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Variability and reproducibility of flow-mediated dilatation in a multicentre clinical trial

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Aims The aim of this study was to assess the reproducibility of flow-mediated dilatation (FMD) in a multicentre setting.

Methods and results This study was performed as part of the dal-VESSEL trial in which FMD was measured in 19 vascular imaging centres in six European countries. A subgroup of patients who were allocated in the placebo group and scanned twice at each trial time point (substudy) was analysed. Intra-sonographer variability was calculated from FMD measurements 48 h apart. Centre variability and short-, medium-, and long-term reproducibility of FMD were calculated at 48 h and at 3 and 9 months intervals, respectively. Intra- and inter-reader variability was assessed by re-analysing the FMD images by three certified readers at two time intervals, 7 days apart. Sixty-seven patients were included. Variability between centres was comparable at 48 h and 3 months interval but almost doubled at 9 months. The mean absolute difference in %FMD was 1.04, 0.99, and 1.45% at the three time intervals, respectively. Curves were generated to indicate the number of patients required for adequate power in crossover and parallel study designs.

Conclusion This study demonstrates for the first time that in a multicentre setting reproducible FMD measurements can be achieved for short- and medium-term evaluation, which are comparable with those reported from specialized laboratories. These findings justify the use of FMD as an outcome measure for short- and medium-term assessment of pharmacological interventions.

Keywords FMD • Reproducibility • Coronary artery disease • Power curve

Introduction

Atherosclerosis begins in early life. Strategies to study and manage 'lifetime' risk from atherosclerosis require robust intermediate phenotypes, on the causal pathway for disease.[1,2] Endothelial function is linked to cardiovascular (CV) risk factors,[3,4] provides prognostic information,[4–6] and can be studied non-invasively by measurement of flow-mediated dilation (FMD).[7] Flow-mediated dilation reflects local nitric oxide bioavailability and enables both examination of mechanisms involved in the initiation and progression of pre-clinical vascular disease and the impact of acute and long-term interventions.[7]

The 'dynamic' nature of the technique is also responsible for some of its limitations. Flow-mediated dilatation is known to be affected by a wide range of biological, environmental, and methodological factors.[8] To ensure accurate assessment of endothelial function, such parameters need to be considered and controlled for carefully in studies utilizing FMD as an outcome measure. Reliable evidence on the reproducibility and variability of FMD is essential for experimental protocol design. Our group have reported such data in the setting of a single experienced FMD laboratory.[8] We subsequently demonstrated similar findings in a very large, single-centre epidemiological study in children.[9]

Evaluation, however, of the long-term effects of an intervention on endothelial function necessitates a multicentre approach. This introduces additional sources of variability in FMD, which have not yet been quantified. dal-VESSEL is the first multicentre trial with FMD as its primary endpoint to assess the safety and effect on endothelial function of the cholesteryl ester transfer protein inhibitor dalcetrapib in patients with coronary heart disease.[10] This study provided the opportunity to assess the short-, medium-, and long-term variability of

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FMD in a multicentre setting but also enabled construction of power curves to assist in the design of clinical trials where endothelial function is used as outcome measure to evaluate the effects of various therapeutic interventions.

Methods

Study design

This study was performed as part of the dal-VESSEL trial (clinicaltrials.gov number NCT00655538).[11] In brief, in a randomized double-blinded parallel trial, the effects of dalcetrapib and placebo treatment on brachial artery flow-mediated dilatation were compared after 3 and 9 months duration in 476 patients. The study protocol was approved by the institutional review boards or ethical committees from participating institutions. Informed consent was obtained from all patients before entering the study. Principle findings have already been reported.[11]

Prior to recruitment, a formal training programme was developed incorporating a common equipment platform and scanning protocol and two centralized training sessions run by experienced teachers. All sonographers were certified by a pre-specified programme developed at a central core FMD laboratory (London Core Lab, London, UK). Thirty-six sonographers were certified and performed the endothelial function measurements in 19 vascular imaging centres in six European countries.

To assess FMD variability and reproducibility, a subset of up to 35% of dal-VESSEL trial participants were scanned twice, at each trial time-point (baseline, 3 months, 9 months; see 'Study Protocols' for details). All scans included in the variability and reproducibility 'substudy' of the dal-VESSEL trial incorporated patients who were assigned to the placebo group. The scans were analysed by three expert readers (London Core Lab: M.C., S.P.L.; A.M.C. Vascular Imaging Amsterdam: J.W.) to assess inter- and intra-reader variability (see 'Study Protocols' for further details).

Brachial B-mode ultrasound imaging

Prior to FMD assessment, patients were asked to adhere to the preparation requirements for the scan. All the patients were requested to fast, starting at least 12 h prior to the scan, as well as refrain from strenuous exercise during that period. Caffeine, smoking, and intake of vitamin C were not allowed from 6 h prior to the scan.[12] Regular medication was continued, but no other medication was allowed prior to the FMD scan. Female participants were postmenopausal. Measurements took place in quiet, temperature-controlled (20–24°C) rooms. Subjects remained at rest in the supine position for 15 min prior to the start of the scan.

B-mode ultrasound scans of the right brachial artery were obtained using a Sonix SP ultrasound machine (Ultrasonix, Vancouver, Canada) equipped with a 7.5 MHz linear array transducer. An especially designed arm-rest and probe holder was constructed to optimize standardization of the position of the ultrasound probe. A blood pressure cuff was placed in the forearm ~1 cm below the antecubital fossa. Measurements were obtained during 1 min, after which the blood pressure cuff was inflated to 250 mmHg in order to interrupt blood supply. Following 5 min of forearm ischaemia, the cuff was released and brachial diameter measurements were continuously recorded for 3 min after cuff release Blood flow velocity and heart rate were continuously monitored by pulsed-wave Doppler and displayed as a spectral Doppler curve. Image acquisition was ECG gated on the R-wave and the ultrasound images saved in a Digital Imaging and Communications in Medicine clip. To ensure the secure and regulatory compliant transmission of data from the image acquisition sites to the centralized server, a standard device data transfer box was utilized (CERTU Medical, Amsterdam, The Netherlands).

Image analysis

Offline analysis of acquired images was performed in a 'blinded' fashion using customized software (Brachial Analyser, Medical Imaging Applications, Iowa, USA). For image analysis, the reader selected a region of interest in the longitudinal image of the brachial artery. Upon definition of the correct interfaces of the scan, the Brachial Analyser software performed an automated tracing of the lumen–wall boundaries of the near and far wall of the images of the clip of the scan.[13] The software quantified a brachial artery lumen diameter for each image of the scan, before and after cuff occlusion. Average baseline diameter and the maximum post-cuff deflation diameter were used to calculate the absolute (peak diameter − baseline diameter) and percentage FMD [(peak diameter − baseline diameter)/baseline diameter] * 100.

The velocity-time integral (VTI) was measured from spectral Doppler curves, using automated flow analysis software (Medical Imaging Applications, Iowa, USA). The VTI was calculated for each R-wave-triggered cardiac cycle as a per cent increase from baseline. Measurements were made three times at baseline and during reactive hyperaemia (peak VTI following cuff release). Blood flow and reactive hyperaemic response were also calculated (Supplementary material online, formulae). Flow-mediated dilatation corrected for shear stress was obtained by dividing %FMD by the hyperaemic velocity-time integral.

Sources of variability

Sonographer performance and intra-sonographer reproducibility

Sonographers were eligible to scan patients in the study following successful certification. This involved attendance in at least two training sessions organized by the Core Lab, and completion of certification process which involved 10 repeat scans with $<2\%$ variability in %FMD. The number of accepted and rejected scans per sonographer was closely monitored and re-certification was performed when rejection of >2 consecutive scans were identified from the same sonographer. To assess intra-sonographer repeatability, a subset of 35% of trial participants were scanned twice by the sonographers 48 h apart.

Centre performance and variability

Centre performance was established by assessing the recruitment process and the number of accepted and rejected scans in the efficacy phase. Centre variability was established by analysing repeat measurements of FMD at each of the dal-VESSEL trial outcome points from the patients who agreed to participate in the substudy. This variability incorporates inter-sonographer variability, as repeat measurements were not always performed by the same sonographer. Variability was calculated for centres where >3 patients were recruited from the placebo group. Analysis of the FMD images were performed by two readers (S.L. and M.C.) and calculation of short-, medium-, and long-term variability per centre was performed.

Short-term variability of flow-mediated dilatation

To assess 'short-term' variability, FMD measurements were performed twice in 'substudy' participants within 48 h. This took place at the start of the dal-VESSEL trial, during baseline assessment of FMD and other relevant clinical parameters (visit 1; V1). We compared brachial artery baseline diameter, FMD (absolute and percentage), and flow stimulus measurements between the two scans. Subjects without a valid pair of scans at V1 were excluded from the present and all other protocols.

Medium- and long-term variability of flow-mediated dilatation

Study participants completing short-term variability, underwent repeat measurements of FMD (within 48 h) at each of the dal-VESSEL trial

outcome time-points (3 and 9 months). Images from repeat FMD scans were used to assess 'medium-' (within 3 months) and 'long-term' (within 9 months) FMD variability. We compared brachial artery baseline diameter, FMD (absolute and percentage), and flow stimulus measurements between: (i) scans from patients in the dal-VESSEL trial placebo group at baseline (V1) and at 3 months (visit 2; V2) ('medium-term' variability); (ii) scans from patients in the dal-VESSEL trial placebo group at V1 and at 9 months (visit 3; V3) ('long-term' variability).

Intra- and inter-reader variability of flow-mediated dilatation

To assess 'reader' reproducibility, 95 images were re-anonymized, and analyses were performed at least 7 days apart. We compared brachial artery baseline diameter and FMD (absolute and percentage) measurements between analyses of individual scans: (i) evaluated by a single reader ('intra-reader' variability); (ii) evaluated by all three readers ('inter-reader' variability).

Statistical analysis

All measures are expressed as mean ± standard deviation or median (range) unless otherwise stated. Flow-mediated dilatation (and other measured parameter) variability ('short-', 'medium-', and 'long'-term and centre variability) was expressed as the absolute difference in pairs of measurements between time-points. Reproducibility of different measurements was assessed by using intraclass correlation coefficient (ICC), technical error of measurement (TEM), and coefficient of variation (CV). Coefficient of variation within such pairs was defined as standard deviation of the difference between paired values divided by the mean and divided by the $\sqrt{2}$.[8] The formulae used are available in the Supplementary material online. Mountain plot and Bland–Altman plots for the various measurements were generated to further assess FMD variability between the various study time-points. Similar statistical methods were used to assess 'intra-' and 'inter-' reader reproducibility.

To assess the number of subjects required for a parallel and crossover multicentre study for 80% statistical power and 5% significance, and to assess the effect of varying CV between centres, power curves were constructed from between and within subject variances as previously reported.[8] All statistical analyses were performed using the STATA 12.1 software (STATAcorp, TX, USA).

Results

Study participants

Four hundred and seventy-six patients were randomized to participate in the study in 19 clinical centres. From those 375 had valid FMD assessment at baseline and 3 months (visit 2) and 310 had valid FMD measurements at 9 months (visit 3).

Sixty-seven patients who participated in the substudy assessment and were in the placebo group were included to assess methodological variability (14% of the total number of patients randomized in the dal-VESSEL trial).[10] Data collected from these patients were used to assess centre, intra-sonographer variability, and the 'short', 'medium', and 'long-term' variability of FMD. Demographic and clinical characteristics of all participants in our study are summarized in *Table 1*. There was no difference in the demographic and clinical characteristics of the participants over the 9-month follow-up period. No changes in patient's regular medication were made over the study period.[10]

Sources of variability

Sonographer performance and intra-sonographer variability

Thirty-six sonographers were certified and eligible to scan patients in the study. The median accepted number of scans per sonographer over the 9-month period of the study was 24 (1, 175) scans, whereas the median rejected number was 12 (0, 54) scans.

The intra sonographer variability was calculated in 10 sonographers from patients in the placebo group. The range for absolute TEM was 0.5–1.1%.

Centre performance and variability

A total number of 2195 scans were performed in 19 centres. The number of scans performed in different centres varied from 17 to 291 for the one with the highest recruitment rate. The total number of rejected scans was 662 (30% of total number of scans). Reasons for rejection were mostly technical (i.e. inadequate image quality due to patient movement, inadequate ECG triggering), which precluded reliable image analysis. The mean rejection rate was 8.7% at baseline and remained at the same level 9% at 3-month evaluation (visit 2), whereas increased to 15% at 9-month assessment (visit 3).

There were 12 centres where serial FMD measurements were performed in the placebo group. From those, only six centres had recruited ≥3patients from the placebo group and consistently followed them up in the serial assessment. For all centres the short- and medium-term variability was better than the long-term one. The short- and medium-term reproducibility were comparable between centres (absolute TEM ranged from 0.4 to 1.4%) while

Table 1 Baseline characteristics of participants in the variability study of the dal-VESSEL trial

Study participant characteristics	
Male (%)	83 (88.3)
Placebo (%)	47 (50.0)
Age (years)	61.3 ± 8.1
Body mass index (kg/m^2)	28.4 ± 4.6
Coronary heart disease (%)	65 (69.4)
Smoking (%)	25 (26.6)
Type II diabetes mellitus (%)	35 (37.5)
Fasting glucose (mg/dL)	6.4 ± 1.6
HbA1c (%)	6.3 ± 0.9
Total cholesterol (mg/dL)	145.6 ± 22.4
HDL cholesterol (mg/dL)	38.1 ± 7.6
LDL cholesterol (mg/dL)	80.2 ± 7.6
Brachial artery baseline diameter (mm)	4.6 ± 0.7
Reactive hyperaemia (%)	450 ± 191.7
Absolute FMD (mm)	0.2 ± 0.1
FMD (%)	4.1 ± 0.9
Reactive blood flow (mL/min)	20.5 ± 7.3
Delta blood flow (mL/min)	16.4 ± 6.3

Variables expressed as number (percentage) or mean ± standard deviation.
FMD, flow-mediated dilatation.

increased up to 2.5% at 9-month interval. There was no difference in the reproducibility for %FMD between centres with higher and lower recruiting rate.

Short-term variability of flow-mediated dilatation

The mean absolute difference in %FMD between values obtained at V1, 48 h apart, was 1.04% and there was good correlation between values (ICC: 0.8) (*Table 2*). A Bland–Altman plot of the 'short-term' %FMD variability data is shown in *Figure 1A*. Similar variability results were obtained for absolute FMD (FMDAbs). The reproducibility for brachial artery diameter (BLD) was superior to %FMD (*Table 2*), whereas poor intraclass correlation was seen for reactive hyperaemia (*Table 2* and Bland–Altman plots in the Supplementary material online). The FMD/VTI had lower reproducibility compared with %FMD and FMDAbs. No association was noted between %FMD and reactive hyperaemia.

Medium- and long-term variability of flow-mediated dilatation

The median absolute difference in %FMD between V1–V2 (3 months) and V1–V3 (9 months) was 0.99 and 1.45%, respectively (*Table 2*). There was good correlation between %FMD values at V1 and V2 (ICC: 0.74), which was comparable with that observed for short-term variability. Correlation was poorer between %FMD values at V1 and V3 (ICC: 0.58). Technical error of measurement and CV results for %FMD over 3 and 9 months are summarized in *Table 2*. Bland–Altman plots of 'medium-'and 'long-term' FMD% variability data are shown in *Figure 1B* and *C*, respectively. Similar variability results were obtained for FMDAbs. Baseline diameter reproducibility was excellent during the course of the study (*Table 2* and Bland–Altman plots in the Supplementary material online). High variability for the reactive hyperaemic response was seen in medium- and long-term assessment (Bland–Altman plots in the Supplementary material online). The reproducibility for FMD/VTI was also poor.

We constructed power curves from the data recorded at 3 months and 9 months apart for crossover and parallel studies. The graphs clearly demonstrate that adoption of a complex crossover design will significantly reduce the number of patients required for the clinical trial (*Figure 2*).

Intra- and inter-reader variability of flow-mediated dilatation

'Intra-' and 'inter-reader' reproducibility for FMD% was excellent. There was no systematic bias between the first and second read of the same reader. The ICC range was 0.84–0.99 for the intra-rater reproducibility and 0.82–0.87 for the inter-rater reliability.

Discussion

This study demonstrates, for the first time, that with optimal sonographer training and adherence to strict and standardized protocols, endothelial function can be assessed serially using flow-mediated dilatation in a multicentre setting in patients with coronary artery disease. The short- and medium-term reproducibility of %FMD approximate those reported from single centres. The power curves which have been constructed incorporating the biological and technical variability of the method suggest that a relative small number of patients are required to detect differences in %FMD both for a crossover and parallel study design trial. These results validate the use of this method as an intermediate endpoint for short- and medium-term

Table 2 Reproducibility of flow-mediated dilatation in a multicentre setting

	FMD abs (mm)	FMD (%)	%FMD/VTI peak (%/cm)	BLD (mm)	React hyper (%)
V1	0.18 (0.08)	4.1 (1.9)	6.7 (4.0)	4.6 (0.7)	555.8 (197.4)
V1 + 48 h	0.18 (0.08)	4.1(2.2)	6.7 (5.9)	4.5 (0.7)	586.5 (216.0)
V2	0.19 (0.09)	4.2 (2.2)	6.7 (4.0)	4.6 (0.7)	596.6 (205.5)
V3	0.19 (0.10)	4.1 (2.5)	5.1 (3.4)	4.7 (0.7)	572.8 (217.4)
Absolute TEM					
V1–V148 h	0.042	0.92	3.3	0.15	5.6
V2–V1	0.047	1.1	2.5	0.09	5.2
V3–V1	0.067	1.5	2.5	0.06	5.3
Coefficient of variation (%)					
V1–V148 h	16.2	15.6	33.9	2.4	22.9
V2–V1	17.7	18.3	26.3	1.4	15.5
V3–V1	25.0	24.9	31.2	0.9	23.4
Intraclass correlation coefficient					
V1–V148 h	0.73	0.80	0.57	0.95	0.19
V2–V1	0.73	0.74	0.60	0.98	0.66
V3–V1	0.52	0.58	0.49	0.99	0.11

Values are expressed as mean (SD) unless otherwise stated.
FMD, flow-mediated dilatation; BLD, baseline diameter; VTI, velocity-time integral; TEM, technical error of measurement; React Hyper, reactive hyperaemia (%): (delta blood flow/blood flow) × 100.

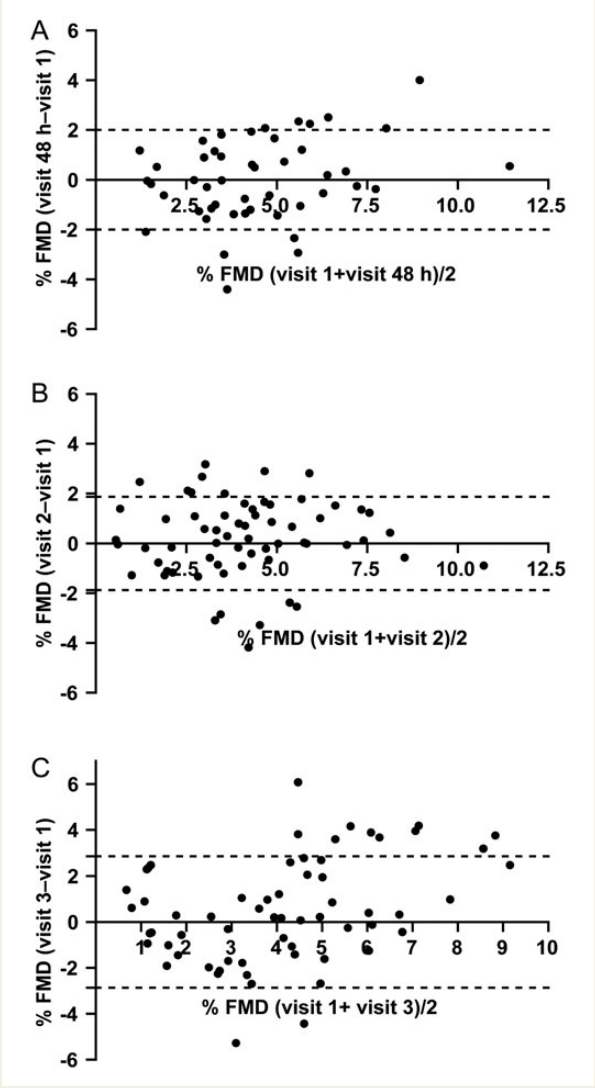

Figure 1 (*A*) Bland–Altman plot showing no systematic bias and good reproduciblity for short-term (within 48 h) assessment of flow-mediated dilatation. Dotted lines represent 2 SD of the differences. (*B*) Bland–Altman plot showing no systematic bias and good reproduciblity for medium-term (within 3 months) assessment of flow-mediated dilatation. Dotted lines represent 2 SD of the differences. (*C*) Bland–Altman plot showing no systematic bias and greater variability for long-term (within 9 months) assessment of flow-mediated dilatation. Dotted lines represent 2 SD of the differences.

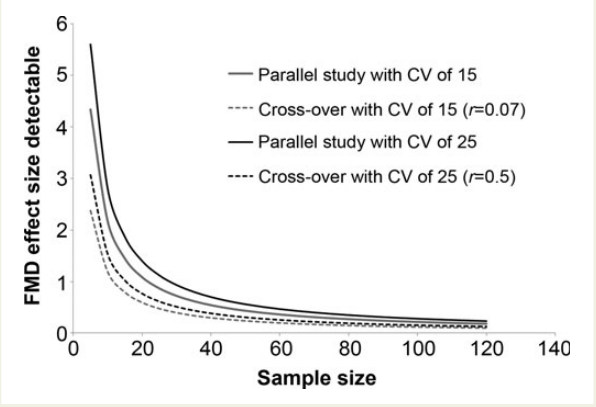

Figure 2 Relationship between effect on maximum % change in flow-mediated dilatation and number of subjects required in various trial scenarios with different coefficient of variation (CV: 15% and CV: 25%) between centres assuming 80% power and 5% significance for a parallel and crossover trial design.

clinical trials to test the safety and efficacy of pharmacological interventions.

Disturbance of endothelial function is an early event in the atherosclerotic process and clinical studies have demonstrated its predictive power for later CV events.[14–16] Endothelial function assessment by flow-mediated dilatation has been widely used in clinical practice since the early 1990s.[17] It has been shown to be accurate, to have relevance to biology and to be associated with CV outcome.[17–19] However, it has been criticized for being technically demanding and operator dependent. Advances in the methodology (use of stereotactic probe holder and analysis software for accurate edge detection diameter) have greatly diminished the latter,[20] however the cost of ultrasound machine and inherent technical challenges affecting the reproducibility of the method in addition to the paucity of expertise has restricted its use to the clinical research setting.

The issue of FMD reproducibility has been a matter of intense debate since CVs up to 51% have been reported among different groups depending on the site of measurement and protocol used.[21] By standardizing the protocol and technical aspects of the method, high reproducibility figures have been reported in specialized laboratories in healthy populations and in those with disease state under controlled conditions.[8] However, limited information exists for the reproducibility of the method in a multicentre setting under less controlled conditions. Recently, Ghiadoni *et al.*[22] documented the short-term reproducibility of FMD in a multicentre setting among healthy volunteers. In the current study, we have expanded these findings to provide information for the reproducibility of the method over a longer time interval but also for coronary artery disease patients who are likely to be the target population for many clinical trials.

Characterization of the flow-mediated dilatation signal has been a matter of debate in different studies. Flow-mediated dilatation is typically expressed as percentage change in vessel diameter following reactive hyperaemia. However, absolute change in diameter and FMD normalized for shear rate have also been reported.[12,23] In this reproducibility study, we were able to demonstrate comparable variability between %FMD and FMDAbs at short-, medium-, and long-term evaluations. However, FMD/VTI was more variable over time. No association was noted between reactive hyperaemia and %FMD and poor reproducibility was noted for reactive hyperaemia. Previous studies have indicated that the measurement of the area under the flow curve (AUC) rather than peak VTI might be more informative as a stimulus for FMD response;[24] however, other studies failed to demonstrate the validity of AUC in FMD assessment.[8]

A number of physiological factors can impact on reactive hyperaemia including arterial stiffness, flow pattern, and blood viscosity,

and these may affect its reproducibility.[25] However, these parameters were not measured in this study and as such the explanation for the variability of the reactive hyperaemia remains speculative. Among the different measures, baseline diameter was the most reproducible. Yeboah *et al.*[16] have demonstrated that BLD may have similar predictive value to FMD in older subjects. However, there is no evidence to suggest that vessel size is nitric oxide dependent or that its measurements are responsive to interventions. Its clinical usefulness, thus, remains limited.

Dal-VESSEL is the first clinical trial to use FMD as a primary endpoint and was set out to establish the vascular impact of an HDL raising drug, dalcetrapib in a population with coronary artery disease.[10] As expected lower absolute and percentage FMDs were found in these patients with coronary artery disease and higher range of CV risk factors compared with values reported for healthy adults.[11] We investigated all the participants who took part in the substudy and were allocated to the placebo group to assess the variability of the method. The reproducibility figures for short- and medium-term measurements were comparable with single-centre values and suggest that FMD can be a valuable tool for clinical trials. The long-term variability of the technique was higher. Although no significant changes in the risk factor profile were noted in the participants with time, it is possible that physiological variability becomes more significant over time. This can only partially be controlled for factors known to affect endothelial function such as changes in temperature, exercise, and infection.[12] Some technical aspects for repeat FMD measurements might also be difficult to control over long-time intervals. For instance, identification of the same arterial segment with comparable baseline diameters is necessary for serial FMD measurements. In addition, declining in the scanning quality over time due to detraining might also be considered. Sonographer variability was performed only once for this study and although close monitoring was performed for the quality of the data, deskilling with time for the centres with low recruitment might be a possibility. The higher rejection rate of the scans at 9 months and the reproducibility results from this study suggest that over longer time interval, identification, and recognition of the same arterial segment might be more challenging even for experienced sonographers. As such this higher variability should be carefully considered when sample size calculations are performed for clinical trials. In addition, previous studies have demonstrated that FMD varies according to sex and to menstrual cycle in females and that lower FMD values were reported in African Americans compared with Caucasians.[12] In this study, however, we were unable to assess the reproducibility between different sexes and ethnic backgrounds as our population were mostly males and of Caucasian origin.

A successful multicentre study using FMD requires adherence to a single-scanning protocol and the use of standardized equipment. Close monitoring of the involved centres and continuing training for operators from centres with low recruitment rate is also necessary. Centralized analysis by experienced readers is also important to minimize the variability of the method. In this study, all the images were analysed by three trained and certified readers who had high intra-rater and inter-rater reproducibility figures. Therefore, the influence of the analysis process in the overall variability of the method was minimized. The major component of FMD variability is between subjects. Interestingly, the power curves which have been constructed suggest that relatively small number of patients is adequate to detect a difference in %FMD in both parallel and crossover trials. Therefore, FMD can be an attractive endpoint in clinical trials for the assessment of pharmacological interventions.

In conclusion, this study suggests that FMD is reproducible and practical to use in a multicentre setting for short- and medium-term pharmacological interventions providing that optimal training and monitoring during the trial time period is performed. Our findings indicate that longer-term assessment with FMD is more challenging as physiological parameters are difficult to control over time. However, the relatively small numbers required to detect an effect in %FMD and its ability to detect early disturbances in endothelial physiology, relevant to atherosclerotic disease progression, suggest that this technique is an attractive option to assess safety and effectiveness of a new pharmacological regime before embarking in large-scale expensive outcome trials.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: none declared.

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