Impact of volunteer-related and methodology-related factors on the reproducibility of brachial artery flow-mediated vasodilation

Impact of volunteer-related and methodology-related factors on the reproducibility of brachial artery flow-mediated vasodilation: analysis of 672 individual repeated measurements

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Objectives: Brachial artery flow-mediated dilation (FMD) is a popular technique to examine endothelial function in humans. Identifying volunteer and methodological factors related to variation in FMD is important to improve measurement accuracy and applicability.

Methods: Volunteer-related and methodology-related parameters were collected in 672 volunteers from eight affiliated centres worldwide who underwent repeated measures of FMD. All centres adopted contemporary expert-consensus guidelines for FMD assessment. After calculating the coefficient of variation (%) of the FMD for each individual, we constructed quartiles ($n = 168$ per quartile). Based on two regression models (volunteer-related factors and methodology-related factors), statistically significant components of these two models were added to a final regression model (calculated as β-coefficient and R^2). This allowed us to identify factors that independently contributed to the variation in FMD%.

Results: Median coefficient of variation was 17.5%, with healthy volunteers demonstrating a coefficient of variation 9.3%. Regression models revealed age ($\beta = 0.248$, $P < 0.001$), hypertension ($\beta = 0.104$, $P < 0.001$), dyslipidemia ($\beta = 0.331$, $P < 0.001$), time between measurements ($\beta = 0.318$, $P < 0.001$), lab experience ($\beta = -0.133$, $P < 0.001$) and baseline FMD% ($\beta = 0.082$, $P < 0.05$) as contributors to the coefficient of variation. After including all significant factors in the final model, we found that time between measurements, hypertension, baseline FMD% and lab experience with FMD independently predicted brachial artery variability (total $R^2 = 0.202$).

Conclusion: Although FMD% showed good reproducibility, larger variation was observed in conditions with longer time between measurements, hypertension, less experience and lower baseline FMD%. Accounting for these factors may improve FMD% variability.

Keywords: Doppler, endothelial function, flow-mediated dilation, reproducibility, ultrasonography

Abbreviations: BP, blood pressure; CV, coefficient of variation; FMD, flow-mediated dilation; Q, quartile

INTRODUCTION

Cardiovascular disease (CVD) remains the world's leading cause of morbidity and mortality. Previous studies have provided convincing evidence that endothelial dysfunction is an early manifestation of CVD [1,2], contributing to development and/or acceleration of the atherosclerotic process. Based on the detrimental role of endothelial dysfunction in this common disease process, studies have attempted to develop and validate (noninvasive) methods and biomarkers to assess endothelial function in humans. The conceptual idea is that identification of endothelial dysfunction, in symptomatic as well as asymptomatic volunteers, is related to increased risk for future development of cardiovascular events [3,4].

A frequently used, noninvasive technique to examine endothelial function in humans *in vivo* is flow-mediated dilation (FMD) [5]. This measurement adopts high-resolution ultrasonography to measure the conduit artery diameter dilatation in response to marked elevation in blood flow (and therefore shear stress) after a 5-min period of distal limb ischaemia [6]. Studies

Journal of Hypertension 2016, 34:1738–1745

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Received 16 February 2016 **Revised** 3 May 2016 **Accepted** 18 May 2016

DOI:10.1097/HJH.0000000000001012

have provided evidence that the FMD response is endothelium dependent [7] and largely mediated by nitric oxide [8], an important and potent vasodilator and antiatherogenic molecule. The measurement of endothelial function using FMD has become popular in clinically orientated studies, likely because of its non-invasive nature, ability to predict cardiovascular events [4,9–11] and correlation to coronary artery endothelial function [2,12].

Despite its valid conceptual basis, a number of factors influence the variability of FMD [13,14]. Previous studies found that FMD is influenced by lifestyle factors (e.g. smoking, physical activity), methodology (e.g. cuff placement, duration of ischaemia), intake of food and beverages, hormonal changes and method of analysis [8,11]. Although many of these factors are currently being controlled for through adopting expert-consensus guidelines [11,15], variation in FMD remains. These sources of variation may be volunteer-dependent and/or methodology-dependent, but this has not yet been systematically studied. Identification of such factors will contribute to the control of measurement error, which will help to appropriately power studies and aid in the construction of rigorous and standardized guidelines [11,16].

The purpose of this study was to identify volunteer-related and methodology-related factors that contribute to FMD variation in humans. To this end, we combined data from previous studies (from eight research centres) that performed repeated measurements within volunteers of brachial artery FMD in a total of 672 individuals. All included studies were performed according to expert-consensus guidelines [11]. Subsequently, we assessed volunteer-related and methodology-related factors that contributed to brachial artery FMD variability.

METHODS

Study population

The International Working Group on Flow-Mediated Dilation (IWG-FMD) originates from eight different research groups in four different countries. All groups provided written consent to contribute their data. We compiled volunteer-level data from all participating research centres (see Supplementary list, http://links.lww.com/MD/B22), including a total of 19 different studies. All affiliated researchers provided details on methodology of included studies in a specifically designed questionnaire. These details were crosschecked with earlier published and/or unpublished data. All centres received an outline of the datasheet to enhance sufficient and complete data collection. A total of 84 parameters were explored. Data from a total of 672 individuals with measurement of the brachial artery FMD, assessed on at least two separate occasions, obtained by B-mode ultrasound systems were available for data analyses. When studies included more than one repeated measurement, only the first and the second measurements were included prior to statistical analyses. All subsequent repeated measurements were rejected to prevent distortion of included parameters.

Brachial artery flow-mediated dilation measurements: methodological considerations

We included data from participants whose FMD data were collected on two separate occasions without an intervention between the two measurements. These measurements were limited to the brachial artery (measurements of e.g. the radial, femoral or popliteal arteries were excluded), in either the right or left arm (consistent for both measurements). To examine brachial artery FMD, participants extend the scanned arm following a short (10–15 min) resting period in the supine position. A rapid inflation and deflation pneumatic cuff was positioned on the forearm of the imaged arm distal to the olecranon process to provide a stimulus of forearm ischaemia [11,15]. With an ultrasound system, B-mode images of the brachial artery in the distal third of the upper arm (above the antecubital fossa in the longitudinal plane) were made. When an optimal image was obtained, the ultrasound probe was held stable (manually or by using a clamp), and ultrasound parameters were set to optimize the longitudinal B-mode image. At least 1 min of baseline diameter was recorded, after which the pneumatic cuff was inflated to at least 50 mmHg above SBP to occlude arterial inflow for a standardized length of time (i.e. standardized time of 5 min of occlusion). Subsequent cuff deflation induced a brief high-flow (hyperaemic) state that increased wall-shear stress at the brachial artery, causing it to dilate. To assess flow velocity, a mid-artery pulsed Doppler signal was obtained during the protocol [11,15]. Although all study centres used slightly different protocols to collect the repeated FMD measurements, all followed the above described expert-consensus guidelines.

Different types of ultrasound systems were used across the different centres, including the TerasonT3000 (Terason, Aloka, United Kingdom; 10-MHz multifrequency linear array transducer, $n = 136$), Sonos 5500 (Hewlett-Packard, 7.5-MHz linear array transducer, $n = 20$), ESAOTEMyLab25 (ESAOTE, Florence, Italy; 10-MHz linear array transducer, $n = 54$), ESAOTE Picus Just 4D (ESAOTE, Maastricht, The Netherlands, 7.5-MHz linear array transducer, $n = 60$), ESAOTE MyLab70 (ESAOTE, Maastricht, The Netherlands; 7.5-MHz linear array transducer, $n = 51$), VIVID E9 (VIVID E9, General Electric, Waukesha, Wisconsin, USA, 15-MHz linear array transducer, $n = 109$) and AU5 Armonic system (ESAOTE, Florence, Italy; 7.0-MHz linear array transducer, $n = 136$). One included study is a multicentre study consisting of seven substudies, which used a range of devices (ESAOTE, Philips, Siemens and General Electric, 7.5–10 MHz linear array transducer, $n = 110$).

All studies used (semi)automatic analysis software. However, different software were used across the centres: Custom-made MyFMD software, V2012.2, Prof A.P.G. Hoeks, Department of Biomedical Engineering, Maastricht University, Maastricht, The Netherlands ($n = 130$); Custom-made software [17], Pisa, Italy ($n = 135$); Custom-made DICOM software for edge detection ($n = 135$) [18,19] and FMD Studio, Cardiovascular Suite, Clinical Physiology, National Research Council, Pisa, Italy ($n = 272$) [20,21]. All centres collected continuous measurements of the diameter and recorded these (on either videocassette

recorder or digitally) for poststudy analyses. No study used fixed time points for diameter estimation.

Sources of variation

Our primary outcome parameter was the variation between both FMD measurements, for which we calculated the coefficient of variation for each individual's repeated measurements, calculated as [(sdFMD/meanFMD) × 100]. Furthermore, we recorded FMD (%), baseline diameter (cm), maximal diameter (cm) and time between measurements (categorized in <24 h, 1–7 days, 8–14 days, 2–4 weeks or >4 weeks).

Measurement of volunteer-related factors

We included the following volunteer-related factors that were all presented using a continuous scale: age (inclusion ≥18 years, range 18–82 years), weight (range 45–171 kg), height (range 1.55–1.94 m), BMI [calculated as weight (kg)/height^2 (m), range 17.6–55.8 kg/m^2], SBP and DBP (in mmHg) and calculated mean arterial pressure [MAP, calculated as (2 × DBP + SBP)/3, range 64–139 mmHg] and blood-specific parameters (i.e. total cholesterol (TC), high-density lipoprotein, low-density lipoprotein, triglycerides, glucose; all in mmol/l). All original parameters were rescaled to the same metric or most frequently used units (i.e. cholesterol and glucose values converted from mg/dl to mmol/l) [22].

We also presented volunteer-related factors using categorical scales: sex (male/female), presence of hypertension [conform current guidelines defined as SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg, or using blood pressure (BP) lowering drugs, yes/no], the presence of diabetes (type 1 or type 2), smoking status (yes/no/history of smoking), presence of dyslipidemia (yes/no, as specified by each contributing centre) and history and/or presence of cardiovascular disease.

Measurement of methodology-related parameters

All assessments followed the expert-consensus FMD guidelines, ensuring that the protocol involved cuff placement around the forearm, occlusion for 5 min and cuff inflation at least 50 mmHg above SBP. Furthermore, we assessed the following factors: use of a probe holder (yes/no), lab experience (total number of peer-reviewed publications that included measurement of FMD from contributing principal investigator through a Pubmed-based search using the search term '[author] AND flow mediated dilation'), mention of the laboratory's own reported coefficient of variation (mentioned as coefficient of variation percentage reported), use of continuous and/or ECG-gated diameter recording, measurement of artery diameter across the cardiac cycle and the time between measurements (<24 h, 1–7 days, 8–14 days, 2–4 weeks and >4 weeks). The Supplementary material, http://links.lww.com/MD/B22 provides details of the questionnaire used to assess these factors.

Missing values

As missing data were present for all of the 82 individual parameters, we used multiple imputation chained equations to impute parameters. We performed this procedure with a maximum up to 30%, as previously described [23,24]. Parameters, for which 31% or more data were missing, were excluded from analyses and are not further mentioned. A more detailed outline of the imputation model can be found in the Supplementary material, http://links.lww.com/MD/B22.

Statistical analysis

All data are presented as *N* (%) or mean ± SD unless stated otherwise. The main outcome measure for the reproducibility of the FMD is the coefficient of variation calculated for the mean difference between both FMD measurements. All descriptive data were examined in the pooled dataset and in quartiles of variation in FMD (i.e. coefficient of variation). Based on the coefficient of variation, we qualified the reproducibility as excellent (0–10%), good (10–20%), moderate (20–30%) or poor (>30%) [25]. In multiple linear regression analyses, we used the (log transformed) FMD coefficient of variation as the dependent variable to identify factors that independently contributed to the variability of the FMD measurement, using backward regression analysis. A total of four models were constructed: Model 1a – Volunteer-related factors (continuous scale), Model 1b – Volunteer-related factors (categorical scale, i.e. presence of hypertension), Model 2 – Methodology-related factors and Model 3 – Significant factors from previous models 1a–1b–2. Details of all regression models are given in the Supplemental information, http://links.lww.com/MD/B22. All statistical analyses were performed using the Statistical Package for Social Sciences, version 20.0 (SPSS, Inc., Chicago, Illinois, USA).

RESULTS

A median coefficient of variation of 17.5% was observed for the entire population of 672 volunteers, whereas a median coefficient of variation of 9.3% was observed for volunteers without cardiovascular risk factors ($n = 109$). We observed substantial variation between volunteers regarding the individual coefficient of variation for the FMD% (Fig. 1). When dividing volunteers into four quartiles, we calculated the coefficient of variation for each quartile (mean

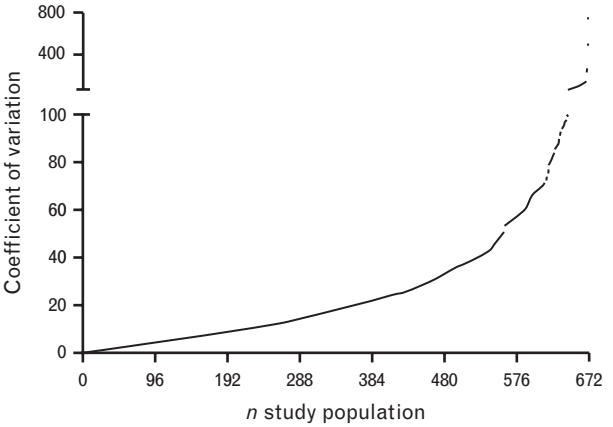

FIGURE 1 Individual reproducibility in brachial artery flow-mediated dilation. Data of all volunteers ($n = 672$) relating to the individual reproducibility of the brachial artery flow-mediated dilation across two repeated measurements.

TABLE 1. Volunteer-related factors

Continuous scale	Pooled (29.9 ± 46.5)	Quartile 1 (3.25%)	Quartile 2 (11.74%)	Quartile 3 (24.76%)	Quartile 4 (61.03%)	*P* value
Age (years)	46 ± 17	40 ± 16	42 ± 15	46 ± 16[a]	54 ± 16[a]	<0.001
	655	*163*	*164*	*164*	*164*	
Sex (% male)	66	64	67	68	67	0.895
	671	*168*	*168*	*167*	*168*	
Weight (kg)	77.4 ± 13.1	75.9 ± 12.1	76.7 ± 11.8	78.6 ± 14.4	78.3 ± 14.1	0.210
	636	*163*	*161*	*160*	*152*	
Height (cm)	1.75 ± 0.1	1.76 ± 0.1	1.76 ± 0.1	1.75 ± 0.1	1.75 ± 0.1	0.657
	637	*163*	*161*	*160*	*152*	
BMI (kg/m^2)	25.3 ± 3.7	24.6 ± 3.4	24.9 ± 3.3	25.8 ± 4.2[a]	25.9 ± 3.5[a]	0.003
	657	*164*	*165*	*164*	*164*	
SBP (mmHg)	129 ± 15	127 ± 13	131 ± 14[a]	130 ± 16[a]	128 ± 15	0.023
	645	*161*	*163*	*159*	*162*	
DBP (mmHg)	79 ± 11	78 ± 11	81 ± 12[a]	79 ± 12	76 ± 11	<0.001
	645	*161*	*163*	*159*	*162*	
Mean BP (mmHg)	96 ± 12	94 ± 11	98 ± 12[a]	96 ± 13	94 ± 11	0.002
	655	*135*	*165*	*163*	*164*	
Cholesterol (mmol/l)	5.3 ± 1.0	5.1 ± 1.0	5.2 ± 1.0	5.4 ± 1.0[a]	5.6 ± 0.9[a]	<0.001
	544	*135*	*134*	*134*	*141*	
HDL (mmol/l)	1.4 ± 0.4	1.4 ± 0.3	1.4 ± 0.3	1.4 ± 0.3	1.4 ± 0.4	0.414
	508	*127*	*126*	*124*	*131*	
LDL (mmol/l)	3.5 ± 0.8	3.3 ± 0.8	3.3 ± 0.8	3.5 ± 0.9[a]	3.7 ± 0.8[a]	<0.001
	466	*115*	*109*	*112*	*130*	
Triglycerides (mmol/l)	1.4 ± 1	1.3 ± 0.8	1.4 ± 1.3	1.4 ± 0.9	1.3 ± 0.8	0.924
	529	*129*	*130*	*130*	*140*	
Glucose (mmol/l)	5.1 ± 0.7	5.0 ± 0.7	5.0 ± 0.9	5.0 ± 0.7	5.4 ± 0.7[a]	<0.001
	466	*132*	*132*	*114*	*88*	

Volunteer-related factors for whole group ($n = 672$) and quartiles (of $n = 168$ each) with median coefficient of variation reported per quartile. Data are reported as mean ± SD with total number of volunteers available for analysis presented below in italic. *P* value refers to an ANOVA.
[a]Post-hoc significantly different from Quartile 1 at $P < 0.05$.

coefficient of variation 29.9 ± 46.5, range 0.14–745.33; median coefficient of variation Quartile-1: 3.25%; Quartile-2: 11.74%; Quartile-3: 24.76% and Quartile-4: 61.03%). We found an excellent, good or moderate coefficient of variation in 33% ($n = 221$), 22% ($n = 147$) and 14% ($n = 94$) of the sample, respectively. A poor coefficient of variation was observed in 31% of the cases ($n = 210$).

Volunteer-related factors

Age, BMI, TC and glucose levels showed a gradual increase across quartiles, with Q3 and Q4 (i.e. large variation in FMD) showing significantly higher values than Q1 (Table 1). SBP, DBP and mean BP were highest in Q2–3, whereas this difference was lost in Q4 (Table 1). When volunteer-related factors were presented using a categorical scale, hypertension and dyslipidemia had significant impact on the reproducibility of the FMD (presence of hypertension Q1 15.5%, Q2 30.4%, Q3 32.1% and Q4 21.4%; diabetes Q1 0%, Q2 0%, Q3 1.2% and Q4 0.6%; both $P < 0.001$), but not sex, smoking status, diabetes mellitus and CVD.

Methodology-related factors

FMD% and baseline diameter were significantly different across quartiles of the coefficient of variation (Table 2). Volunteers in Q4 had a lower FMD and a larger baseline diameter (Table 2). We found that all factors related to the practical performance of the FMD, except the use of a probe holder, were significantly different between quartiles (Table 2). Larger variation in coefficient of variation FMD% (i.e. Q3–4) was associated with absence of ECG-gated recording, no measurement of the diameter across the cardiac cycle, longer time between tests, less experience of the research centre in FMD measurements and absence of reporting the coefficient of variation of the laboratory in manuscripts (Table 2).

Regression analyses

Model 1a – volunteer-related factors (continuous)

After including all volunteer-related factors that significantly differed across quartiles, this model showed an $R^2 = 0.087$ and adjusted $R^2 = 0.086$. We found that only age predicted variation in FMD% coefficient of variation [$\beta = 0.248$, ratio of 28.1%, confidence interval (CI) (0.020–0.035), $P < 0.001$].

Model 1b – volunteer-related factors (categorical)

Including all volunteer-related factors that differed across quartiles, we found an $R^2 = 0.112$ and adjusted $R^2 = 0.108$. We identified hypertension [$\beta = 0.104$, ratio of 11%, CI (0.095–0.533), $P = 0.005$], dyslipidemia [$\beta = 0.331$, ratio of 39.2%, CI (0.813–1.275), $P < 0.001$] and sex [$\beta = -0.069$, ratio of −6.7%, CI (−0.390–0.010), $P = 0.063$) as significant predictors for the reproducibility of the FMD%.

Model 2 – methodology-related factors

The model showed an $R^2 = 0.198$ and adjusted $R^2 = 0.184$, when including methodology-related factors that differed across quartiles. The model identified time between measurements [$\beta = 0.318$, ratio of 37.5%,CI (0.179–0.298),

TABLE 2. Methodological-related factors

Continuous scale	Pooled (29.9 ± 46.5)	Quartile 1 (3.25%)	Quartile 2 (11.74%)	Quartile 3 (24.76%)	Quartile 4 (61.03%)	*P* value
Baseline diameter (mm)	4.3 ± 0.8	4.1 ± 0.8	4.3 ± 0.7[a]	4.4 ± 0.8[a]	4.4 ± 0.8[a]	<0.001
	672	*168*	*168*	*168*	*168*	
Maximal diameter (mm)	4.5 ± 0.8	4.3 ± 0.8	4.5 ± 0.7[a]	4.6 ± 0.9[a]	4.5 ± 0.8[a]	<0.001
	672	*168*	*168*	*168*	*168*	
FMD (%)	5.4 ± 3.0	6.1 ± 2.8	5.8 ± 2.4	5.7 ± 2.8	4.1 ± 3.6[a]	<0.001
	672	*168*	*168*	*168*	*168*	
Laboratory experience (papers per PI)	29.2 ± 24.8	35.6 ± 21.9	35.1 ± 22.9	30.9 ± 25.3[a]	15.4 ± 23.6[a]	<0.001
	672	*168*	*168*	*168*	*168*	
CV reported (%)	16.8 ± 9.5	14.7 ± 6.9	14.6 ± 6.7	16.5 ± 9.5	22.2 ± 12.4	<0.001
	612	*155*	*160*	*158*	*139*	
Categorial scale						
Analysis by laboratory	96	99	99	95[a]	92[a]	<0.001
	672	*168*	*168*	*168*	*168*	
ECG-gated recording	28	25	38[a]	35[a]	13[a]	<0.001
	672	*168*	*168*	*168*	*168*	
Cardiac cycle (%)	84	87	88	87	73[a]	<0.001
	672	*168*	*168*	*168*	*168*	
Probe holder (%)	80	77	79	77	86	0.110
	672	*168*	*168*	*168*	*168*	
Time: <24 h (%)	53	69	69	52	21	<0.001
1–7 days (%)	6	6	9	6	4	
8–14 days (%)	7	5	5	10	8	
2–4 weeks (%)	9	9	6	8	11	
>4 weeks (%)	25	11	11	24	56	
	672	*168*	*168*	*168*	*168*	

Methodological-related factors presented for whole group ($n = 672$) and quartiles ($n = 168$ each) with median CV reported per quartile. Data are reported as mean ± SD with the total number of volunteers available for analysis presented below in italic. *P* value refers to an ANOVA. CV, coefficient of variation; FMD, flow-mediated dilation; PI, principal investigator.
[a]Post-hoc significantly different from Quartile 1 at $P < 0.05$.

$P < 0.001$], FMD% at baseline [$\beta = -0.124$, ratio of −11.7%, CI (−0.098 to −0.021), $P = 0.002$], baseline diameter [$\beta = 0.082$, ratio of 8.6%, CI (0.007–0.270), $P = 0.039$) and lab experience [$\beta = -0.133$, ratio of −12.4%, CI (−0.011 to −0.003), $P = 0.001$) as significant contributors to the variation in FMD% coefficient of variation.

Model 3 – overall model

Factors identified by models 1a, 1b and 2 were included in the overall model which resulted in an $R^2 = 0.208$ and adjusted $R^2 = 0.202$. Backward linear regression analysis identified time between measurements [$\beta = 0.291$, ratio of 33.8%, CI (0.156–0.273), $P < 0.001$], hypertension [$\beta = 0.096$, ratio of 10.1%, CI (0.068–0.501), $P = 0.010$], baseline FMD% [$\beta = -0.142$, ratio of −13.3%, CI (−0.105 to −0.030), $P < 0.001$] and lab experience [$\beta = -0.131$, ratio of −12.3%, CI (−0.012 to −0.003), $P = 0.001$] as significant contributors to the variation in FMD% across two repeated measurements (Fig. 2). Baseline diameter demonstrated a borderline significant association with FMD% reproducibility [$\beta = 0.070$, ratio of 7.2%, CI (0.015–0.242), $P = 0.084$].

DISCUSSION

The study included 672 repeated measurements of the brachial artery FMD, involving data from different research centres and various populations. This allowed us to comprehensively explore factors contributing to the within-volunteer variability of brachial artery FMD%, when measured according contemporary guidelines [11]. We present the following observations. First, the majority of the measurements showed an excellent-to-good reproducibility. For asymptomatic volunteers, the median coefficient of variation was 9.3%. This demonstrates that FMD is a reproducible tool to assess endothelial function *in vivo*. Second, we also found substantial variation between individuals in the coefficient of variation of FMD%. In particular, the presence of hypertension contributed to a larger variation in FMD%, independent of other factors. Third, we found that a poorer reproducibility of the FMD was associated with the presence of a lower baseline FMD%, a higher baseline brachial artery diameter, a longer time period between repeated measurements and less experience of the laboratory with the FMD measurement. Taking these factors into consideration for sample size calculations in future studies will help to decrease chances of type II errors.

Volunteer-related factors

Several previous studies have explored and described reproducibility of brachial artery FMD and presented mixed results, ranging from an excellent to poor reproducibility [13,26,27]. The overall median coefficient of variation percentage in our analysis of 17.5% in the whole study population and 9.3% in volunteers without cardiovascular risk/disease are in line with findings of most previous studies that reported a good reproducibility [14,16,28–30]. An important strength of our analysis is the large number of repeated measurements, which allowed us to identify between-volunteer and laboratory-related factors contributing to the variation in brachial artery FMD% within an individual. Interestingly, we found that older age, dyslipidemia and presence of hypertension were related to larger

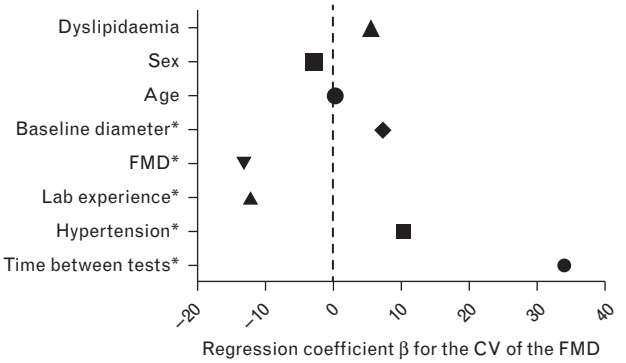

FIGURE 2 Regression analysis. Plot for regression coefficient β for the coefficient of variation of the flow-mediated dilation. *Implies a statistical significant contribution in final model.

variation in FMD%. This suggests, in agreement with previous work [28], that reproducibility of the FMD may be lower in populations with clinical symptoms than in healthy, young volunteers.

An explanation for the larger variation in clinical populations could be the presence of a lower baseline FMD% that is typically observed in older volunteers [31] and in those with hypertension [32], CVD [33] or dyslipidemia [14]. Indeed, we found that baseline FMD% is a strong and independent predictor for larger variability. Therefore, baseline FMD% was added to the statistical model to explore its impact on variability in FMD% independent of older age, hypertension and dyslipidemia. Interestingly, in this model, the impact of age and dyslipidemia disappeared, suggesting that the lower baseline FMD% in older volunteers is at least partly responsible for the larger variation with increasing age. In contrast, the impact of hypertension remained significant, indicating that other factors play a role in the larger variation in repeated measurements of brachial artery FMD%. Possibly, this poorer reproducibility may relate to higher stiffness of the vessels in clinical populations, compared with healthy volunteers [34]. Craiem *et al.* [28] also found that volunteers with CVD, despite comparable baseline FMD% values, demonstrate a larger coefficient of variation compared with healthy controls.

Methodology-related factors

Identification of methodology-related factors that contribute to the variation in FMD is highly relevant because such factors can potentially be controlled for. Several previous studies have highlighted the importance of methodological factors, which formed the basis for the FMD expert-consensus guidelines [11]. The present study identified time between measurements and lab experience as independent determinants of the variation in FMD%, with more time between FMD measurements leading to a higher coefficient of variation. Most studies that explored FMD reproducibility included fixed time points between measurements, which makes direct comparisons of the duration between testing difficult. Interestingly, Charakida *et al.* [35] explored FMD reproducibility after a few hours, 2 day, 3 months and 9 month. In agreement with our findings, this study also demonstrates a poorer coefficient of variation with increased time between retesting. In contrast, Sorensen *et al.* [27] found no difference in reproducibility when FMD was repeated after 1–2 days, 1–2 weeks or 2–4 months. However, this study did not apply FMD measurements according to current guidelines, which may have affected the results. Although longer time between repeated measures may be associated with increased variability due to purely methodological variation, it is also likely that true biological variability is greater under circumstances in which the repeated measure is more distant in time.

Laboratories that provided data for this analysis adopted expert-consensus guidelines to perform and analyse FMD. This makes it difficult to explore the importance, for reproducibility, of the individual aspects within these guidelines. Nonetheless, our analysis showed that laboratory experience with FMD measurements independently contributes to the variation in FMD measurement. More specifically, the greater the experience of a laboratory with the FMD technique, the smaller the variation between repeated FMD measurements. This somewhat self-evident finding is nonetheless important, as it should guide laboratories who adopt the technique in attaining the level of practice and experience required before robust measures can be assumed. Nonetheless, limited experience of FMD did not completely invalidate assessment: the subgroup of healthy volunteers without cardiovascular risk/disease that showed a coefficient of variation of $9.3 \pm 19\%$ ($n = 109$) included data from both experienced and less-experienced laboratories, demonstrating the feasibility of a low coefficient of variation in FMD measurements. This is in accordance with previous multicentre studies [16]. These data demonstrate the importance of adherence to the expert-consensus guidelines in addition to a priori practice and experience with the FMD technique.

Practical relevance

The study demonstrates that, in addition to adopting current guidelines, some factors should be considered that might affect the variation of the FMD. For example larger FMD reproducibility is observed when the time between measurements increases and/or in the presence of hypertension, and low resting FMD%. These factors should be taken into consideration when performing a sample size calculation and in the design of the study. Furthermore, the data of this study also emphasize that, in addition to fair reproducibility of the FMD in less-experienced laboratories, training and gaining more experience is likely to minimize measurement error of the FMD technique.

Limitations

One limitation of our study is that it was not prospectively designed to address FMD reproducibility. This may have introduced some error, especially relating to controlling physical activity and/or dietary instructions for the time between testing. However, all data were collected as in a 'real-world' study rather than being set up as a reproducibility study. Therefore, our study possesses ecological validity and can be extrapolated to various research settings. Another limitation is that all data in our analysis derive

from laboratories adopting current guidelines for FMD measurement. Therefore, we were unable to address the relative importance of individual aspects included in these guidelines. In addition, although all centres indicated that they adhered to the expert-consensus guidelines, we have no specific data on the internal control of adherence and/or small variation within these guidelines between centres (e.g. differences in analysis software, ultrasound machines). Such differences may in part contribute to the inherent variability of the FMD.

In conclusion, we have shown in a large dataset of repeated measurements that the majority of FMD measurements show an excellent-to-moderate reproducibility. Despite adopting expert consensus guidelines, several volunteer-related and methodology-related factors have independent impact on the variation in FMD% between two measurements. These include the presence of hypertension, a lower resting FMD%, a larger baseline artery diameter, a longer time between subsequent measurements and less laboratory experience with the measurement. Future studies should take these volunteer-related and methodology-related factors into consideration to improve sample size calculation. Such procedures will importantly decrease variability of the FMD and, consequently, decrease chances for type II errors in studies that rely on FMD as their primary outcome parameter.

ACKNOWLEDGEMENTS

We thank all individual researchers that contributed to data collection at Radboud University Medical Center (Dr Tim Schreuder, Dr Joost Seeger, Dr Constantijn Wouters), Liverpool John Moore's University (Dr Helen Jones, Dr Gurpreet Birk, Dr Thomas Bailey), The University of Western Australia (Dr Ceri Atkinson, Dr Louise Naylor, Dr Howard Carter), University of Pisa (Dr Rosa Maria Bruno), Maastricht University (CARIM; Dr Frank van Bussel, Dr Yvo Kusters, NUTRIM; Dr Peter Joris), and Wageningen University (Dr Lieke Gijsbers, Dr James Dower). We thank Dr Lian van Engelen for her valuable contribution to the set-up, performance and interpretation of the statistical analysis.

Sources of funding: Ms Anke van Mil is financially supported by a Top Institute for Food and Nutrition-grant.

D.H.T. is financially supported by The Netherlands Heart Foundation (E Dekker-stipend, 2009T064). Professor Green receives Fellowship and grant funding from the National Heart Foundation of Australia (APP1045204).

Conflicts of interest

Disclosures: A.G. and P.L.Z. are employed by Unilever R&D Vlaardingen B.V. No conflicts of interest, financial or otherwise, are declared by the remaining author(s).

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Reviewers' Summary Evaluations

Reviewer 1

This study describes a comprehensive analysis of the methodological issues that are related to the variability of forearm endothelial function using the flow-mediated dilation technique. The technique is challenging, and this study provides meaningful results from studies conducted in various centers, taking into account realistic aspects such as variability of devices and operator experience, suggesting that the technique can give reliable results in approximately two thirds of the cases. However, there is nonuniform availability of data from all centers, and this presents a potential limitation of the study.

Reviewer 2

Strength: Although the study design was retrospective, it reflects the 'real life' and thus is consistent in underlying the difficulties in flow-mediated vasodilatation (FMD) measurement.

Weaknesses: The study was not prospectively designed. No standardized control has been done of activities preceeding the measurements that can heavily act on FMD, such as smoking and physical activity.