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1 **ADHERENCE TO GUIDELINES STRONGLY IMPROVES REPRODUCIBILITY OF**
2 **BRACHIAL ARTERY FLOW-MEDIATED DILATION**

3
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24 **ABSTRACT**

25 **Background:** Brachial artery FMD is widely used as a non-invasive measure of endothelial
26 function. Adherence to expert guidelines is believed to be of vital importance to obtain
27 reproducible measurements. We conducted a systematic review of studies reporting on the
28 reproducibility of the FMD in order to determine the relation between adherence to current expert
29 guidelines for FMD measurement and its reproducibility.

30 **Methods:** Medline-database was searched through July 2015 and 458 records were screened for
31 FMD reproducibility studies reporting the mean difference and variance of repeated FMD
32 measurements. An adherence score was assigned to each of the included studies based on
33 reported adherence to published guidelines on the assessment of brachial artery FMD. A Typical
34 Error Estimate (TEE) of the FMD was calculated for each included study. The relation between
35 the FMD TEE and the adherence score was investigated by means of Pearson correlation
36 coefficients and multiple linear regression analysis.

37 **Results:** Twenty-seven studies involving 48 study groups and 1,537 subjects were included in the
38 analyses. The adherence score ranged from 2.4 to 9.2 (out of a maximum of 10) and was strongly
39 and inversely correlated with FMD TEE (adjusted $R^2=0.36$, $P<0.01$). Use of automated edge-
40 detection software, continuous diameter measurement, true peak diameter for %FMD calculation,
41 a stereostatic probe holder, and higher age emerged as factors associated with a lower FMD TEE.

42 **Conclusions:** These data demonstrate that adherence to current expert consensus guidelines and
43 applying contemporary techniques for measuring brachial artery FMD decreases its measurement
44 error.

45 **Keywords:** cardiovascular disease; atherosclerosis; endothelial function; reproducibility;
46 methodology

47 INTRODUCTION

48 The endothelium is a key regulator of vascular homeostasis and endothelial dysfunction is an
49 early manifestation of atherosclerosis ¹. Currently, the most widely used technique to study
50 endothelial function *in vivo* is the flow-mediated dilation (FMD) of the brachial artery. This is a
51 non-invasive, ultrasound-based method which correlates with endothelial function of the
52 coronary arteries ^{2,3} and independently predicts cardiovascular disease (CVD) ^{4,5}. The technique
53 is attractive as a surrogate end-point, especially since changes in FMD can be detected across a
54 relatively short timeframe ⁶. Despite its popularity, minor changes in the methodological
55 approach may critically impact variability and decrease reproducibility of the FMD response ⁷⁻⁹.

56
57 Previous expert consensus guidelines have made important contributions to standardize the
58 technical approach and to set minimum standard requirements for FMD measurements ^{10, 11}.
59 However, not all studies on FMD apply these recommendations, or only in part. The impact of
60 adherence to these guidelines on the reproducibility of FMD measurements is currently unclear,
61 but may importantly contribute to the measurement error of the FMD technique. Furthermore,
62 little is known about the relative importance of the individual aspects of the expert-consensus
63 guidelines to contribute to the reproducibility of the FMD. Better quantitative data on this matter
64 can help reduce variation within and between studies, which will increase the statistical power of
65 studies on FMD to detect changes and, subsequently, decrease chances for type II errors.

66
67 In light of these considerations, we hypothesized that adherence to expert consensus guidelines is
68 related to better reproducibility of FMD measurements ^{10, 11}. Therefore, we performed a
69 systematic search for published studies that reported data on reproducibility of FMD
70 measurements, and investigated the relation between (full or partial) adherence to current expert

71 consensus guidelines and reproducibility of the FMD. Secondly, we explored which subject- and
72 methodology-related factors were related to FMD reproducibility.

73

74 **METHODS**

75 **Search Strategy**

76 The MEDLINE bibliographic database was searched (January 2000 through July 2015) for
77 studies that assessed the reproducibility of the FMD using the following search terms: "*flow*
78 *mediated dilation*", "*flow mediated dilatation*", "*flow mediated vasodilation*", "*flow mediated*
79 *vasodilatation*", "*endothelial function*", "*endothelial dysfunction*", "FMD", "FMV", "*brachial*
80 *artery*", "reproducibility", "reliability", "repeatability", "*coefficient of variation*", "CV", and
81 "*variance*". The search was limited to studies in human adults published in the English language.
82 Additionally, we supplemented the search by hand-searching references of included studies and
83 relevant reviews and meta-analyses on this topic.

84

85 **Selection of Studies**

86 Included studies were identified by means of a two-step selection process. During the first step,
87 two reviewers (ACCMvM, AG) independently screened titles, abstracts and keywords of
88 publications to identify potentially eligible studies. Studies were included if the mean difference
89 and variance of repeated FMD measurements of the brachial artery were reported. During step 2
90 of the selection, both reviewers examined the full text of these publications to gauge eligibility
91 based on two additional inclusion criteria: FMD was determined through noninvasive ultrasound
92 imaging, and a reactive hyperaemia protocol (with an ischemia duration of 4 to 5 minutes) was
93 used to elicit the shear stress stimulus required for FMD. Thus, studies that adopted (ischemic)
94 hand-grip exercise, passive movement and/or skin warming protocols to elicit (brachial) artery

95 dilation were not included in our analysis. In cases of discrepancy between the reviewers,
96 eligibility was discussed along with a third reviewer (DHJT) until consensus was reached.

97

98 **Data extraction**

99 *Study and subject characteristics:* A standardized data collection sheet was used to extract
100 general publication details (author, year of publication, country) and specific study- and subject
101 characteristics: number of subjects, mean age (in years); CVD risk status of the study population
102 (defined as presence of diagnosed CVD, hypertension or diabetes); baseline brachial artery
103 diameter (in mm); % brachial artery FMD and its associated variance for each repeated
104 measurement; and the mean absolute difference between repeated FMD measurements and its
105 associated variance.

106

107 *Adherence to guidelines:* We extracted information from the methods sections of the individual
108 papers to assess the adherence to current expert-consensus guidelines. Based on recent guidelines
109 ¹¹, we scored each individual study on the reporting of 19 different factors which were divided
110 over 4 categories. The categories were related to: 1. Subject preparation (10 items), 2. Image
111 acquisition (4 items), 3. Data analysis (3 items), and 4. Laboratory (2 items). Before performing
112 the systematic literature search, values were assigned to each factor proportional to its perceived
113 importance for valid assessment of the FMD. This was done through expert consensus discussion
114 within the Working Group (AG, LG and DHJT) (see online data supplement). The “*Adherence*
115 *Score*” that could be assigned to a study ranged from 0 to 10 points, depending on how many of
116 the 19 different factors that were reported. In addition, we counted the number of previous studies
117 on FMD published by the principal author of each study included in the systematic review. This

118 number served as a measure of the perceived experience in FMD measurements for each centre at
119 the time of publication of the reproducibility data included here.

120

121 **Statistical analysis**

122 Reported measures of FMD reproducibility varied between studies. Many studies presented the
123 coefficient of variation (CV) of repeated measurements, although this measure was calculated in
124 a number of different ways, precluding direct comparisons. Measures of reproducibility included
125 the technical error of the measurement (TEM), Pearson- and intraclass correlation coefficients
126 (ICC), and limits of agreement. In order to make valid comparisons between studies, we defined
127 as primary outcome measure the typical error of estimate (TEE) of FMD ,which is calculated as
128 standard deviation of the paired differences/ $\sqrt{2}$ ¹².

129

130 Data are presented as mean \pm SD or median (range) as appropriate for continuous variables and
131 as frequencies for categorical variables. FMD TEE data were highly skewed (Shapiro-Wilk test,
132 $P<0.0001$) and were log transformed prior to the analyses. Relations between log-FMD TEE and
133 continuous variables were determined by Pearson correlation coefficients analysis. For
134 categorical variables, the statistical significance of differences in FMD TEE between different
135 levels were assessed by the Mann-Whitney U test. Significant correlates were entered in a
136 multivariate linear regression analyses with backward elimination to identify independent
137 predictors of FMD TEE. All analyses were conducted using JMP version 11.0 (SAS Institute
138 Inc., Cary, NC, USA).

139

140 **RESULTS**

141 Our systematic search identified 446 potentially relevant publications and an additional 12 were
142 obtained through review of references of included studies, relevant reviews and meta-analyses.
143 Twenty-seven studies¹³⁻³⁹ with 48 relevant study groups met our inclusion criteria and were
144 included in our analysis (Figure 1). Characteristics of the included study groups are presented in
145 Table 1. The 48 study groups comprised a total of 1,537 subjects (mean sample size 32; range, 8-
146 135) with a mean age of 41.5 years (range, 22-79 years). Eleven study groups included subjects
147 with increased CVD risk, i.e. presence of diagnosed CVD, hypertension or diabetes. The other
148 remaining 37 study groups consisted of healthy subjects. The time between repeated FMD
149 measurements ranged from 25 minutes to 9 months. Mean baseline brachial diameter was 3.9 mm
150 (range, 3.5 to 4.7 mm) and mean baseline FMD (i.e. this first of the two repeated measurements)
151 was 7.1% (range, 1.8 to 19.9%). The FMD TEE ranged from 0.33 to 4.83% across study groups,
152 with a mean value of 1.4%. The level of experience for each centre at the time of publication of
153 the reproducibility study in question varied widely (number of previously published studies on
154 FMD ranging from 0 to 71, median of 3).

155

156 **Methodology-related factors *versus* variation in FMD**

157 There was considerable variation in the methodological factors between studies. Adherence
158 scores ranged from 2.4 to 9.2, with a mean of 5.3 (out of a maximum of 10). The adherence score
159 was inversely correlated with log FMD TEE (adjusted $R^2=0.36$, $P<0.01$, Figure 2).

160

161 To explore the impact of the different aspects of the adherence score on the FMD TEE, we
162 compared the FMD TEE between adherence (Yes vs No) to various methodological variables.
163 Statistically significant differences in FMD TEE were found for use of the true peak diameter to

164 calculate %FMD, continuous brachial artery diameter measurement over the cardiac cycle, use of
165 automated edge detection software and smoking cessation prior to measurements (Table 2).

166

167 **Subject-related factors versus variation in FMD**

168 For the remaining methodology related factors and subject characteristics, there were weak, but
169 statistically significant correlations of log FMD TEE with age (adjusted $R^2 = -0.18$, $P < 0.01$) and
170 with baseline FMD (adjusted $R^2 = 0.11$, $P = 0.013$). In addition, FMD TEE was significantly smaller
171 in the subgroup of studies that applied a stereostatic probe holder, and in studies performed by
172 groups with more experience according to number of earlier publications on FMD. The %FMD
173 TEE of studies above and below the median duration between repeated measurements (7 days) was
174 not significantly different (Table 3), and there was no correlation between %FMD TEE and the
175 time between repeated measurements (adjusted $R^2 = -0.02$, $P < 0.75$).

176

177 We constructed a stepwise multivariate regression model with log FMD TEE as the dependent
178 variable and all factors that significantly influenced FMD TEE based on the individual analyses
179 (adherence score, age, baseline FMD, probe holder and previous experience). The stepwise
180 multivariate regression model predicted 51% of the variability in log FMD TEE. Adherence score
181 ($\beta = -0.16$), age ($\beta = -0.01$) and probe holder ($\beta = -0.19$) remained as statistically significant
182 ($P < 0.05$) predictors in the model (Table 4).

183

184 **DISCUSSION**

185 Measurement of the FMD of the brachial artery has obtained in the recent years a well-
186 established predictive capacity for future CVD events. Despite this and its relatively

187 straightforward and non-invasive approach, the clinical use of the FMD is hampered by its
188 sensitivity to variations in methodology.

189
190 Our systematic analysis of previous studies that explored the FMD reproducibility provides us
191 with a number of novel observations. First, we found considerable variation in the methodology
192 applied to measure FMD and consequently, differences between studies in the adherence to
193 current expert consensus guidelines. Secondly, these data show a robust inverse association
194 between adherence to the guidelines and FMD reproducibility, with higher adherence to
195 guidelines being related to smaller variation in FMD. Thirdly, we identified methodological
196 factors that were associated with smaller variation in FMD. Specifically, the use of automated
197 edge detection software, continuous measurement of brachial artery diameter over the cardiac
198 cycle, calculating %FMD by means of the true peak diameter and use of a stereostatic probe
199 holder were related to a better reproducibility. Taken together, our study provides strong
200 scientific data that highlight the importance of rigorous application of standardized contemporary
201 methodology to reduce measurement error of the FMD and, consequently, improve its use in
202 (pre)clinical studies.

203
204 To our knowledge, no previous study has explored the (relative) importance of adherence to
205 expert consensus guidelines for measures of vascular health, including frequently used techniques
206 like intima-media thickness, pulse wave velocity, and finger photoplethysmography. Taking all
207 studies on the reproducibility of the FMD together, involving 1,537 subjects, we found a TEE of
208 1.4% based on an average FMD of 7.1%. This indicates an overall good-to-acceptable
209 reproducibility of the FMD. However, significant variation was observed between studies, with
210 adherence to the expert consensus guidelines representing an important determinant of this

211 variation. Our data suggests that roughly 36% of the variation in FMD reproducibility can be
212 explained through adherence to the guidelines alone. The presence of a linear relation between
213 adherence to the guidelines and variation of the FMD suggests that measurement error would be
214 further reduced with stricter adherence to the guidelines. Our data also indicate that even with full
215 adherence to current expert consensus guidelines, some level of measurement error remains
216 present. Nonetheless, a significant amount of variation in the FMD can be prevented by strong
217 adherence to guidelines.

218
219 Our analysis provides further insight into methodological factors that determine within-person
220 error of the FMD measurement. For example, we found that taking the true peak artery diameter
221 (rather than a fixed time point), continuous diameter measurement and automated edge-detection
222 contribute to minimizing measurement error. The importance of these methodological factors
223 have already been acknowledged in previous work. For example, Black *et al.* found that the peak
224 diameter following cuff release differs between young and older subjects ⁷. Consequently,
225 calculating the FMD% at an arbitrary time point (e.g. 60 seconds) may lead to misleading
226 conclusions compared to a more sophisticated approach in which diameter of the brachial artery is
227 recorded continuously, allowing for the detection of the true peak dilation. Furthermore, previous
228 work demonstrated that the adoption of edge-detection software to perform (observer-
229 independent) analysis leads to smaller variation compared to the application of manual calipers (a
230 technique highly sensitive for measurement bias) ^{35, 40, 41}. Whilst these studies highlight the
231 importance of considering these factors for valid use of FMD, the present study highlights the
232 importance of considering these factors to lower variation. Therefore, our study provides an
233 additional rationale to perform continuous assessment of the diameter and the adoption of edge-
234 detection software when performing valid and reproducible assessments of the FMD.

235
236 Another important observation in our study was that previous experience of a laboratory with the
237 FMD resulted in a smaller variation in FMD. A potential explanation for this finding is that
238 experienced laboratories are more likely to demonstrate better adherence to the expert consensus
239 guidelines. Indeed, when all factors were included in the final regression analysis (including
240 adherence to the guidelines), previous experience of a laboratory with FMD did not emerge as an
241 independent predictor of FMD reproducibility. Another factor that contributed to a smaller
242 variation of the FMD was the use of a probe holder. The use of such devices is largely dependent
243 on the personal preference of the laboratory and the effect on measurement reproducibility is a
244 complex topic, since highly skilled operators with years of experience are able to conduct FMD
245 measurements with exceptional reproducibility, regardless of the use of a probe holder¹⁸. One
246 may speculate that sonographers' learning curves will likely differ depending on whether a probe
247 holder is used or not and also depending on the design and construction of the probe holder itself.
248 Therefore, despite the significant inverse association in our analysis, it remains difficult to
249 ascertain whether use of a probe holder leads to a smaller variation in FMD *per se*. Further
250 studies are needed to confirm the importance of using a probe holder to reduce variability of the
251 FMD.

252
253 Of the subject-related factors (age, diameter and baseline FMD), only age contributed
254 independently to the variation in FMD. Notably, higher age of subjects was associated with a
255 smaller variation in FMD. Older age is typically associated with a lower FMD^{42, 43}, which may
256 contribute to a smaller (biological) variation and/or less ability to change in response to
257 hemodynamic stimuli, consequently leading to a smaller measurement error. However, previous
258 work suggests the presence of larger variability for measurements of vascular health in clinical

259 groups. For example, Craiem *et al.* found that subjects with CVD, despite comparable baseline
260 FMD% values, demonstrate a larger coefficient of variation compared to healthy controls ²¹. At
261 least, our data suggest that the reproducibility of the FMD may differ between (clinical) groups.

262
263 Interestingly, the time duration between repeated measurements did not significantly affect FMD
264 reproducibility in our analyses. This might seem counterintuitive as poorer reproducibility is
265 expected as the time duration between repeated measurements increases. Indeed, a recent study
266 specifically designed to determine FMD reproducibility over short (48 hours), medium (3
267 months) and long (9 months) time frames did find poorer reproducibility at 9 months between
268 repeated measurements ¹⁴. Reproducibility was comparable for the shorter time periods however,
269 which is in agreement with a recent Italian multicenter study which found no differences in FMD
270 reproducibility up to 30 days between measurements ¹⁵. It should be noted that there was a large
271 heterogeneity in time between measurements in the included study groups, with the majority
272 ranging between one and 15 days (n = 32) and some up to 30 (n=11), 90 (n = 4) and 270 days (n
273 = 1). Excluding these last 16 studies from the analyses did not appreciably change our findings
274 our findings however (data not shown).

275
276 *Limitations.* An obvious limitation of our systematic review is that the degree of adherence to
277 expert consensus guidelines was assessed from information as provided in the papers. If a
278 methodological description omitted one or more of the 19 different scoring factors, no points
279 were assigned for those factors. As a consequence some studies with sparse methodological
280 descriptions received lower scores. Inconsequent reporting of methodological details might
281 therefore have confounded our outcomes. It should also be acknowledged that our estimation of
282 the experience of a laboratory with FMD measurements does not necessarily reflect the

283 experience of an individual sonographer. However, a laboratory more experienced in performing
284 FMD measurements will generally require a level of skill and training for their sonographers that
285 will meet at least the standard of their previous work. This highlights the importance of the level
286 of experience in performing studies with FMD as an outcome variable. Another limitation is that
287 our analysis on the relative importance of individual subject- and/or methodology-related factors
288 could only be based on a between-study comparison of factors contributing to the reproducibility
289 of the FMD. Various other factors may have influenced this analysis. Therefore, future studies
290 are necessary to further explore the importance of (some of) the methodology-related factors,
291 including the effects factors which we could not examine with the current dataset such as the
292 observer/analyst, the time of cuff occlusion and changes in baseline brachial artery diameter.

293
294 In conclusion, this systematic review shows that adherence to current expert consensus guidelines
295 significantly reduces measurement error when assessing brachial artery FMD in humans.
296 Moreover, when adopting the guidelines, we found that the use of contemporary techniques (i.e.
297 continuous diameter recording, edge-detection and wall-tracking software and possibly also the
298 use of a probe holder) is crucial to improve reproducibility of the FMD measurement.
299 Considering these factors will importantly decrease measurement error of the FMD and,
300 consequently, decrease chances for type II errors in studies that rely on FMD as their primary
301 outcome parameter. In other words, ignoring current expert-consensus guidelines causes
302 significant variability of the FMD and, consequently, may lead to spurious conclusions. This
303 study delivers important insight that should be taken into account when developing future updates
304 to expert-consensus guidelines.

305

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311

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313 AG and PLZ are employed by Unilever R&D Vlaardingen B.V. No conflicts of interest, financial
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315

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- 423

424 **FIGURE LEGENDS:**

425 **Figure 1.** Flow diagram of the study selection procedure.

426

427 **Figure 2.** Linear correlation between the Typical Error of the Flow Mediated Dilation Estimate
428 (FMD TEE) and adherence to expert guidelines (Adherence Score) in 27 studies (involving 48
429 study groups) of FMD reproducibility.

430

431

432 **TABLES:**

433

Table 1. General characteristics of the FMD reproducibility studies included in the systematic review.

Source	Health status	Number of subjects	Mean age (years)	Mean baseline FMD (%)	Mean baseline diameter (mm)	Time between measurements (days)	TEE
Kanahara 2014 ¹³	Healthy	32	40	7.90	3.83	14	1.28
Charakida 2013 ¹⁴		67	61	4.10	4.55	2	0.94
Charakida 2013	CVD, Diabetes	67	61	4.10	4.60	90	1.04
Charakida 2013		67	61	4.10	4.65	270	1.47
Ghiadoni 2012 ¹⁵		135	32	6.52	3.53	1 hour	0.83
Ghiadoni 2012	Healthy	135	32	6.52	3.55	30	1.15
Onkelinx 2012 ¹⁶		18	68	6.80	3.92	0.5 hour	0.94
Onkelinx 2012	CVD	18	68	7.13	3.91	2	0.88
Lima 2010 ¹⁷	Healthy	31	25	13.17	3.57	2	2.91
Thijssen 2009 ¹⁸	Healthy	10	24	6.83	4.28	0.5 hour	0.89
Donald 2008 (true peak diameter) ¹⁹	Healthy	32	43	8.10	3.70	6 hours	0.79

Source	Health status	Number of subjects	Mean age (years)	Mean baseline FMD (%)	Mean baseline diameter (mm)	Time between measurements (days)	TEE
Donald 2008 (true peak diameter)		34	43	7.50	3.70	7	0.79
Donald 2008 (true peak diameter)		37	43	8.10	3.75	30	0.53
Donald 2008 (true peak diameter)		35	43	7.80	3.80	90	0.74
Donald 2008 (60 sec)		32	43	7.30	3.70	6 hours	1.08
Donald 2008 (60 sec)		34	43	6.70	3.70	7	0.95
Donald 2008 (60 sec)		37	43	7.50	3.75	30	0.63
Donald 2008 (60 sec)		35	43	7.10	3.80	90	0.87
Simova 2008 ²⁰	CVD, Hypertension	40	62	6.05	3.84	0.25 hour	0.85
Craiem 2007 ²¹	Healthy	10	32	7.60	3.95	1 hour	0.80
Craiem 2007		10	32	8.10	3.89	7	0.91

Source	Health status	Number of subjects	Mean age (years)	Mean baseline FMD (%)	Mean baseline diameter (mm)	Time between measurements (days)	TEE
Craiem 2007	CVD	26	44	6.98	3.97	1 hour	1.34
Craiem 2007		26	44	5.66	4.15	30	0.96
Harris 2007 ²²	Healthy	9	57	7.80	4.11	2	1.32
Meirelles 2007 ²³	Healthy	10	33	19.90	3.50	1.5 hours	2.70
Meirelles 2007		13	33	16.50	3.55	3	2.50
Donald 2006 ²⁴	Healthy	16	28	7.30	3.55	1	1.63
Harris 2006 ²⁵	Healthy	16	23	9.88	3.74	2 hours	0.71
Leeson 2006 ²⁶	Healthy	17	32	4.74	4.05	20	1.22
Elsen 2005 ²⁷	Healthy	15	23	4.61	4.04	1	0.63
Sejda 2005 ²⁸	Healthy	18	28	5.95	4.04	7	3.89
Sejda 2005		18	28	4.23	4.15	7	1.63
Stoner 2004 ²⁹	Healthy	9	23	10.20	3.90	2	3.26
West 2004 ³⁰	Diabetes	18	55	5.57	4.01	7	0.81
West 2004		18	55	5.57	4.01	14	1.07

Source	Health status	Number of subjects	Mean age (years)	Mean baseline FMD (%)	Mean baseline diameter (mm)	Time between measurements (days)	TEE
Sidhu 2002 ³¹	Healthy	12	36	5.38	3.94	20	0.37
Sidhu 2002	CVD	12	62	1.80	4.29	20	0.33
Beux 2001 ³²	Healthy	38	44	6.62	4.41	1 hour	1.97
Beux 2001		38	44	4.32	4.41	1 hour	1.22
De Roos 2001 ³³	Healthy	34	27	4.13	3.90	25	2.01
Herrington 2001 ³⁴	Healthy	127	79	2.63	4.53	7	0.79
Herrington 2001		30	45	7.87	4.35	7	1.46
Woodman 2001 ³⁵	Healthy	24	55	6.60	4.06	7	0.71
Lind 2000 ³⁶	Healthy	10	22	7.40	3.55	2 hours	2.19
Lind 2000		10	22	7.40	3.55	21	2.82
Preik 2000 ³⁷	Healthy	8	28	10.60	3.62	20	1.06
Liang 1998 ³⁸	Healthy	30	44	10.80	3.84	18	2.01
Hardie 1997 ³⁹	Healthy	19	36	3.00	3.78	90	4.83

435

Table 2. Relationship of individual components of the adherence score with FMD TEE

Adherence Score Characteristic	Median (IQR) %FMD TEE					
	n	No	n	Yes	p	
Subject preparation						
Fasting state (>6h)	21	1.08 (0.83-2.10)	27	0.96 (0.80-1.47)	0.38	
No smoking/tobacco consumption prior to measurement (>6h)	22	0.89 (0.73-1.22)	26	1.30 (0.89-2.55)	< 0.01	
No habitual exercise prior to measurement (>48h)	31	1.22 (0.89-1.97)	17	0.87 (0.72-1.36)	0.07	
No food/beverages that contain alcohol and/or caffeine for >12 h	31	1.06 (0.79-1.97)	17	1.04 (0.82-1.40)	0.6	
No polyphenol-rich food/beverages (cocoa, tea, fruit juices) for >18 h	45	1.04 (0.79-1.63)	3	1.15 (0.83-2.91)	0.6	
No vitamins for at least 72h	44	1.05 (0.80-1.63)	4	0.99 (0.68-2.47)	0.8	
Vasoactive medications withheld/noted on the morning of the study	26	1.01 (0.79-1.98)	22	1.06 (0.84-1.51)	0.8	
Supine position; ≥15 min rest in a quiet, temperature controlled room	30	1.01 (0.80-1.72)	18	1.10 (0.82-1.60)	1.0	
Repeated measurements standardised to timing of the menstrual cycle	36	1.01 (0.79-1.89)	12	1.06 (0.84-1.59)	0.7	
Repeated measurements done in fixed time windows (same time of day)	7	1.22 (0.94-1.47)	41	0.96 (0.79-1.80)	0.5	
Image acquisition						
Diameter measurements recorded continuously over the cardiac cycle	35	1.22 (0.85-2.01)	13	0.88 (0.75-0.95)	< 0.01	
Diameter measurements obtained during end diastole only	15	0.89 (0.79-1.15)	33	1.22 (0.83-2.01)	0.06	

Adherence Score Characteristic	Median (IQR) %FMD TEE						
Simultaneous acquisition of pulse-wave Doppler velocity signal for quantification of shear stimulus	20	1.40	(0.86-2.15)	28	0.94	(0.79-1.21)	0.05
Image analysis							
Analysis using automated edge detection and wall tracking software	13	2.19	(1.47-2.87)	35	0.91	(0.79-1.22)	<0.01
FMD calculation point (true peak diameter)	17	1.63	(0.94-2.76)	31	0.91	(0.79-1.28)	<0.01
Lab data							
Use of experienced sonographers reported	20	1.09	(0.73-2.38)	28	1.05	(0.82-1.47)	0.7
Same sonographers paired to same subjects for repeated measurements	8	1.10	(0.86-1.44)	40	1.01	(0.79-1.89)	0.8

437 **Table 3.** Relationship of subject- and methodology-related characteristics with FMD TEE

Continuous variables	Adjusted Pearson	
	R ²	P-values
Age (years)	-0.18	<0.01
Baseline FMD (%)‡	0.11	0.01
Baseline diameter (mm)	-0.02	0.15
Number of subjects (n)	-0.001	0.33

Categorical variables	Median (IQR) %FMD TTE				
	n	No	n	Yes	P-values
CVD risk	37	1.15 (0.79-2.01)	11	0.94 (0.85-1.07)	0.31
Distal occlusion cuff placement	5	2.01 (0.91-2.6)	43	1.04 (0.79-1.47)	0.17
Stereostatic probe holder	18	1.82 (1.02-2.85)	30	0.92 (0.73-1.22)	<0.01
Experienced centre*	23	1.32 (0.88-2.5)	25	0.91 (0.80-1.19)	0.01
Time between repeated measurements above median†	18	0.94 (0.81-1.72)	30	1.06 (0.71-1.61)	0.77

438 ‡ Baseline FMD refers to the first of the two repeated measurements

439 *Centre experience was defined as the number of previous studies on FMD published by the
440 principle author of each included study. The effect of centre experience was examined by
441 comparing the %FMD TEE of studies below (No) and above (yes) the median number of
442 previously published FMD studies.

443 †The effect of the time duration between studies was examined by comparing the %FMD TEE of
444 studies below (no) and above (yes) the median duration of 7 days.

445

446 **Table 4.** Relation of the adherence score, subject- and methodological factors with the
 447 reproducibility of the FMD measurement

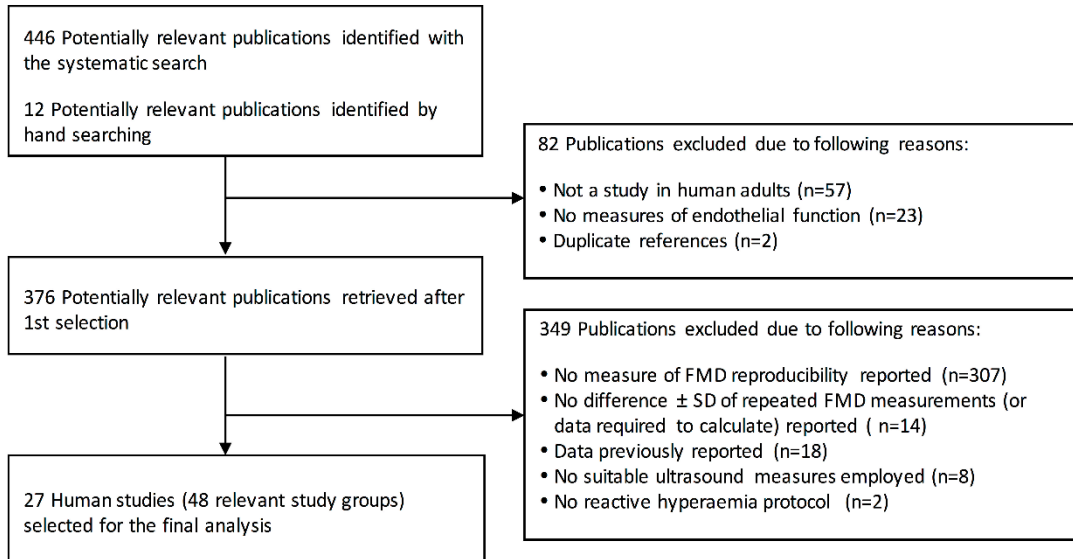
Stepwise Regression Analysis (model Adj R²=0.51)			
Variable	β	95% CI	P-value
Adherence Score (unit)	-0.16	-0.24; -0.07	<0.01
Age (year)	-0.01	-0.02; -0.001	0.03
Stereostatic probe holder (yes)	-0.19	-0.06; -0.33	<0.01

448 The regression coefficient β represents the increase in the log FMD TEE per unit increase in each
 449 factor. Baseline FMD and Centre experience did not remain in the model

450

451 **FIGURES:**

452 **Figure 1:**



453

454

