Original Article

Assessment of flow-mediated dilation reproducibility: a nationwide multicenter study

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Objective: Impaired flow-mediated dilation (FMD) is associated with cardiovascular risk factors and provides prognostic information. Despite the noninvasive nature of this technique, a major limitation to its widespread use is low reproducibility. The aim of this study was to evaluate impact of methodological standardization among different investigation sites on brachial artery FMD reproducibility.

Methods: Seven Italian centers recruited 135 healthy volunteers, aged 20–60 years. FMD was assessed by high-resolution ultrasound equipped with a stereotactic probe-holding device. Certified sonographers recorded brachial artery scans at baseline (day 1a), 1 h after (day 1b), and 1 month later (day 30). Endothelium-independent vasodilation (EIVD) to sublingual glyceril-trinitrate was recorded at day 1 and day 30. FMD and EIVD were blindly evaluated at the coordinating center by an automated edge detection system. The intra-session (day 1a versus 1b) and inter-session (day 1a versus 30) coefficients of variation were calculated.

Results: FMD was not significantly ($P = 0.91$) different at day 1a, day 1b and day 30 (6.52 ± 2.9, 6.42 ± 3.1, $6.57 \pm 2.8\%$, respectively). The FMD intra-session coefficient of variation was $9.9 \pm 8.4\%$ (from 7.6 to 11.9% across centers). The FMD inter-session coefficient of variation was $12.9 \pm 11.6\%$ (from 11.6 to 16.1% across centers). Inter-session coefficient of variation for EIDV was $19.7 \pm 16.8\%$.

Conclusions: This study shows a homogeneous coefficient of variation for FMD among different centers. The inter-session coefficient of variation was similar to the intra-session coefficient of variation, representing the intrinsic FMD variability. We demonstrate for the first time that rigorous and standardized procedure may provide reproducible FMD assessment to study endothelial function in multicenter clinical trials.

Keywords: endothelial function, flow-mediated dilation, multicenter study, reproducibility

Abbreviations: ANOVA, analysis of variance; BP, blood pressure; CI, confidence intervals; EIDV, endothelium-independent vasodilation; FMD, flow mediated dilation; GTN, glyceril trinitrate; SD, standard deviation

INTRODUCTION

Endothelial function is considered the first step to atherosclerosis and plays an important role in the pathogenesis of cardiovascular disease [1,2]. Hence, it has been recently investigated as a putative prognostic tool [3–6] and a possible therapeutic target [7]. Endothelium-dependent vasomotion can be measured both invasively and noninvasively in coronary and peripheral circulations, but the wide heterogeneity in the methods has not allowed firm conclusions to be reached regarding the optimal endothelial vasomotor test [8]. However, over the two past decades, a noninvasive technique has evolved to evaluate flow-mediated dilation (FMD) in the brachial artery [9]. An increased flow following distal forearm ischemia represents a physiological stimulus for brachial artery vasodilation assessed by high-resolution ultrasound. FMD is almost completely abolished by inhibitors of nitric oxide synthase, demonstrating its dependence on local nitric oxide availability [10]. Impaired FMD occurs in the presence of cardiovascular risk factors [11] and is independently associated with cardiovascular events [3–6,12,13], although negative results were also reported [14,15]. Assessment of brachial FMD in clinical investigation has increased because of its apparent simplicity, efficiency and noninvasive nature. Owing to the biological and technical variability of the measurement, several caveats should be considered in designing a study in which FMD is investigated. These include not only proper study design and sample size but also efforts to achieve a uniform technique and to minimize operator-dependency, including adoption of

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probe-holding devices, automated systems to measure brachial artery diameter changes and adequate operator training [2,16–18]. As of today, only few experienced research centers apply these procedures to achieve a high standard of accuracy and reduce FMD variability [19]. The lack of a uniform and such a rigorous methodology represents one of the major limitations for the application of FMD assessment in large multicenter studies. Therefore, the present study was designed to determine the impact of methodological standardization among different centers on FMD reproducibility. To this aim, we evaluated the time-dependent variability of FMD measurements obtained in healthy volunteers by trained operators according to a uniform technique with centralized analysis by an automated edge detection system, composed of a special-purpose hardware/software device for measuring changes of the brachial artery diameter [20,21].

METHODS

Patients

One hundred and thirty-five healthy volunteers were included in the study. Exclusion criteria were: smoking, hypertension, dyslipidemia, diabetes mellitus, history of cardiovascular or systemic disease, and clinical signs of inflammatory disease. Individuals were also excluded if they experienced inter-current illness, including viral infections, since they can transiently impair FMD. Women were studied in the follicular phase, and those on hormonal or contraceptive therapy were excluded. All medications, including anti-inflammatory drugs, β_2-adrenergic agonists and local vasoconstrictors, were withdrawn, if present, 1 week before examination, as well as food or beverages containing antioxidants which might affect FMD [22]. On the day of the study, individuals were in a fasting state, refrained from caffeine-containing beverages, and avoided exercise for at least 12 h prior to the experiment.

The protocol was approved by the local Ethics Committees, and, in accordance with institutional guidelines, all volunteers were aware of the investigational nature of the study and gave written consent for their participation.

Experimental procedures

Studies were performed in the morning at rest with volunteers in the supine position from at least 15 min, in a quiet air-conditioned room (22–24°C), to minimize the possible negative effect of environmental and physiological influences, including stress [23]. Endothelial-dependent vasodilation was assessed as dilation of the brachial artery in response to increased blood flow in accordance with current guidelines [16,18], as previously described [17,24]. At each center the investigators used for the tests the echo-Doppler machine available at their Institution (Esaote, Philips, General Electric, Siemens). The right brachial artery was located and scanned longitudinally between 5 and 10 cm above the elbow using a linear array transducer with a frequency ranging from 7.5 to 10 MHz. The transducer was held in the same position throughout the scan by an adjustable stereotactic clamp to ensure greater image stability.

A sphygmomanometer blood pressure (BP) cuff was positioned on the right forearm 2 cm below the elbow. Right brachial artery was scanned longitudinally between 5 and 10 cm above the elbow, capturing images starting 1 min prior to cuff inflation. The cuff was inflated for 5 min at 250 mmHg and then deflated to induce reactive hyperemia. Endothelium-independent vasodilation (EIVD) was elicited by the administration of low dose (25 μg) of sublingual glyceril-trinitrate (GTN) [17,24].

Volume blood flow was calculated by multiplying duplex flow velocity (corrected for the angle) by heart rate and vessel cross-sectional area (πr^2). Flow velocity was measured at baseline and within 15 s after cuff release. Peak reactive hyperemia was calculated as the maximum percentage increase in brachial artery flow after cuff release as compared to baseline flow.

Blood pressure and heart rate were measured prior to each study by an automatic sphygmomanometer (mean of at least two values measured over 5 min in the sitting position).

Experimental design

Training

All sonographers had previous experience with FMD studies. A training program with 20 supervised scans was performed at the coordinating center (Pisa, by L.G.) to standardize the ultrasound measurements and familiarize with the probe-holding device and the real-time edge detection system. At the end of the training, operators were asked to send recorded scans. Quality certification was obtained when the core reading lab approved five consecutive scans from each center. The scans were validated if the image of the artery was clear and stable during the whole recording, anatomical markers were well recognized, mean diameter evaluated in the first minute was stable [standard deviation (SD) <2%], and all timings of the procedure were respected.

Vascular scan recordings

Recording time frames were 10 min for FMD studies (1 min for baseline, 5 min of ischemic period, 4 min for assessing changes in diameter following reactive hyperemia) and 6 min for EIDV studies (1 min for baseline, 5 min for assessing changes in diameter following GTN administration).

Figure 1 shows the timetable of the study. In the first day, sequences of B-mode images of the brachial artery were recorded by the certified operator at each center for baseline FMD (day 1a) and repeated 1 h after, maintaining the probe in the same position (day 1b). A third sequence for FMD was obtained 1 month apart (day 30). EIVD to GTN was also evaluated at day 1 and day 30. An interval of 60 min was left between the last FMD assessment and GTN administration.

Identifiable anatomical markers were requested to ensure consistency between scans at day 1a and 1b and at day 30.

Reading procedure

All scans were recorded in the internal memory of the ultrasound equipment, exported to CD, DVD or USB devices and transferred to the core laboratory (Pisa) by regular

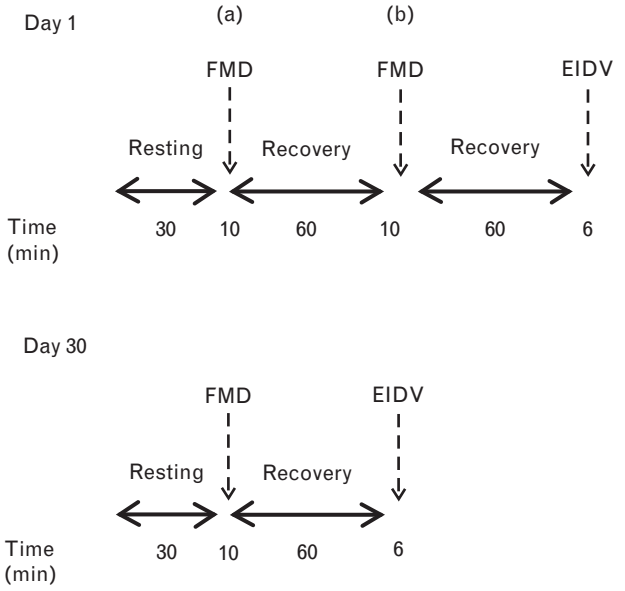

FIGURE 1 Time-lines of experimental design at day 1 and 30. FMD, flow-mediated dilation; EIDV, endothelium-independent vasodilation.

mail. Recorded scans were analyzed at the core lab by a trained operator (F.F.), who was blind to the study's subject and phase. FMD and response to GTN were calculated as the maximal percentage increase in diameter above baseline (mean of measures obtained during the first minute), using an automatic edge detection system (FMD Studio system, Institute of Clinical Physiology, National Research Council, Pisa) [20,21].

Scans were rejected in case of poor quality and/or instability of the images due to inconsistency of clear artery borders and anatomical markers.

Statistical analysis

Both FMD and response to GTN data showed a distribution not significantly different from normal, according to Kolmogorov–Smirnov test. Differences in the studied parameters of the population were calculated using analysis of variance (ANOVA) for repeated measures. The agreement between ultrasound measurements at different times was evaluated by Bland–Altman analysis and correlation coefficients. The mean differences for repeated measurements were reported as bias in the Bland–Altman analysis. The coefficients of variation were calculated on measurements obtained in each individual at day 1a versus day 1b (intra-session variability) and at day 1a versus day 30 (inter-session variability). In addition, tests of equivalence were performed with indifference margins fixed at values of 10% of the mean FMD value obtained at day 1a. Results are expressed as mean ± SD and as median with 95% confidence intervals (CI). A P value less than 0.05 was considered statistically significant. Statistical software IBM SPSS Statistic 18.0 (© 2009 IBM Corporation) was used for data analysis.

RESULTS

Table 1 shows the clinical characteristics of the healthy volunteers participating in the study. These volunteers had normal BMI, BP values, plasma glucose levels and lipid profile, with age ranging from 22 to 48 years. FMD correlated inversely with age ($P < 0.01$), and brachial artery diameter ($P < 0.01$); female volunteers had significantly smaller FMD than male volunteers ($P < 0.01$).

TABLE 1. Clinical characteristics of the study population ($n = 135$ healthy volunteers)

Sex (males/females)	70/65
Age (years)	31.5 ± 6.7
BMI (kg/m^2)	21.4 ± 1.5
Waist circumference (cm)	80.1 ± 8.2
Total cholesterol (mg/dl)	176.9 ± 20.5
HDL cholesterol (mg/dl)	53.0 ± 11.6
Triglycerides (mg/dl)	127.8 ± 24.7
Glycemia (mg/dl)	89.7 ± 3.8
Serum creatinine (mg/dl)	0.85 ± 0.18
SBP (mmHg)	121.2 ± 5.9
DBP (mmHg)	78.6 ± 6.8
Heart rate (b.p.m.)	68.9 ± 6.8

Data are shown as mean ± SD.

The rejection rate of recorded scans was 2% for the assessment of FMD intra-session variability and 9% for inter-session FMD variability, respectively. A similar 8% rate of rejection was observed for inter-session EIDV variability.

Baseline brachial artery diameter was 3.54 ± 0.69 mm at day 1a, 3.51 ± 0.58 mm at day 1b and 3.56 ± 0.87 mm at day 30 ($P = 0.384$). Correlation coefficients for brachial artery diameter were 0.931 and 0.969 (for day 1a versus day 1b and day 1a versus day 30, respectively; $P < 0.0001$ for both). The intra-session and inter-session coefficients of variation for the diameter were 1.83 ± 1.76 and 4.84 ± 3.39%, respectively. The intra-session bias was 0.03 mm and the inter-session bias was 0.02 mm; SDs of the differences were 1.74 and 0.73 mm, respectively.

Reactive hyperemia was 495 ± 29% at day 1a, 429 ± 38% at day 1b and 465 ± 36% at day 30 ($P = 0.567$). Correlation coefficients for reactive hyperemia were 0.475 and 0.498 (for day 1a versus 1b and day 1a versus 30, respectively; $P < 0.001$ for both). The intra-session and inter-session coefficients of variation for reactive hyperemia were 28.2 ± 38.6 and 25.5 ± 24.6%, respectively.

Flow-mediated dilation was not significantly different at day 1a (6.52 ± 2.9%), day 1b (6.42 ± 3.1%) and day 30 (6.57 ± 2.8%) ($P = 0.91$). This result did not change ($P = 0.84$) when analysis was adjusted for reactive hyperemia. Correlation coefficients of FMD values were 0.914 and 0.834 for assessments obtained at day 1a versus day 1b ($P < 0.0001$) and at day 1a versus day 30 ($P < 0.0001$), respectively (Fig. 2).

Figure 3 shows Bland–Altman plots of FMD assessment at day 1a versus day 1b and at day 1a versus day 30. Bias of differences for FMD at day 1a versus day 1b was not significantly different from that at day 1a versus day 30 (0.06 and 0.05%, respectively; $P = 0.94$). The SDs of the differences were 1.17 and 1.63%, respectively. The mean differences of FMD measurements were 0.84 and 1.09% for intra-session and inter-session values, respectively.

The tests of equivalence confirmed that measures at different times were comparable. Absolute changes in

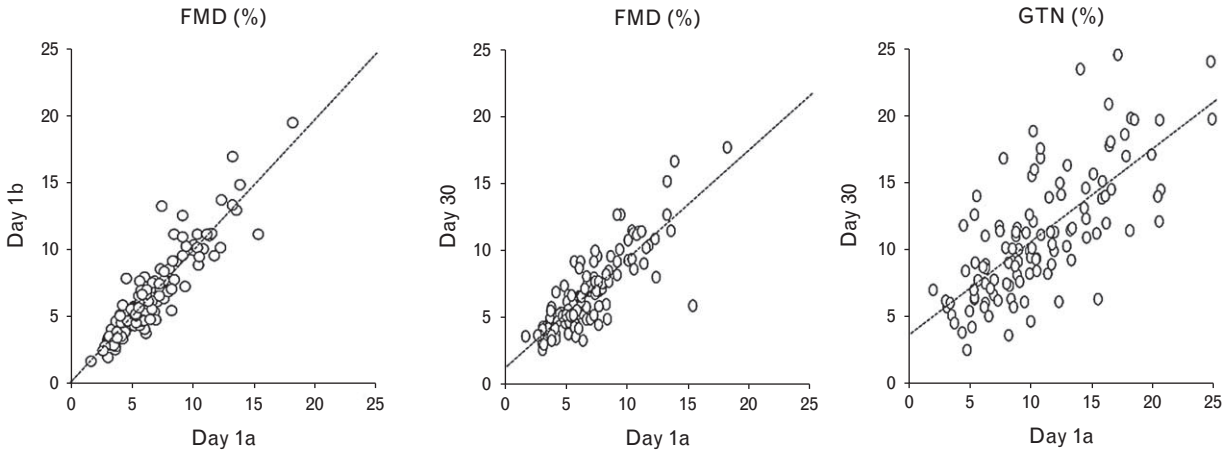

FIGURE 2 Graphs show correlations of percentage changes in brachial artery diameter induced by flow-mediated dilation (FMD%) evaluated at baseline (day 1a) versus that obtained after 1 h (day 1b) or 30 days (day 30) and glyceryl-trinitrate (GTN%) at baseline versus 30 days. According to the rate of rejection, based on poor quality and/or instability of images, data refer to 131, 125 and 126 individuals, respectively.

FMD were 0.06% (95% CI −0.16/+0.28%) for assessments obtained at day 1a versus day 1b, and 0.02% (95% CI −0.26/+0.30%) for those obtained at day 1a versus day 30, both resulting within the fixed indifference margins of ±0.66% (Fig. 4).

Overall, coefficients of variation were 9.9 ± 8.4 and 12.9 ± 11.6% for the intra-session and inter-session FMD measures, respectively. The coefficients of variation across the centers are shown in Table 2.

Response to GTN was 10.4 ± 4.9 and 10.9 ± 4.7% at day 1a and day 30, respectively. The inter-session coefficient of variation of GTN response between assessments obtained at day 1a and day 30 was 19.7 ± 16.8% (from 12.1 to 25.4% across different centers), with a correlation coefficient of 0.722 ($P < 0.0001$) (Fig. 2).

DISCUSSION

The endothelium plays a primary role in vascular homeostasis and a healthy endothelium is indeed protective against the development and clinical manifestations of atherosclerosis [1,3]. Brachial artery FMD is widely used to study endothelial function of conduit artery [1,2]. This technique is attractive because it is noninvasive, uses standard high-resolution ultrasound equipment, but it is not simple to performed. Indeed, the reliability of its results depends on the accurate application of the test protocol and on adequate skill of the operator [19]. Underestimation of practical challenges associated with FMD might explain some of the discrepancies reported in the literature and represent a major limitation to a widespread application of this method in clinical studies [2,8,16–18].

The present multicenter study of FMD assessment by a uniform methodology was designed to determine its reproducibility. We report for the first time that adherence to a rigorous protocol, with certified operator training as well as defined experimental settings (adjustable stereotactic probe-holding device, automated computer-assisted brachial artery measurements), is feasible in different research centers, ensures high-quality examinations and, most of all, provides an optimal time-dependent reproducibility of FMD. Our results demonstrate the opportunity, afforded by

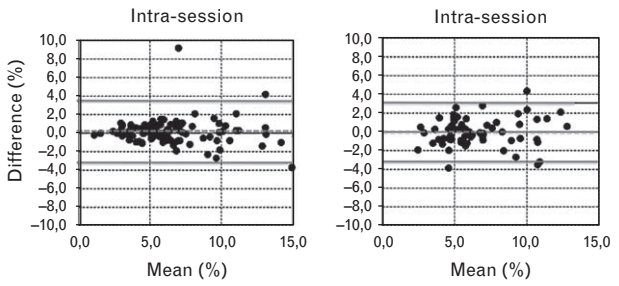

FIGURE 3 Bland–Altman plots of intra-session (within day 1, left panel) and inter-session flow-mediated dilation (FMD) assessments (day 1 versus day 30, right panel). The X-axis shows the mean (%) FMD and the Y-axis the difference between pairs. The dotted lines represent the bias and the continuous lines represent the 95% confidence intervals. According to rejection rate, data refer to 131 and 125 individuals, respectively.

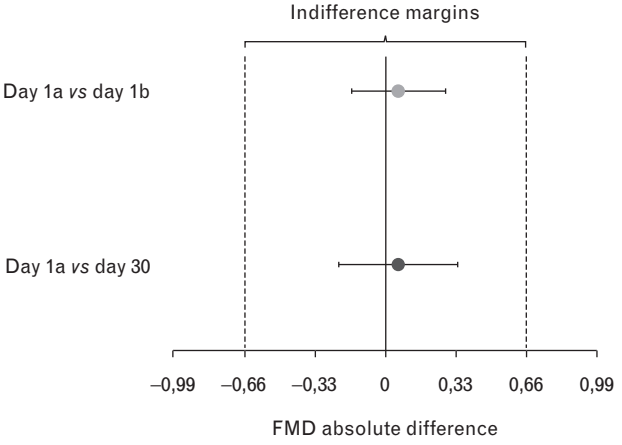

FIGURE 4 Graph show testing of equivalence for intra-session (within day 1, top) and inter-session flow-mediated dilation (FMD) assessments (day 1 versus day 30, bottom). The X-axis shows the absolute difference in the mean FMD between the sessions. Data are shown as median value and 95% confidence intervals; vertical continuous lines represent the indifference margins, defined as the 10% of mean FMD value obtained at day 1.

TABLE 2. Coefficients of variation (mean values and 95% confidence intervals) for flow-mediated dilation in the different centers

Center	Intrasession (day 1a versus day 1b)	Intersession (day 1a versus day 30)
1	7.6 (0.3–10.9)	11.6 (2.1–13.2)
2	9.5 (0.3–12.1)	12.8 (4.7–17.2)
3	10.1 (0.4–15.1)	13.6 (5.0–14.3)
4	9.9 (0.1–11.3)	13.7 (5.1–18.8)
5	9.8 (0.3–11.1)	11.6 (3.6–16.4)
6	11.9 (6.8–14.7)	16.1 (8.6–20.4)
7	9.9 (2.1–12.4)	12.6 (5.4–17.6)

current ultrasound methodology, to evaluate endothelial function in a reliable manner in clinical studies.

The major finding of our study is that FMD assessment over time is highly reproducible in a group of healthy volunteers. In contrast with the debated poor reproducibility of the technique and its high operator-dependency, the present findings show a similar coefficient of variation for intra-session (1 h apart) and inter-session (1 month apart) FMD assessment. Moreover, absolute changes in FMD were within the selected margins of indifference of 10% both for day 1a versus day 1b and day 1a versus day 30. It is also noteworthy that the overall FMD variability is comparable with that observed by the authors who originally described the noninvasive method for FMD using a similar methodology [19]. Importantly, our results do not show any significant difference in FMD variability among the centers participating in the study. These data are of relevance as the intra-session assessment, investigating FMD under similar environmental setting, aimed to test the intrinsic variability of endothelial response. The variability, already demonstrated for other functional parameters such as carotid artery distensibility [25], may be due to continuous changes occurring in the arterial wall as a consequence of intrinsic mechanical/structural properties, independently from the stimuli. Otherwise, it could be explained by blood flow and pressure fluctuations. Concerning other sources of variability, individual differences are dependent on sex and age, as shown not only for FMD [19,26] but also for other arterial variables, such as aortic pulse wave velocity (PWV) [27]. Furthermore, the observed variability of peak reactive hyperemia might be related to the lower accuracy of blood flow velocity assessment [18], whereas inter-session baseline diameter variability was to be expected because of the design of our study.

Finally, we documented a satisfying reproducibility of EIVD induced by sublingual administration of low-dose GTN.

The issue of FMD reproducibility in a single center was raised by Sorensen *et al*. [26], who reported an overall variation of approximately 25% in repeated FMD measures. Accordingly, current guidelines indicate that FMD coefficient of variation should be below 20–30% [16]. Such variability, clearly suboptimal for application in clinical practice, was reduced in single experienced centers by the introduction of stereotactic probe holders and computerized analysis systems [18–21,28]. To our knowledge, no other studies evaluated FMD reproducibility in a multicenter setting, except the Cardiovascular Health Study (CHS) [4], which reported a lower correlation coefficient (0.67 versus 0.83 of the present study) for repeated FMD measures obtained on two separate days (more than 2 weeks apart) in 80 participants. This difference might be related to the substantial improvement of our automated, computer-based analysis. Such a system might be more suitable for centralized readings, because of greater reliability against noise by working at 25 frames per second and by analyzing a greater number of frames for diameter measurements. Moreover, it would offer consistent performances in terms of coefficients of variation, regardless of the different ultrasound equipment used. The real-time characteristic represents an advantage in terms of time-saving for study processing analysis and reduced operator's learning curve [29], which is a major challenge for FMD assessment [16]. The low number of rejected examinations because of poor quality and/or instability of the images in the repeated scans proves the good level of training achieved by the operators trained in our core laboratory.

Several tests have been developed to study endothelial function, but they are still not considered valuable prognostic tools, either because of their invasiveness or insufficient sensitivity and specificity. Hence, increasing interest exists in determining the clinical usefulness of brachial artery FMD as the endothelium vasomotor test for integrating the risk factor burden. Although most evidence highlights a strong correlation among impaired FMD, cardiovascular damage [30–32] and major events [4–6,33], negative results are also reported [14], possibly due to differences in the methodology of FMD assessment [2,18]. Furthermore, several studies have demonstrated that FMD improves with modifications of cardiovascular risk factors and with the use of drugs known to reduce cardiovascular risk [22,24,34]. Thus, in parallel with more established variables such as carotid intima–media thickness [19] or aortic PWV [35,36], FMD testing could be included in clinical trials as a surrogate end-point [2] also in consideration of the fact that FMD changes, as a result of drug treatment, occurs over a much shorter time (few months) compared to changes of other vascular markers.

A possible limitation of our study is represented by the inclusion of only healthy volunteers for testing FMD reproducibility. Patients with cardiovascular risk factors and disease have lower FMD and we cannot exclude that they might present different, possibly higher, coefficients of variation. This issue deserves careful assessment in appropriate future studies and most of all in clinical trials investigating the effect of treatment on endothelial dysfunction. Another challenge to the diffusion of this methodology is represented by the costs of the equipment (high-resolution ultrasound, stereotactic probe-holder device, automated software analysis system).

In conclusion, our study shows for the first time that adherence to a rigorous protocol, including operator training, standardized experimental settings and automated B-mode image edge detection system, provides accuracy and time-dependent reproducibility for this noninvasive assessment of endothelial function also in a multicenter pattern of investigation.

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Conflicts of interest

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