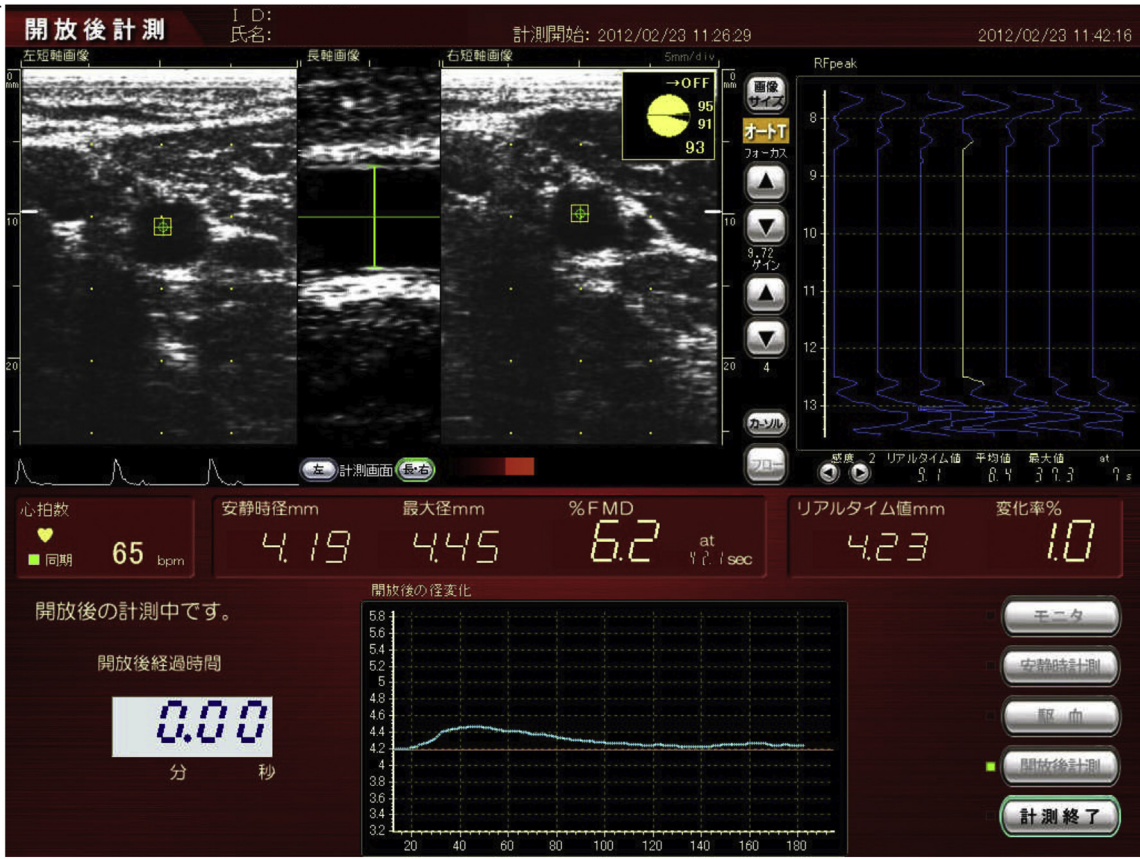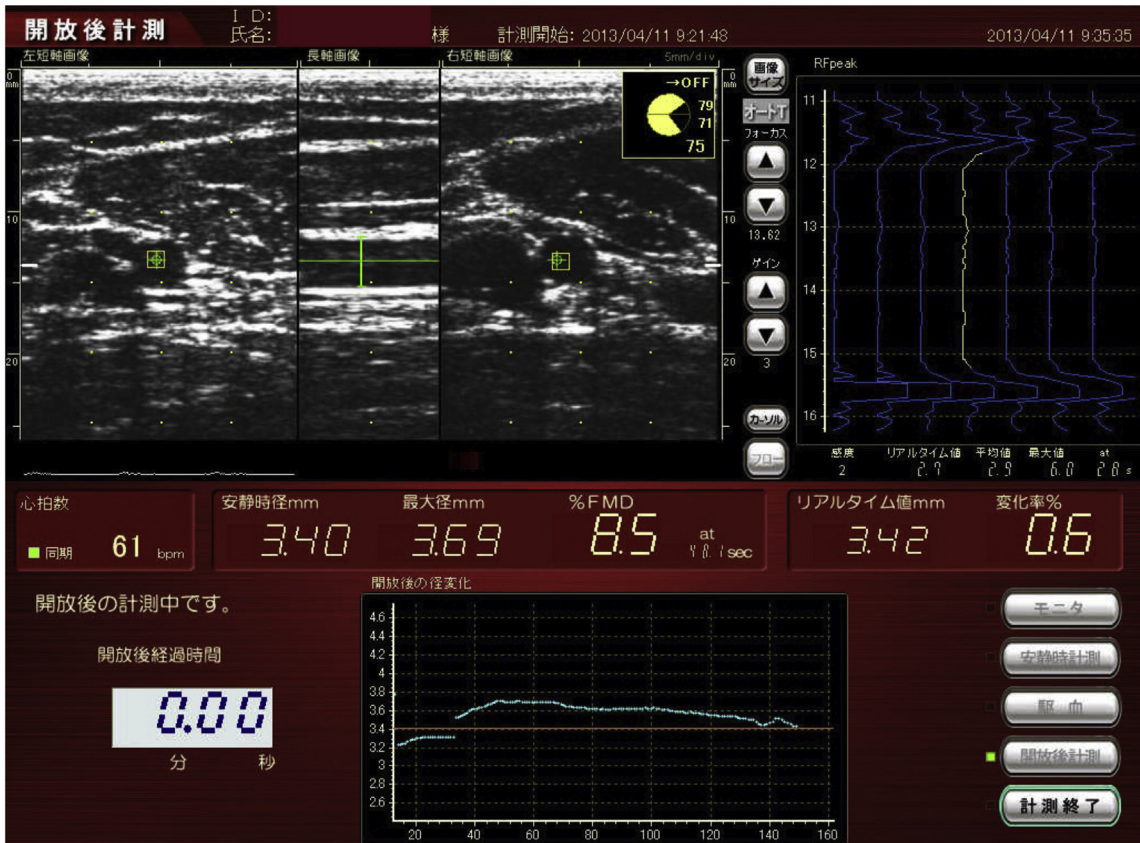
Fig. 2. Images of flow-mediated vasodilatation with/without clear intima–media complex signals.

important confounder in the determination of the predictive value of FMD for future cardiovascular events, the allometric scaled FMD (DIAmax/DIAbase 0.89) was also calculated [15].

2.4. Training and certification for the assessment of FMD

See Supplementary file 3.

2.5. Statistics

The data were expressed as mean ± SD or median with 95% confidence intervals (CI). The relationships among the variables were assessed by univariate linear regression analysis and stepwise multiple linear regression analysis. Bland—Altman plots were used to assess the variability of the FMD measurements between the institutions and the COLB. For assessing the inter-rater reliability, the intra-class correlation coefficient (ICC) was calculated. In all the analyses, a p value of less than 0.05 was considered to indicate a statistically significant difference. ROC analysis was conducted to determine the discriminative power of FMD for the risk of CV disease in the subjects, and the area under the curve (AUC) was calculated. All the analyses were conducted using the IBM/SPSS (22.0J for Windows, IBM/SPSS Inc., Chicago, IL).

3. Results

3.1. Study 1: validation of the reliability of FMD assessment across the participating institutions

A total of 981 brachial artery scans obtained for FMD assessment were sent to the COLB from the 18 participating institutions (Study 1-1: Fig. 1).

The reliability of the assessment of FMD from these 981 records at the individual institutions was assessed. The clinical characteristics of the study subjects are shown in Table 1. Based on the reports registered on the WEB, the scans were deemed as adequate in 880 of the 981 subjects (Study 1-2: Fig. 1), the remaining falling under exclusion criterion 1 {unclear images of the brachial artery interfaces (n = 79) or patient movement (n = 22)}, and the correlation coefficient of the FMD values improved after exclusion of the inadequate scans (from R = 0.725 to R = 0.838) (Fig. 3).

Of these 880 scans, clear IMC signals were obtained in 814 (Study 1-3: Fig. 1), the remaining falling under exclusion criterion 2, but no further improvement of the correlation coefficient was observed after exclusion of the latter scans (Fig. 3). The National Defense Medical college and Hiroshima University participated in the FDR study, and in the present study, the correlation co-efficients between the FMD-COLB and FMD values analyzed at these two institutions and their ICC values were found to be acceptable {R = 0.868, P < 0.001, ICC = 0.862 (0.811—0.900) and R = 0.862, P < 0.001, ICC = 0.964 (0.787—0.914)}. Bland—Altman analysis showed no systemic bias in the FMD measurement vari-ability between the institutions and the COLB (Fig. 3). The stan-dard deviation of the difference of the FMD values between each of the institutions and the COLB was 0.25 ± 1.55%. The ICC values of two institutions were not satisfactory (i.e., ICC <0.60) (Supplementary Table 1). As compared to the other institutions for which the ICC values were determined to be ≥0.60 (i.e., good inter-rater reliability), the number of study subjects enrolled from these two institutions was small (n = 9 each), and both in-stitutions participated in only Study A. Correlations between the diameters of the brachial artery analyzed at each participant institution and the core laboratory and the Bland—Altman plots

Table 1
Clinical characteristics of the subjects in each study.

Study subjects	Study 1	Study 2-1s	Study 2-2
Number of subjects	981	6660	729
Gender (men/women)	660/321	5269/1391	620/109
Age (y)	63 ± 9	50 ± 10	63 ± 9
SBP (mm Hg)	131 ± 18	127 ± 16	128 ± 17
DBP (mm Hg)	77 ± 10	80 ± 12	77 ± 10
HR (beats/min)	64 ± 10	64 ± 10	63 ± 10
Current smoker (%)	124 (12.6)#1	2982 (31.3)#2	119 (16.3)#3
BMI	25.0 ± 3.6	23.4 ± 3.3	24.8 ± 3.6
TC (mmol/L)	4.7 ± 0.9	5.3 ± 0.8	4.5 ± 0.9
HDL (mmol/L)	1.4 ± 0.4	1.5 ± 0.4	1.3 ± 0.4
TG (mmol/L)	1.5 ± 1.0	1.4 ± 1.1	1.6 ± 1.1
FBG (mmol/L)	6.2 ± 1.7	5.5 ± 1.0	6.4 ± 1.9
Crnn (μmol/L)	72 ± 21	72 ± 16	77 ± 33
eGFR (mL/min/1.73m²)	72 ± 17	78 ± 14	71 ± 17
FMD-INST (%)	4.87 ± 2.63	6.38 ± 3.07	4.82 ± 2.70
FMD-INSTallo (%)	5.68 ± 3.08	7.40 ± 3.51	5.63 ± 3.13
FMD-COLB (%)	4.63 ± 2.78	—	—
DIAbase-INST (mm)	4.20 ± 0.66	4.03 ± 0.58	4.26 ± 0.62
DIAbase-COLB (mm)	4.19 ± 0.65	—	—
DIAmax-INST (mm)	4.40 ± 0.65	4.28 ± 0.57	4.46 ± 0.61
DIAmax-COLB (mm)	4.38 ± 0.64	—	—
Medications [n (%)]			
For hypertension	917 (93.5)	1492 (22.4)	601 (82.4)
For dyslipidemia	618 (63.0)	604 (9.1)	570 (78.2)
For diabetes mellitus	212 (21.6)	299 (4.5)	211 (28.9)
CVD (%)	469 (47.8)	0	729 (100)
FRS		3.9 ± 4.6	6.5 ± 3.2
FRSclass (L/M/H) (%)		4424/1630/481 (67.7/24.9/7.4)	290/255/119 (43.7/38.4/17.9)
Not Calc FRS		125	65

Abbreviations: Study 1 = study subjects for examination of the reliability of FMD assessment in each participant institute; Study 2-1s = study subjects without car-diovascular disease for examination of FMD in subjects without cardiovascular disease of different FRS classes and of age-based normal/reference values of FMD; Study 2-2 = study subjects with a history of cardiovascular disease for examination of FMD; SBP = systolic blood pressure; DBP = diastolic blood pressure; HR = heart rate; Current Smoker = number of current smokers; BMI = body mass index; TC = serum level of total cholesterol; TG = serum level of triglycerides; HDL = serum level of high-density lipoprotein cholesterol; FBG = fasting blood glucose level; Crnn = serum level of creatinine; eGFR = estimated glomerular filtration rate; FMD = flow-mediated dilatation of the brachial artery; FMD-INSTallo = allometric scaled FMD-INST; DIAbase = baseline diameter of the brachial artery; DIAmax = diameter of the brachial artery at maximum dilatation; -COLB = analyzed by the core laboratory: -INST = analyzed at each participant institution; Medication: number of subjects receiving medications; CVD = number of subjects with cardio-vascular disease; FRS = Framingham risk score; FRS class = risk classification based on the Framingham risk score; L = low risk; M = intermediate risk; H = high risk; Not Calc FRS = number of subjects in whom the Framingham risk score could not be calculated because of data loss/age over 75 years; #1 = smoking history was not available for 18 subjects; #2 = smoking history was not available for 22 subjects; #3 = smoking history was not available for 23 subjects.

are depicted in supplementary Figs. 1 and 2.

3.2. Study 2: establishment of reference FMD values and evaluation of the discriminative power of FMD for the presence of CV disease

The flow-chart for the study subject selections is shown in Fig. 1. Among the data of a total of 7627 subjects without CV disease from study B, study C and the FDR study (data from two institutions with ICC values of <0.60 were not included), clear images of the brachial artery interfaces were obtained in 6660 subjects (87.3%) (Fig. 1). The clinical characteristics of the study subjects are shown in Table 1. The FRSs were calculated in 6535 of the 6660 subjects (after exclusion of subjects aged over 75 years). In these 6535 subjects, a stepwise multivariate linear regression analysis demonstrated that the age (beta = −0.105, p < 0.001), gender (men = 1 and women = 0) (beta = −0.204, p < 0.001), and FRS (beta = −0.085, p < 0.001) were significantly associated with the FMD, independent
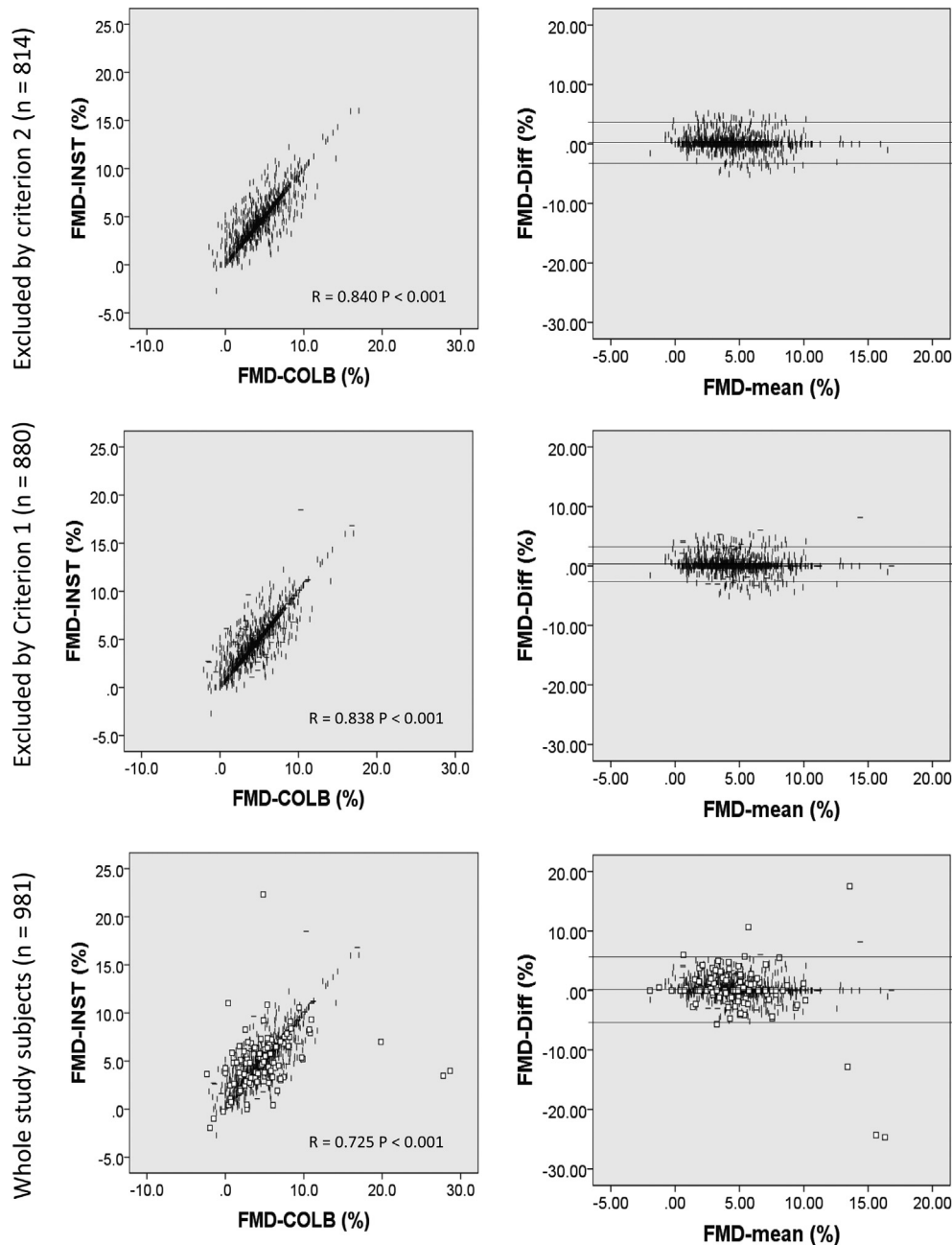
Fig. 3. Correlations between flow-mediated dilatation of the brachial artery analyzed at each participant institution and the core laboratory and Bland–Altman plots for variability of the flow-mediated dilatation measurement between each participant institution and the core laboratory. Abbreviations FMD = flow-mediated vasodilatation of the brachial artery; -COLB = analyzed at the core laboratory: -INST = analyzed at each participant institution; -Diff = difference in value between each participant institution and the core laboratory; -mean = mean of the values at each participant institution and the core laboratory; Exclusion criterion 1 = Interfaces in the longitudinal B-mode images not clear and/or inadequate due to patient movement; Exclusion criterion 2 = exclusion criteria 1 plus unclear intima–media complex signal in the A-mode images.

of the DIAbase or history of treatments for hypertension, dyslipidemia, and/or diabetes mellitus (Total R^2 = 0.289). First, the FMD values in these 6535 subjects stratified into different FRS-based CV risk categories were obtained (Study 2-1-1: Fig. 1). Second, among the total of 6660 subjects, the age-based reference values of FMD were examined in 4533 subjects after exclusion of subjects over the age of 75 years and subjects with cardiovascular risk factors, (Study 2-1-2: Fig. 1). Third, the age-based normal values were examined in the remaining 1908 subjects who were under 75 years of age and had no risk factors for cardiovascular disease (Study 2-1-3: Fig. 1). Then, the subjects were divided by the gender and age (classified in

decades). Fourth, among the study A subjects and subjects with CV disease from Study C and the FDR study, clear images of the brachial artery interfaces were obtained in 729 subjects, and the FMD values in these subjects were determined (Study 2-2: Fig. 1). The clinical characteristics of the study subjects are shown in Table 1.

Table 2 shows the FMD and DIAbase values in each group classified by the gender and FRS-based risk category in subjects with and without CV disease. Table 2 also shows the reference and normal values of FMD and DIAbase classified by the gender and age (classified in decades).

When subjects with and without CV disease in each FRS-based

Table 2
Flow-mediated vasodilatation of the brachial artery and brachial artery diameter according to the Framingham Risk Score-based cardiovascular risk class and age-based normal/reference values (median and 95% confidence intervals classified in decades).

	Men	CVD	Women	CVD
	nonCVD		nonCVD	
FRS class				
FRS-L	n = 3239	n = 237	n = 1185	n = 53
FMD (%)	6.50 (2.30–12.10)	5.10 (1.24–10.61)	6.90 (1.97–13.20)	4.86 (0.28–10.28)
FMDallo (%)	7.55 (2.67–13.94)	5.83 (1.45–12.28)	7.87 (2.28–15.00)	5.63 (0.33–11.73)
DIAbase (mm)	4.13 (3.38–5.00)	4.30 (3.47–5.45)	3.37 (2.72–4.26)	3.59 (2.66–4.67)
FRS-M	n = 1486	n = 226	n = 144	n = 29
FMD (%)	5.50 (1.80–10.20)	4.48 (1.06–9.45)	4.72 (0.22–9.32)	4.50 (0.61–8.67)
FMDallo (%)	6.50 (2.01–11.94)	5.26 (1.24–10.97)	5.51 (0.26–10.56)	5.14 (0.71–9.86)
DIAbase (mm)	4.26 (3.50–5.10)	4.35 (3.48–5.44)	3.61 (2.88–4.63)	3.73 (2.83–4.43)
FRS-H	n = 462	n = 106	n = 19	n = 13
FMD (%)	5.15 (1.30–9.64)	4.58 (1.02–8.07)	3.25 (−)	3.74 (−)
FMDallo (%)	6.06 (1.59–11.11)	5.32 (1.22–9.35)	3.81 (−)	4.39 (−)
DIAbase (mm)	4.21 (3.52–5.22)	4.29 (3.47–5.44)	3.79 (−)	4.00 (−)
FRS-all	n = 5187	n = 569	n = 1348	n = 95
FMD (%)	6.07 (2.00–11.40)	4.67 (1.11–9.73)	6.60 (1.69–13.01)	4.65 (0.29–9.64)
FMDallo (%)	7.09 (2.33–13.19)	5.48 (1.31–11.35)	7.50 (1.97–14.86)	5.33 (0.33–11.00)
DIAbase (mm)	4.17 (3.43–5.06)	4.31 (3.47–5.44)	3.40 (2.74–4.33)	3.73 (2.80–4.70)
Reference values				
Age 30-	n = 669	n = 8	n = 228	n = 2
FMD (%)	7.05 (3.10–13.18)	3.97 (−)	8.80 (4.50–14.41)	9.66 (−)
FMDallo (%)	8.21 (3.65–15.31)	4.64 (−)	9.97 (5.16–16.52)	11.03 (−)
DIAbase (mm)	3.99 (3.30–4.77)	4.18 (−)	3.12 (2.66–3.73)	3.49 (−)
Age 40-	n = 1483	n = 51	n = 291	n = 2
FMD (%)	6.73 (2.60–12.30)	5.77 (1.07–12.02)	7.90 (2.99–15.37)	7.33 (−)
FMDallo (%)	7.82 (3.01–14.24)	6.62 (1.27–13.81)	9.06 (3.46–17.45)	8.47 (−)
DIAbase (mm)	4.11 (3.37–5.00)	4.27 (3.46–5.32)	3.29 (2.66–4.04)	4.00 (−)
Age 50-	n = 1228	n = 149	n = 282	n = 17
FMD (%)	5.98 (1.90–10.90)	5.00 (1.27–9.73)	6.31 (1.90–12.39)	5.08 (−)
FMDallo (%)	6.99 (2.25–12.59)	5.92 (1.50–11.35)	7.24 (2.10–13.99)	5.87 (−)
DIAbase (mm)	4.18 (3.43–4.99)	4.30 (3.55–5.43)	3.40 (2.71–4.22)	3.74 (−)
Age 60-	n = 275	n = 412	n = 77	n = 88
FMD (%)	5.60 (1.78–10.61)	4.46 (1.02–9.46)	5.45 (2.36–10.61)	4.39 (0.27–8.60)
FMDallo (%)	6.55 (2.07–12.26)	5.20 (1.22–11.03)	6.29 (2.70–11.93)	5.02 (0.31–9.89)
DIAbase (mm)	4.15 (3.29–5.07)	4.32 (3.46–5.45)	3.53 (2.82–4.26)	3.73 (3.02–4.78)
Reference all	n = 3665	n = 620	n = 878	n = 109
FMD (%)	6.50 (2.40–11.86)	4.63 (1.10–9.66)	7.40 (2.70–14.09)	4.51 (0.41–9.50)
FMDallo (%)	7.55 (2.82–13.74)	5.41 (1.30–11.28)	8.46 (3.15–15.90)	5.26 (0.47–10.69)
DIAbase (mm)	4.12 (3.38–4.97)	4.30 (3.47–5.43)	3.30 (2.68–4.09)	3.73 (2.89–4.72)
Normal values				
Age 30-	n = 326	–	n = 170	–
FMD (%)	7.15 (3.14–13.57)		8.82 (4.50–13.99)	–
FMDallo (%)	8.26 (3.75–15.71)		9.97 (5.17–15.89)	
DIAbase (mm)	3.92 (3.20–4.63)		3.11 (2.65–3.73)	–
Age 40-	n = 565	–	n = 183	–
FMD (%)	7.30 (2.61–12.64)	–	7.90 (3.32–16.17)	–
FMDallo (%)	8.53 (3.06–14.63)		9.06 (3.74–18.12)	
DIAbase (mm)	4.01 (3.30–4.94)		3.26 (2.71–3.95)	–
Age 50-	n = 394	–	n = 142	–
FMD (%)	6.40 (1.90–11.80)	–	6.37 (1.05–11.49)	–
FMDallo (%)	7.49 (2.26–13.59)		7.27 (1.15–13.12)	
DIAbase (mm)	4.13 (3.31–4.94)		3.41 (2.75–4.08)	–
Age 60-	n = 90	–	n = 38	–

Table 2 (*continued*)

	Men	CVD	Women	CVD
	nonCVD		nonCVD	
FMD (%)	5.73 (1.57—10.41)	—	5.56 (2.38—10.67)	—
FMDallo (%)	6.71 (1.88—12.03)		6.35 (2.73—12.23)	
DIAbase (mm)	4.22 (3.44—5.11)	—	3.64 (2.96—4.39)	—

nonCVD = subjects without cardiovascular disease; p-value = p-value between subjects with and without cardiovascular disease; FRS-L = subjects classified according to the Framingham risk scores in the low risk category; FRS-M = subjects classified according to the Framingham risk scores into the intermediate risk category; FRS-H = classified according to the Framingham risk scores into the high risk category; FRS-all = All subjects included in the Framingham risk class stratification; Age 30- = subjects aged 30—39 years; Age 40- = subjects aged 40—49 years; Age 50- = subjects aged 50—59 years; Age 60- = subjects aged over 60 years; reference all = all study subjects included to obtain reference values; (—) = number of subjects was not enough to calculate the 95% confidence intervals; other abbreviations are as described in the footnote for Table 1.

risk category and in each age category were combined, ROC analysis revealed a significant power of the FMD to discriminate between subjects with and without CV disease (Supplementary Table 2); the area under the curve (AUC) of the ROC was larger in the FRS-based low-risk category than in the FRS-based intermediate- or high-risk category (Supplementary Table 2). In all study subjects included to obtain these reference values, the AUC revealed that the discriminative power of FMD for subjects with CV disease was greater than that of DIAbase (Supplementary Table 2). In addition, the allometric scaled FMD did not improve that discriminative power (Supplementary Table 2). When the subjects with CAD registered from the two institutions (Study A subjects) with ICC values of <0.60 (n = 19) were excluded (Supplementary Table 1), the AUCs to discriminate between patients with and without CV disease were almost similar (data not shown).

4. Discussion

The novelties of the present study are that we attempted to validate the reliability of assessment of FMD (brachial artery scan recording and its analysis) across multiple participant institutions using a semi-automatic device, and to establish the normal and reference values of FMD based on the FMD data from multiple institutions measured under a uniform protocol using the semi-automatic device.

There were two issues concerning the reliability of FMD assessment at individual institutions that needed to be clarified. One is the wide variability of FMD values assessed from A-mode images. Donald et al. reported that FMD values measured by A-mode wall tracking vary to a greater degree than those assessed using the B-mode edge detection system, because of the difficulty in maintaining positional stability of a single M-line without 2-dimensional image guidance [16]. In the present study, however, adequate A-mode images were obtained under stereotaxic guidance from the B-mode 2-dimensional images. Therefore, this issue was thought to be overcome by the use of the dedicated ultrasonographic device in the present study. Another issue is inter-reader variability of FMD analysis. Some previous studies have reported a closer relationship between the results of different methods of FMD analysis (e.g., A-mode analysis vs. B-mode analysis) from the same scan [17,18]. In these studies, however, the analysis of the scans was conducted by same reader. Furthermore, while recent multicenter studies reported that use of a standardized procedure using a computer-assisted software to analyze the brachial artery scans after optimal training yielded reproducible FMD values in some multicenter studies [7,8], the recorded scans were analyzed at the COLB by a few well-trained operators using such software. In the present multi-center study, we examined the inter-reader variability for FMD analysis (i.e., between the reader at each of the institutions and the reader at the COLB).

The Dal-VESSE study reported that the intra-class correlation coefficient range of the inter-reader reliability of the FMD values, as assessed by a B-mode device, among the readers of the core laboratory was 0.82—0.87 [7]. Brooks et al. reported that the standard deviation of difference of the FMD values assessed by a B-mode device between two readers was 2.32% [19]. In these studies, the subjects with inadequate images of the vessel interfaces were excluded. In the present study, in which the operators conducted the measurement after obtaining certification (optimal training under a uniform protocol using the same semi-automatic device was provided to the operators) [9], the results revealed a good correlation between the FMD assessment conducted at each institution and the FMD-COLB when cases with inadequate brachial artery scan images were excluded. The correlation co-efficient of FMD value (r = 0.838) and the standard deviation of difference of the FMD values as assessed by A-mode imaging (1.55%) between each institution and the COLB were similar to those for the B-mode images. While all the participating institutions had received certification as to their ability for accurate assessment of FMD based on a training session provided to them before they started patient registration for the FMD-J study (Supplementary file 3), the reliability was found to be unsatisfactory for two institutions which received only a small number of subjects for the assessment of FMD. Thus, as described in the guideline [6], after training for the assessment of FMD, attending periodic refresher courses in FMD assessment may be needed for the maintenance of competency.

In addition to the aforementioned instability of maintenance of position of a single M-line without 2-dimensional image guidance, we hypothesized that unclear signals of the IMC in the A-mode images may be another reason for the wide variability of FMD value assessed by the A-mode device. In the present study, however, the correlation coefficient between the FMD values measured at each institution and the COLB and the distributions of the Bland—Altman plots of the difference of the FMD values between each institution and the COLB were similar after exclusion based on exclusion criterion 1 and exclusion criterion 2. Thus, the unclear signal of the IMC in the A-mode images may not be a significant cause of the wide variability of the FMD value.

It is known that the protocol used (i.e., technical aspects such as lower arm/upper arm occlusion or occlusion duration) can affect the FMD value [20]. Even so, the protocols for the assessment of FMD in previously conducted multicenter studies were diverse [4,5,7—9,20], and the number of study subjects in these studies ranged from 2000 to 3,000, and all of these data were analyzed by the COLB. The strength of the present study was that reference values of FMD were established based on the data of more than 6000 study subjects obtained using a uniform protocol at each participating institution that fulfilled the criteria for certifying the reliability of the assessment.

Consistent with the results of previous studies [10—12], age, gender and FRS were significant determinants of FMD in the present study. Then, with the patients classified by the FRS-based CV risk category and by the gender, and normal/reference values were set according to the age (classified in decades) in both genders.

While the number of women was relatively small, the AUC to discriminate CV disease was larger in the FRS-based low-cardiovascular risk category as compared to that in the FRS-based intermediate- or high-risk group in both genders. Similar findings were reported by Witte et al., therefore, FMD may be related to the estimated 10-year risk for CAD, especially in subjects with a low risk for CV disease [21]. In addition, the AUC to discriminate CV disease was significant in subjects in whom the FMD values were in the present range of reference values. These results suggest that FMD values in the FRS-based risk classes (especially the low-risk group) and the age-based reference range may represent a state without advanced atherosclerotic vascular damage.

The present study had some limitations, as follows; 1) The reported rejection rate of scans from previous studies is in the range of 10—30% [7,8]. In the present study, the rejection rate was 10%. Thus, it is necessary to recognize that, in the present state, this FMD assessment method may not be applicable to all subjects. For such cases, reactive hyperemia peripheral arterial tonometry is available for the assessment of endothelial function [22], but it should be taken into account that FMD and peripheral tonometry reflect different facets of the pathophysiological abnormalities of vascular endothelium [22,23]; 2) Further studies are needed to clarify whether the reference and normal FMD values determined in the present study might also be applicable to other ethnicities; 3) The edge-detection system improves the accuracy of determination of the diameter by multiple-point detection [24]. The device in the present study determined the diameter at 21 points in a 3-mm segment of the longitudinal B-mode image [9]; 4) The present study did not validate some technical aspects that might affect the FMD value (e.g., cuff occlusion pressure, area of placement of the cuff subject preparation) [6,20]; 5) DIAbase has also been reported as a marker to predict future CV events [25], and in the present study also, DIAbase allowed discrimination between subjects with and without CV disease. Therefore, in the prospective study arms of the FMD-J study, the comparison of the usefulness of FMD with that of DIAbase to predict the outcomes is proposal; 6) The Doppler flow values to obtain the shear rate [26] is automatically calculated by the device used in the present study, therefore, the reliability of Doppler flow values measured by the present device was not evaluated in the present study; 7) Endothelial-independent vasodilatation was not assessed in the FMD-J study.

5. Conclusion

When the analysis was limited to cases with clear FMD recordings (about 90% of cases), the reliability of the FMD assessment (scan and its analysis) with a semi-automatic device conducted in individual institutions appeared to be acceptable, but attending periodic refresher courses in FMD assessment may be needed for the maintenance of competency. Reference FMD values (lower cuff occlusion) for the Japanese population are proposed based on reliable data derived from multiple institutions, and the reference values may identify patients with values in the reference ranges may be in a state without advanced atherosclerotic vascular damage.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2015.08.001.

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