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Reference Intervals for Brachial Artery Flow-Mediated Dilation and the Relation With Cardiovascular Risk Factors.

REFERENCE INTERVALS FOR BRACHIAL ARTERY FLOW-MEDIATED DILATION AND THE RELATION WITH CARDIOVASCULAR RISK FACTORS

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ABSTRACT

Endothelial function, assessed using brachial artery flow-mediated dilation (FMD), predicts future cardiovascular disease (CVD) risk. This study established age- and sex-specific reference intervals for brachial artery FMD in healthy individuals, and examined the relation with CVD risk factors. In a retrospective study design, we pooled brachial artery FMD (acquired according to expert-consensus guidelines for FMD protocol and analysis) and participant characteristics/medical history from 5,362 individuals (4-84 years; 2,076 females). Healthy individuals (n=1,403 [582 females]) were used to generate age-/sex-specific percentile curves. Subsequently, we included individuals with CVD risk factors, without overt disease (un-medicated n=3,167 [1,247 females], and medicated n=792 [247 females]). Multiple linear regression tested the relation of CVD risk factors (body mass index, blood pressure, cholesterol, diabetes, dyslipidaemia and smoking) with FMD. Healthy males showed a negative, curvilinear relation between FMD and age, whilst females revealed a negative linear relation that started higher, but declined at a faster rate than males. Age- and sex-specific differences in FMD relate, at least partly, to baseline artery diameter. FMD was related to CVD risk factors in un-medicated (e.g. systolic-/diastolic blood pressure) and medicated individuals (e.g. diabetes/dyslipidaemia). Sex mediated some of these effects ($P<0.05$), with normalisation of FMD in medicated men, but not women with dyslipidaemia. In conclusion, sex alters the age-related decline in FMD, which may partly be explained through differences in baseline diameter. Sex also alters the influence of some CVD risk factors and medication on FMD. This work improves interpretation and future use of the FMD technique.

Key words: ageing, sex differences, flow-mediated dilation, reference intervals, risk factors.

Abbreviations and acronyms

BMI = body mass index

CVD = cardiovascular disease

FMD = flow-mediated dilation

FP = fractional polynomial

HDL = high-density lipoprotein cholesterol

LDL = low-density lipoprotein cholesterol

SD = standard deviation

1. INTRODUCTION

The vascular endothelium plays a key role in the maintenance of vascular homeostasis (1). Since endothelial dysfunction contributes to the development and progression of atherosclerosis, ultimately leading to cardiovascular disease (CVD) (2), studies have explored strategies to assess endothelial dysfunction as an early biomarker of CVD (3,4). In 1992, Celermajer *et al.* introduced the flow-mediated dilation (FMD) approach; a non-invasive assessment of endothelial function using ultrasonography (5). Brachial artery FMD has now become a popular research tool, likely due to its non-invasive nature, responsiveness to interventions (6,7), and correlation with coronary artery endothelial function (4,8,9). Despite the independent prognostic value of FMD (10-14), even in asymptomatic individuals (10-12,15), some limitations hamper widespread use of the technique.

Age- and sex-specific differences in FMD have been consistently reported (16-21), with older age and male sex being associated with lower FMD values. However, marked differences in FMD values are present between studies, prohibiting meaningful comparisons. Variation between laboratories in FMD protocol (e.g. timing, occlusion cuff position) and analysis (manual *versus* automated) limits between-laboratory comparison. Consistent implementation of expert-consensus guidelines (14,22) seems a logical solution to these issues, especially since strict adherence to these guidelines lowers FMD variability (23). Previous work attempted to construct age- and sex-specific reference values (24). However, this work did not control for age-related changes in baseline artery diameter and CVD risk factors, i.e. (patho)physiological indices that importantly contribute to the magnitude and variation of the FMD. This highlights the need and importance of age- and sex-specific reference values for FMD, collected when adhering to protocol guidelines, to facilitate interpretation of FMD outcomes.

We combined FMD observations from six laboratories that all strictly adhered to protocol guidelines (14,25) and performed analysis using automated edge-detection and wall-tracking software. First, using data from 1,403 healthy individuals, we established age- and sex-specific reference intervals for brachial artery FMD across the entire lifespan. This data also allowed us to explore the role of age- and sex-specific differences in baseline artery diameter on FMD. Secondly, we enriched the dataset with 3,959 individuals with established CVD risk factors (i.e. above international cut-off normative values) and explored how these risk factors, as well as medication use, impacted the age- and sex-specific FMD reference values.

2. METHODS

2.1 Study population.

The data that support the findings of this study are available from the corresponding author upon reasonable request. Research groups from the International Working Group on Flow-Mediated Dilation identified eligible studies that included assessment of brachial artery FMD. Studies were included if all measurements were performed with adherence to the expert-consensus guidelines on measuring FMD (14,25) and data collection adhered to the Declaration of Helsinki. All participants provided oral and written informed consent prior to each individual study.

We compiled individual-level brachial artery FMD data with corresponding participant characteristics and medical history from six laboratories (for the list of contributing laboratories and investigators, see supplementary file; Table S1). With permission from principal investigators, we also included unpublished data (32% of total observations). When the original

studies adopted a methodological design with repeated FMD measurements, we included the FMD that was performed first.

For our first objective, i.e. age- and sex-specific reference intervals, healthy individuals (4-84 years; 821 males and 582 females) were selected following stringent inclusion criteria (26), including (when available): (i) systolic blood pressure <140mmHg and diastolic blood pressure <90mmHg, (ii) body mass index (BMI) <25kg/m^2, (iii) waist circumference <102cm for males and <88cm for females, (iv) total cholesterol <4.9mmol/L, (v) Low-density lipoprotein cholesterol (LDL) <3mmol/L, (vi) High-density lipoprotein cholesterol (HDL) >1mmol/L for males and >1.2mmol/L for females, (vii) triglycerides <1.7mmol/L, (viii) glucose <5.6mmol/L, (ix) never smoked, (x) no history of metabolic- or CVD/event, and (xi) not taking any medications or hormone-based contraception/therapy. For the second objective, i.e. impact of CVD risk factors, the remaining participants with one or more risk factor (n=3,959) were stratified into un-medicated (males; n=545, females; n=247), and medicated individuals (males; n=1920, females; n=1247). The medicated subpopulation were taking blood pressure-, lipid- and/or glucose-lowering drugs.

2.2 Flow-mediated dilation: methodological considerations.

We included brachial artery FMD data from research groups strictly adhering to expert-consensus guidelines (14,25). FMD assessments were performed following standardised participant preparation procedures (i.e. fasted state, abstained from exercise, caffeine and alcohol, and timing of menstrual cycle) (27). Following 10-15 minutes of supine rest, brachial artery diameter was assessed via high-resolution duplex ultrasound using a hand-held probe or probe-holder approach. B-mode images were obtained and optimized, and Doppler velocity

was recorded simultaneously. After at least 1 minute of baseline diameter and blood flow velocity measurement, an occlusion cuff, placed distal to the olecranon process, was inflated to suprasystolic pressure (i.e. >50mmHg above the participant's systolic blood pressure) for 5 minutes. Recordings were resumed 30 seconds before cuff deflation, and FMD was recorded for a further 3 minutes post cuff deflation.

FMD data were analysed using an automated edge-detection and wall-tracking software (BloodFlow Analysis [n=3,244] or FMD Studio, Quipu SRL [n=2,118]) which is largely operator independent, and also substantially more reproducible than manual approaches (28). These software packages track the vessel walls and blood velocity trace in B-mode frames via a pixel density and frequency distribution algorithm.(28) An optimal region of interest to be analysed was selected by the sonographer, based on consistent image quality, with a clear distinction between the artery walls and lumen. Despite the initial region of interest selection being operator-determined, the remaining analysis was automated and independent of operator bias (28). Laboratory-specific details of analysis software and ultrasound machines are reported in the supplementary file (Table S1).

2.3 Statistical analysis

Statistical analyses were conducted using IBM SPSS version 25 (SPSS Inc., Chicago, IL) unless stated otherwise.

We used multiple imputation chained equations to impute missing values (29) for weight, BMI, systolic-, diastolic- and mean arterial blood pressure, and baseline- and peak diameter (all

variables had <20% missing data). We generated five imputed datasets, which were used to fit the relevant regression models and results reported were obtained from the pooled analyses on all imputed datasets.

For the definition of age- and sex-specific reference intervals for brachial artery FMD, calculation of age-specific reference intervals were performed in healthy males (n=821) and females (n=582) separately. Initially, to account for differences in analysis software, we performed multiple linear regression including a dummy variable for FMD Studio as an independent determinant of FMD outcome. The regression coefficient for the dummy variable (β=0.166%) was used as a calibration factor to rescale individual FMD values obtained using FMD Studio. To calculate age- and sex-specific reference intervals, we utilised fractional polynomial (FP) regression (30) in STATA software (Stata Corp., College Station, TX, USA) with the xrigls command. Age-specific 2.5th, 10th, 25th, 50th, 75th, 90th and 97.5th percentile curves were calculated as meanFMD + Zp x SD, where Zp assumed the values of -1.96, -1.28, -0.67, 0, 0.67, 1.28, and 1.96, respectively. Age- and sex-specific percentile curves were also calculated for baseline brachial artery diameter. Furthermore, Pearson correlation coefficient was used to assess the relationship between baseline diameter and FMD in both the estimated (derived from the age- and sex-specific percentile curves) and observed (original) outcomes. Fisher r-to-z transformation was used to compare the correlation coefficient between males and females. Sensitivity analyses were conducted whereby age- and sex-specific reference intervals were calculated for males and females ≥9 years and ≥18 years.

We examined the relation with CVD risk factors. Based on the equations computed for healthy individuals, we calculated the expected $mean_{FMD}$ and SD_{FMD} for individuals with CVD risk factors and calculated age- and sex-specific Z-scores as $observed_{FMD}$ -

205 expected$_{FMD}$/SDexpected$_{FMD}$. Z-scores represent the number of SDs above or below the healthy
206 population mean (50th percentile) of the same age and sex.

207

208 Multiple linear regression determined the relation of CVD risk factors with FMD Z-scores in
209 four subpopulations (un-medicated and medicated males and females). Age was included in
210 the regression model to account for any residual effects on outcomes. Sub-analyses were
211 conducted for smoking and cholesterol, since limited available data were present for these
212 variables. We added interaction terms between each risk factor and sex to explore whether the
213 effects of the model predictors are moderated by sex differences.

214

215 **3. RESULTS**

216 Participant characteristics are presented for all males and females in Tables 1 and 2,
217 respectively.

218

219 *3.1 Age-/sex-specific reference intervals for brachial artery FMD in the healthy subpopulation.*
220 The best fitting FPs' powers (p) for mean$_{FMD}$ and SD$_{FMD}$ were both p=1 for females, which
221 represents a linear relation between FMD and age. For males, the mean$_{FMD}$ p=0 and SD$_{FMD}$ p=-
222 0.5, indicating a curvilinear relation (Figure 1). The equations derived for estimated FMD for
223 females were:

224 $$\text{mean}_{FMD}\,(\%) = 9.5947 - 0.0631 \times \text{age}$$

225 $$\text{SD}_{FMD}\,(\%) = 4.5400 - 0.0349 \times \text{age}$$

226 and, for males:

227 $$\text{mean}_{FMD}\,(\%) = 7.9279 - 1.5725 \times \ln(\text{age}/10)$$

228 $$\text{SD}_{FMD}\,(\%) = 1.4008 + 2.3163 \times (\text{age}/10)^{-0.5}$$

Given the large difference in available data between sexes in young children, additional FP regression analyses were performed in individuals ≥9 years and ≥18 years (Supplementary file; Figures S1 and S2, respectively). These analyses confirmed the primary observations of age- and sex-dependent variation in FMD. In individuals ≥9 years and ≥18 years we additionally explored the role of height and found that every 10 cm increase in height is associated with a 0.16 mm (95%CI: 0.14 to 0.18) increase in baseline diameter and a 0.28 % (95%CI: -0.48 to -0.09) decrease in FMD. Importantly, these effects were independent of sex (sex*height interaction for baseline diameter P=0.481; sex*height interaction %FMD P=0.404). In contrast our observations in males, we found a negative linear relation between FMD and age in males ≥18 years. Repeating analysis in women and men ≥30 years confirmed the presence of a negative linear relation between FMD and age in both adult groups (data not shown). Linear regression was used to explore the effect of scan location (i.e. laboratory) on %FMD, with laboratories contributing >200 scans being entered in the analysis. With adjustment for basic demographics (age, sex) and lifestyle (BMI, smoking status) covariates, we found no statistically significant difference (P≥0.1) for laboratory on %FMD.

3.2 Age- and sex-specific differences in baseline brachial artery diameter.

The best fitting FPs' powers (p) for $mean_{BaselineDiameter}$ and $SD_{BaselineDiameter}$ were p=-2 and p=-1 respectively for females, and p=-0.5 and p=-1 respectively for males, indicating a curvilinear relation in both sexes (Figure 1).

The equations derived for estimated baseline artery diameter for females were:

$$mean_{BaselineDiameter}\,(mm) = 3.3764 - 0.6070 \times (age/10)^{-2}$$

$$SD_{BaselineDiameter}\,(mm) = 0.6389 - 0.3195 \times (age/10)^{-1}$$

and, for males:

$$\text{mean}_{\text{BaselineDiameter}}\,(\text{mm}) = 5.8692 - 2.9237 \times (\text{age}/10)^{-0.5}$$

$$\text{SD}_{\text{BaselineDiameter}}\,(\text{mm}) = 0.7172 - 0.3177 \times (\text{age}/10)^{-1}$$

Corresponding reference intervals (percentiles) derived from the above equations for estimated FMD and baseline artery diameter are presented in Table 3.

Correlation analysis demonstrated weak but statistically significant inverse relationships between observed baseline artery diameter and FMD (female r^2=0.163, male r^2=0.149; both P<0.001), which was not different between sex (Fisher's P=0.697; Figure 2). Additional analysis between estimated baseline artery diameter and FMD (derived from the equations above) revealed a strong inverse relation in males (r^2=0.975, P<0.001), whilst a significantly weaker relation was found in females (r^2=0.605, P<0.001; Fisher's P<0.001).

3.3 Relation of CVD risk factors with FMD percentiles compared to healthy age- and sex-matched individuals.

In the un-medicated subpopulation, lower FMD Z-scores (i.e. lower FMD compared to age-/sex-matched healthy reference values) were found for higher systolic blood pressure in both males and females (P=0.015 and P<0.001, respectively). Higher diastolic blood pressure was significantly associated with higher FMD Z-scores in females (P<0.001). Presence of diabetes was significantly associated with lower FMD Z-scores in males (P<0.001; Table S2).

In the medicated subpopulation, presence of dyslipidaemia and diabetes were significantly associated with lower FMD Z-scores in females (both P=0.01). In males, smoking and diabetes were significantly associated with lower FMD Z-scores (P=0.022 and P=0.027, respectively), whilst dyslipidaemia was related to higher FMD Z-scores (P=0.029; Table S2). These observations are largely reinforced when standardised regression coefficients (per SD increase in- or presence of CVD risk factor) are presented in Figure 3.

3.4 Sex differences in the relation of CVD risk factors with FMD Z-scores.

Using sex as an interaction term in the regression model revealed that systolic- and diastolic blood pressure were stronger determinants for FMD in un-medicated females than in males (both P=0.019, Figure 3). In the medicated subpopulation, no sex differences were found for systolic and diastolic blood pressure (Figure 3). Whilst presence of dyslipidaemia was not significantly affected by sex in the un-medicated group, sex altered the effect of dyslipidaemia on FMD Z-score in the medicated group (Figure 3). More specifically, FMD was supra-normalised in medicated males, whilst FMD in females was lower in those with dyslipidaemia compared to healthy age- and sex-matched individuals (P<0.001).

4. DISCUSSION

Following strict adherence to expert-consensus guidelines (14,25), we provide age- and sex-specific reference intervals for brachial artery FMD, where sex altered the age-related decline in FMD. Healthy males demonstrated a negative curvilinear relation between FMD and age, whilst females revealed a linear relation, where FMD started higher, but declined at a faster rate with age compared to males. Importantly, our work revealed that differences in baseline brachial artery diameter may, at least partly, contribute to the age- and sex-related differences

in FMD. This suggests that age- and sex-related differences in FMD in healthy individuals may, in addition to differences in endothelial function, also relate to age- and sex-related differences in structural characteristics (i.e. baseline diameter). Additionally, our work provides insight into how CVD risk factors and (cardiovascular-controlling) medications influence FMD. We found that some CVD risk factors (e.g. blood pressure, diabetes, dyslipidaemia, BMI) alter age- and sex-related FMD Z-scores, both in un-medicated and medicated individuals. Moreover, we found that sex altered the impact of CVD risk factors and medication. Specifically, a larger impact of blood pressure on FMD was evident in un-medicated females compared to males, whilst dyslipidaemia was associated with a lower FMD in medicated females, but not in males. Taken together, these reference intervals for brachial artery FMD importantly contribute to improved interpretation of FMD outcomes, but also extend our knowledge and understanding of factors that influence FMD.

In the past years, reference values have been estimated for other (pre)clinical tests of vascular structure (e.g. stiffness (31,32) and intima-media thickness (33) in large arteries, and media/lumen ratio in small arteries (34)), which contributed to widespread and valid use of the technique. Importantly, in these examples, efforts were made to standardise assessment prior to estimating reference intervals. Similarly, we have pooled data from laboratories that strictly adhere to guidelines for performance and analysis of brachial artery FMD (14,25). The importance of following these guidelines is supported by our dataset, in that we found no between-software or between-laboratory differences in FMD results. Importantly, data were derived from multiple laboratories, different countries, and involved multiple principal investigators and sonographers. This emphasises that adhering to expert-consensus guidelines is essential for the future use of FMD, but also highlights the relevance and robustness of the age- and sex-specific reference intervals presented in our work.

In the healthy population, and in line with most previous work (16,18-20), we observed an age-related decline in FMD in both sexes. Nonetheless, the rate of change differed between sexes. Early work reported a linear decline in both groups that starts around the 4th or 5th decade of life (19). Previous studies, however, are limited by the inclusion of a relatively small age range and/or have included individuals with CVD risk factors. Another limitation is largely ignoring the potential role of age- and sex-specific differences in baseline artery diameter, which is relevant since baseline diameter is inversely related to FMD (35-37). The role of baseline diameter has extensively discussed in expert-consensus FMD guidelines (14,38), and by various others (39). Differences in baseline artery diameter may partly contribute the lower FMD in males compared to females, and may also influence the age-related changes in FMD. Indeed, the age-related decline in FMD in our data set is mirrored by a concomitant increase in baseline diameter. This effect seems stronger in males than in females, supported by the stronger relation between estimated FMD and baseline diameter in males. Furthermore, in children there was a steeper rate of change in males compared to females, which may contribute to the characteristic drop in FMD in males (and not in females) during childhood and adolescence in our data set. This suggests that, in addition to age- and sex-related differences in endothelial function, also baseline diameter may contribute to age- and sex-related differences in FMD. However, further work is required, preferably related to a prospective, within-subject design, to better understand the role of baseline diameter to the age-related changes in FMD.

The higher FMD in females, but also the steeper decline in FMD with age, compared to males may relate to differences in sex hormones, especially since oestrogen has been linked to cardio-protective properties (40). These protective effects of oestrogen may work through

upregulation of nitric oxide (41), or increasing the sensitivity of the endothelium to increases in shear stress (27,42). Conversely, in contrast with previous research (19,43), the characteristic drop in sex hormones associated with menopause did not translate to a steeper decline in FMD in our study. These discrepancies may be attributed to between-study differences in participant inclusion criteria (e.g. blood pressure/BMI cut-off values). Our data suggests that remaining within "normal" ranges for CVD risk factors may be protective against the menopause-related drop in FMD, although the relatively small sample size of women aged >40 years in our study must be considered as a potential limitation. However, previous studies are limited by the cross-sectional nature, making it difficult to untangle the impact of menopause *versus* older age. An alternative explanation for the gradual decline in FMD with age relates to changes in structural characteristics of the artery wall, including increases in intima-media thickness (33) and arterial stiffness (31,32). Also other body characteristics, such as muscle mass or height, may contribute to our findings. Furthermore, age-related increases in retrograde and oscillatory shear (44) inflammation, and oxidative stress (45) may also contribute to the gradual age-related decline in FMD in healthy individuals. Future work is required to better understand the nature and physiological mechanisms underlying this change.

When examining the relation between CVD risk factors and FMD Z-scores, we found that blood pressure and diabetes were negatively associated with FMD in un-medicated individuals. This is not surprising, given previous work related to endothelial dysfunction with the presence of high blood pressure (46) and diabetes (47), whilst these risk factors also impacted sex- and age-specific reference values for carotid intima-media thickness (33) and arterial stiffness (31,32). Moreover, the relation between blood pressure and FMD Z-score disappeared in the medicated subgroup, implying that FMD is not different from healthy controls when using drugs that target these risk factors. These findings are supported by previous work in blood

pressure-lowering medication (48), which found these drugs to (in)directly improve endothelial function in patients. In contrast to our hypothesis, but also conflicting with previous work (49), no significant impact on FMD in un-medicated individuals was found in other well-established risk factors, including BMI, cholesterol and smoking. Our observation does not imply that these traditional risk factors do not alter endothelial function. A potential explanation for these findings may relate to the small proportion of available data for smoking and cholesterol variables. Nonetheless, our data confirms that elevated blood pressure is an important risk factor associated with endothelial dysfunction. A final consideration relates to the potential role of structural characteristics, especially since our work supports a role for the diameter explaining age- and sex-related changes in FMD. Previous work found comparable predictive values of brachial artery diameter and FMD for CVD events in asymptomatic (50) and symptomatic populations (51). Additionally, within- or between-subject differences in wall thickness may also explain differences in FMD, especially since changes in the wall-to-lumen ratio may alter vascular responsiveness in conduit arteries (52). This warrants further work to explore the role of structural indices, including the diameter and wall thickness, in changes in FMD, both with older age and in relation to CVD risk factors.

We also found that sex affects the impact of CVD risk factors and medication on FMD. In un-medicated individuals, systolic- and diastolic blood pressure were stronger determinants of FMD Z-score in females than in males. These findings fit with previous observations, in that untreated hypertensive women showed larger endothelial dysfunction (53) and a stronger relation between hypertension and myocardial infarction incidence compared to men (54). However, the larger FMD Z-scores in women with a higher diastolic blood pressure were unexpected. This observation may relate to the inclusion of unmedicated, healthy individuals who did not present with hypertension. Interestingly, also unmedicated men showed a trend for

this positive relation between FMD Z-score and diastolic blood pressure. Interestingly, sex-specific differences for the effect of blood pressure on FMD disappeared in the medicated group. Additionally, we reported sex differences in the medicated group, with females demonstrating significantly lower FMD Z-scores than males in the presence of dyslipidaemia. In fact, FMD Z-scores for medicated males with dyslipidaemia were supra-normalised (i.e. greater than the healthy population mean of the same age), highlighting the success of drugs targeting dyslipidaemia in males. Whilst the underlying mechanisms for these sex differences remain unclear, these observations are extremely important in contemporary medicine where increased awareness is required that sex differently affects the process of atherosclerosis and CVD development, as well as the impact of pharmacological treatments.

Despite the large number of strengths, i.e. large sample size, and all FMD data obtained with strict protocol guideline adherence, key limitations of this study largely relate to the retrospective study design. More specifically, data on important factors associated with CVD risk and vascular function such as physical activity, cardiorespiratory fitness, ethnicity, sex hormone levels and endothelial markers were not included in the database. These additional data would have complemented the dataset to gain some mechanistic insight underlying our major findings.

5. CONCLUSIONS

In conclusion, we estimated age- and sex-specific percentiles for brachial artery FMD in a healthy population and explored the relation of CVD risk factors on FMD Z-scores. Notably, the FMD data included in the present study were obtained with strict adherence to protocol guidelines (14,25). Despite the large number of studies (and contributing authors) included in

the analyses, between-study variability was low, emphasising the importance of strict guideline adherence. More importantly, this also highlights the feasibility and use of FMD for (pre)clinical work, when guidelines are strictly adhered to. Accordingly, our age- and sex-specific reference values enable better interpretation of FMD outcomes. Moreover, our work also highlights that sex leads to distinct age-related changes in FMD, but also affects the impact of some CVD risk factors in (un)medicated individuals.

6. PERSPECTIVES

Competency in medical knowledge: Sex-specific differences were evident in the age-related decline in endothelial function, whilst sex also altered the relation between cardiovascular risk factors and medications versus endothelial function. These data improve our understanding of endothelial function, highlighting sex-specific differences in the development of cardiovascular disease and impact of risk factor-targeting medications on endothelial function in humans.

Translational outlook: Construction of reference intervals for brachial artery FMD improves interpretation of FMD data, but also emphasizes the importance of adhering to guidelines in future FMD studies. This allows for wider uptake of the FMD technique, whilst this may also facilitate more research to understand the underlying mechanisms of age-, sex- and risk factor-specific differences in endothelial function.

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610 **Novelty and Significance**

611 *What is New?*

- Strong variation between laboratories in performance of the flow-mediated dilation (FMD) hamper widespread use of this technique, and prohibits meaningful between-laboratory comparison.
- Upon strongly adhering to expert-consensus guidelines for FMD, this study established age- and sex-specific reference intervals for brachial artery FMD in healthy individuals, and examined the relation with CVD risk factors.

What is Relevant?

- Men show a negative, curvilinear relation between FMD and age, whilst females revealed a negative linear relation, which is partly related to baseline diameter.
- Some CVD risk factors, including systolic blood pressure, are related to a lower FMD in un-medicated individuals.
- The relation between systolic blood pressure and brachial artery FMD disappeared in medicated individuals.

Summary

- The age-related decline in brachial artery FMD is different between men and women, which is at least partly explained through differences in baseline diameter.
- CVD risk factors impair brachial artery FMD, with sex altering the influence of some CVD risk factors and medication on FMD.

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8. FIGURE LEGENDS

Figure 1: Age-specific percentiles of brachial artery flow-mediated dilation (FMD; percentage change from baseline) and baseline diameter (in mm) in males (FMD (**A**) n=821; baseline diameter (**B**) n=790) and females (FMD (**C**) n=582; baseline diameter (**D**) n=571).

Figure 2: Brachial artery flow-mediated dilation (FMD; percentage change from baseline) and baseline diameter (in mm) in healthy males (**A**; n=796) and females (**B**; n=579). Pearson correlation coefficient was used to determine the relationship between FMD and baseline diameter in males and females separately.

Figure 3: Point estimates and 95% confidence intervals represent the increase in brachial artery FMD Z-score (in SD from the healthy population mean) per SD increase (or presence) in risk factor resulting from a multivariable regression model including all risk factors and age for males (●) and females (○). (**A**) un-medicated males (n=1920) and females (n=1247); (**B**) medicated males (n=545) and females (n=247). BMI – body mass index; SBP – systolic blood pressure; DBP – diastolic blood pressure; HDL – high-density lipoprotein cholesterol

Table 1: Participant characteristics of the total male population, and healthy, un-medicated and medicated male subpopulations.

			CVD risk factors	
Characteristics	**Total**	**Healthy**	**Un-medicated**	**Medicated**
n	3286	821	1920	545
Age (years)	42±19	26±15	45±17	58±11
Body mass index (kg/m^2)	26.3±5.2	22.2±3.8	27.2±4.6	29.4±5.0
Systolic blood pressure (mmHg)	130±17	118±13	133±17	137±16
Diastolic blood pressure (mmHg)	78±13	70±10	81±12	83±11
Mean arterial pressure (mmHg)	95±14	86±10	98±14	101±13
Total cholesterol [mmol/L (*n*)]	5.1±1.0 (1756)	4.2±0.5 (105)	5.3±1.0 (1252)	4.9±1.1 (399)
LDL cholesterol [mmol/L (*n*)]	3.2±0.9 (1562)	2.3±0.5 (87)	3.4±0.9 (1139)	3.0±1.0 (336)
HDL cholesterol [mmol/L (*n*)]	1.2±0.4 (1613)	1.4±0.2 (89)	1.2±0.4 (1181)	1.2±0.4 (343)
Total-to-HDL cholesterol ratio (n)	4.4±1.3 (1612)	3.0±0.5 (89)	4.5±1.3 (1180)	4.3±1.3 (343)
Triglycerides [mmol/L (*n*)]	1.6±1.1 (1670)	0.9±0.4 (92)	1.6±1.1 (1221)	1.7±1.1 (357)
Plasma glucose [mmol/L (*n*)]	5.5±1.5 (1293)	4.7±0.6 (83)	5.3±1.1 (982)	6.6±2.4 (228)
Baseline artery diameter (mm)	4.37±0.86	3.90±0.83	4.46±0.82	4.75±0.72
FMD (%)	5.56±2.91	6.66±3.24	5.45±2.72	4.30±2.42
Current smoker [*n* (%)]	127 (3.9)	0	102 (5.3)	25 (4.6)
Diabetes [*n* (%)]	288 (8.8)	0	107 (5.6)	181 (33.2)
Dyslipidaemia [*n* (%)]	1474 (44.9)	0	1074 (55.9)	400 (73.4)
Blood pressure-lowering medication [*n* (%)]	499 (15.2)	0	0	499 (91.6)
Lipid-lowering medication [*n* (%)]	253 (7.7)	0	0	253 (46.4)
Glucose-lowering medication [*n* (%)]	167 (5.1)	0	0	167 (30.6)

Data are presented as mean±SD. For blood metabolites, *n* represents the number of available data within the respective subpopulation. For categorical data, data are presented as *n* (percentage of subpopulation). HDL – high-density lipoprotein cholesterol; LDL – low-density lipoprotein cholesterol; FMD – flow-mediated dilation.

Table 2: Participant characteristics of the total female population, and healthy, un-medicated and medicated female subpopulations.

			CVD risk factors	
Characteristics	**Total**	**Healthy**	**Un-medicated**	**Medicated**
n	2076	582	1247	247
Age (years)	41±18	28±16	44±16	56±12
Body mass index (kg/m^2)	25.7±6.3	21.6±3.5	26.9±6.1	29.5±7.1
Systolic blood pressure (mmHg)	125±19	113±12	129±18	138±18
Diastolic blood pressure (mmHg)	76±12	68±10	78±12	81±14
Mean arterial pressure (mmHg)	92±15	83±10	94±14	98±17
Total cholesterol [mmol/L (*n*)]	5.3±1.0 (1150)	4.3±0.4 (119)	5.1±1.0 (839)	5.3±1.0 (192)
LDL cholesterol [mmol/L (*n*)]	3.3±0.9 (999)	2.3±0.4 (93)	3.4±0.8 (737)	3.2±1.0 (169)
HDL cholesterol [mmol/L (*n*)]	1.5±0.4 (1026)	1.7±0.3 (99)	1.5±0.4 (755)	1.6±0.4 (172)
Total-to-HDL cholesterol ratio (n)	3.6±1.0 (1025)	2.6±0.4 (98)	3.8±1.0 (755)	3.6±1.0 (172)
Triglycerides [mmol/L (*n*)]	1.2±0.9 (1066)	0.8±0.3 (100)	1.2±0.8 (783)	1.5±1.3 (183)
Plasma glucose [mmol/L (*n*)]	5.0±0.9 (866)	4.6±0.5 (107)	5.0±0.7 (656)	5.8±1.6 (103)
Baseline artery diameter (mm)	3.51±0.66	3.25±0.61	3.58±0.64	3.78±0.66
FMD (%)	6.62±3.47	7.78±3.77	6.36±3.22	5.18±3.13
Current smoker [*n* (%)]	97 (4.7)	0	78 (6.3)	19 (7.7)
Diabetes [*n* (%)]	119 (5.7)	0	37 (3.0)	82 (33.2)
Dyslipidaemia [*n* (%)]	883 (42.5)	0	708 (56.8)	175 (70.9)
Blood pressure-lowering medication [*n* (%)]	216 (10.4)	0	0	216 (87.4)
Lipid-lowering medication [*n* (%)]	73 (3.5)	0	0	73 (29.6)
Glucose-lowering medication [*n* (%)]	81 (3.9)	0	0	81 (32.8)

Data are presented as mean±SD. For blood metabolites, *n* represents the number of available data within the respective subpopulation. For categorical data, data are presented as *n* (percentage of subpopulation). HDL – high-density lipoprotein cholesterol; LDL – low-density lipoprotein cholesterol; FMD – flow-mediated dilation.

Table 3: Age- and sex-specific percentiles of brachial artery FMD (%) and baseline artery diameter (mm) in healthy females and males, derived from the predictive equations.

Characteristics	Age (years)	Females							Males						
		2.5th	10th	25th	50th	75th	90th	97.5th	2.5th	10th	25th	50th	75th	90th	97.5th
FMD (%)	5	0.72	3.69	6.35	9.28	12.20	14.87	17.84	-0.15	3.03	5.89	9.02	12.15	15.00	18.18
	10	0.75	3.60	6.16	8.96	11.77	14.33	17.18	0.64	3.17	5.44	7.93	10.42	12.69	15.21
	15	0.78	3.51	5.96	8.65	11.34	13.79	16.52	0.84	3.08	5.08	7.29	9.50	11.50	13.74
	20	0.80	3.41	5.76	8.33	10.91	13.25	15.86	0.88	2.95	4.80	6.83	8.87	10.72	12.79
	25	0.83	3.32	5.56	8.02	10.47	12.71	15.21	0.87	2.82	4.57	6.49	8.41	10.16	12.10
	30	0.86	3.23	5.36	7.70	10.04	12.17	14.55	0.83	2.70	4.37	6.20	8.03	9.71	11.57
	35	0.88	3.14	5.16	7.39	9.61	11.63	13.89	0.79	2.58	4.19	5.96	7.73	9.34	11.13
	40	0.91	3.05	4.96	7.07	9.18	11.10	13.23	0.73	2.47	4.03	5.74	7.46	9.02	10.76
	45	0.93	2.95	4.77	6.76	8.74	10.56	12.58	0.68	2.37	3.89	5.56	7.23	8.75	10.45
	50	0.96	2.86	4.57	6.44	8.31	10.02	11.92	0.62	2.28	3.76	5.40	7.03	8.52	10.17
	55	0.99	2.77	4.37	6.12	7.88	9.48	11.26	0.57	2.19	3.65	5.24	6.85	8.3	9.93
	60	1.01	2.68	4.17	5.81	7.45	8.94	10.60	0.51	2.11	3.54	5.11	6.68	8.11	9.71
	65	1.04	2.59	3.97	5.49	7.02	8.40	9.95	0.46	2.03	3.44	4.98	6.53	7.94	9.51
	70	1.07	2.49	3.78	5.18	6.58	7.86	9.29	0.41	1.95	3.34	4.87	6.39	7.78	9.33
	75	1.09	2.40	3.57	4.86	6.15	7.32	8.63	0.36	1.88	3.25	4.76	6.26	7.64	9.16
	80	1.12	2.31	3.38	4.55	5.72	6.78	7.97	0.31	1.82	3.17	4.66	6.15	7.50	9.01
Baseline artery diameter (mm)	10	2.14	2.36	2.56	2.77	2.98	3.18	3.40	2.16	2.43	2.68	2.95	3.21	3.46	3.73
	15	2.27	2.56	2.82	3.11	3.39	3.65	3.94	2.49	2.84	3.14	3.48	3.82	4.13	4.47
	20	2.29	2.61	2.90	3.22	3.55	3.84	4.16	2.71	3.09	3.43	3.80	4.18	4.52	4.90
	25	2.28	2.63	2.94	3.28	3.62	3.93	4.28	2.86	3.26	3.62	4.02	4.42	4.78	5.18
	30	2.27	2.63	2.95	3.31	3.67	3.99	4.35	2.98	3.40	3.77	4.18	4.59	4.96	5.38
	35	2.25	2.63	2.96	3.33	3.69	4.03	4.40	3.08	3.50	3.89	4.31	4.73	5.11	5.53
	40	2.24	2.62	2.96	3.34	3.71	4.05	4.43	3.16	3.59	3.98	4.41	4.83	5.22	5.66

45	2.23	2.62	2.97	3.35	3.73	4.07	4.46	3.22	3.66	4.06	4.49	4.92	5.32	5.76
50	2.23	2.62	2.97	3.35	3.74	4.09	4.48	3.28	3.72	4.12	4.56	5.00	5.40	5.84
55	2.22	2.61	2.97	3.36	3.75	4.10	4.49	3.33	3.78	4.18	4.62	5.06	5.47	5.92
60	2.21	2.60	2.97	3.36	3.75	4.11	4.51	3.37	3.83	4.23	4.68	5.12	5.53	5.98
65	2.21	2.60	2.97	3.36	3.76	4.12	4.52	3.41	3.87	4.27	4.72	5.17	5.58	6.03
70	2.20	2.60	2.97	3.36	3.76	4.12	4.53	3.45	3.90	4.31	4.76	5.21	5.62	6.08
75	2.20	2.60	2.97	3.37	3.77	4.13	4.53	3.48	3.94	4.35	4.80	5.25	5.67	6.12
80	2.19	2.60	2.97	3.37	3.77	4.13	4.54	3.51	3.97	4.38	4.84	5.29	5.70	6.16

FMD – flow-mediated dilation

Figure 1

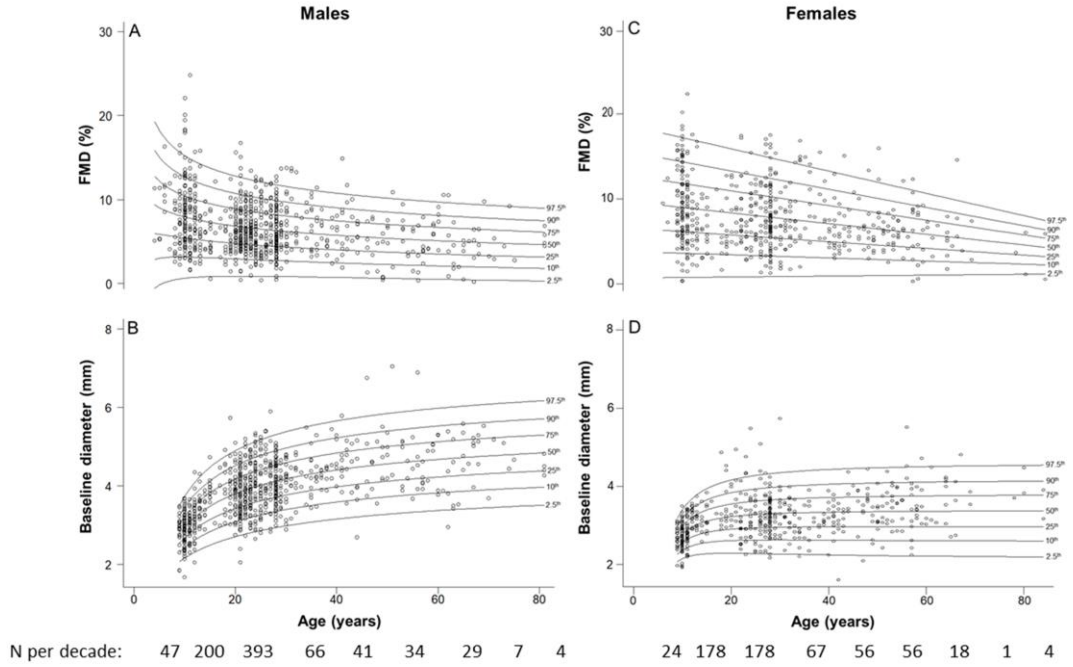


Figure 2.

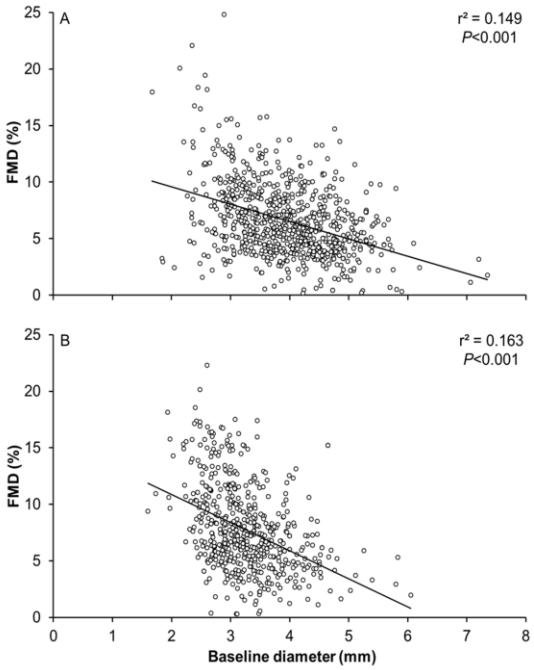
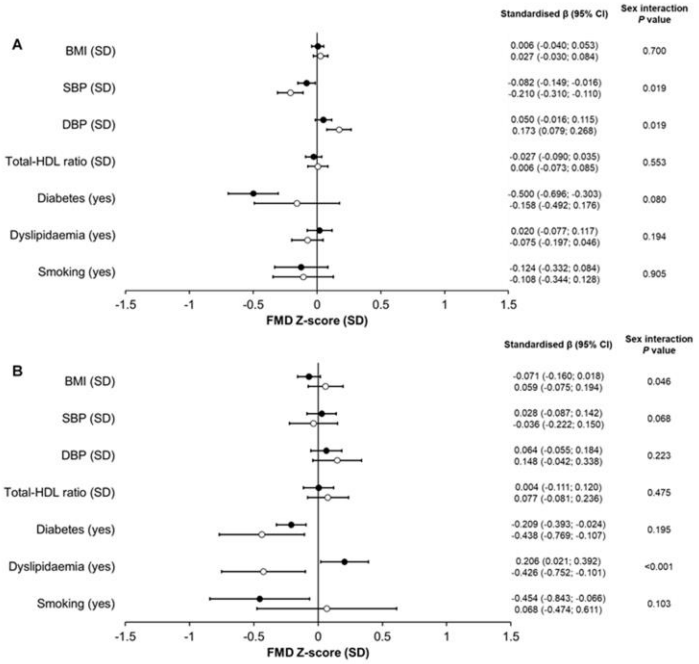


Figure 3



Graphical abstract