Methodological considerations for a vascular function test battery

Abbreviations: acetylcholine chloride (ACh); augmentation index (AIx); area under the curve (AUC); blood pressure (BP); cardiovascular disease (CVD); coefficient of variation (CV); coronary heart disease (CHD); diastolic blood pressure (DBP); digital volume pulse (DVP); endothelial dysfunction (ED); flow mediated dilation (FMD); intra-class correlation (ICC); laser Doppler imaging with iontophoresis (LDI); perfusion units (PU); pulse transit time (PTT); pulse wave analysis (PWA); pulse wave velocity (PWV); reflection index (RI); sodium nitroprusside (SNP); stiffness index (SI); systolic blood pressure (SBP); typical error (TE)

Abstract

There is a dearth of information regarding the reliability of non-invasive measures of vascular function taken in a single testing session. This study aimed to determine the test-retest reliability of a 'test battery' of vascular function measures; automated blood pressure (BP), laser Doppler imaging with iontophoresis (LDI), digital volume pulse (DVP), pulse wave velocity (PWV), augmentation index (AIx) measured by pulse wave analysis (PWA) and flow mediated dilation (FMD) taken within- and between-sessions. Measures were taken in 21 non-smoking males intra-session and again inter-session (one-week apart) to determine repeatability and reproducibility, respectively. There was moderate-excellent repeatability (ICC: 0.53- 0.93; CV = 2.2-18.1%) and reproducibility (ICC: 0.71-0.96; CV 1.9-14.2%) for BP, DVP-stiffness index, PWV, AIx, AIx normalised to heart rate (75 bpm), absolute and percentage FMD. Repeatability of the DVP-reflection index was moderate (ICC: 0.64; CV = 9.5%) but there was poor reproducibility (ICC: 0.17; CV = 15.1%). Moreover, the repeatability and reproducibility of the LDI measures ranged from poor-good (ICC: 0.31-0.84; CV = 28.4-36.7%). These data indicated that there was considerable variability in the repeatability and reproducibility of measurements of endothelial function and arterial stiffness taken in a battery of measurements, which needs careful consideration in future research designs.

INTRODUCTION

Cardiovascular disease (CVD) remains the most common cause of worldwide mortality, accounting for 45% of all deaths in Europe alone [62]. Hypertension is amongst the top five most robust modifiable risk factors for CVD [69] and blood pressure (BP) has been widely targeted by nutritional and exercise interventions [20,33]. In recent years the prognostic and clinical utility of measurements of endothelial function and arterial stiffness are becoming more widespread [2]. Moreover, because endothelial dysfunction and stiffening of the arteries precede hypertension, these measures collectively represent valuable nonpharmacological therapeutic targets in the prevention and management of CVD [23]. Currently, there are now a number of non-invasive methods, that when used in combination, can determine both morphological and functional changes in the micro- and macro-vascular system and these are being widely adopted in sports and exercise science and medical research [29,46,52].

Importantly, endothelial function has been demonstrated to be responsive to both acute and chronic nutritional interventions (e.g. polyphenols [28]) and exercise trials, regardless of modality [4]. However, the effects of such interventions on arterial stiffness is less clear. For example, polyphenol supplementation has been reported to improve arterial stiffness acutely, measured by pulse wave velocity (PWV), but not following chronic supplementation [13]. Conversely, chronic aerobic exercise training ($\geq$ 4 weeks) has been shown to improve arterial stiffness, measured by PWV and augmentation index (AIx), but only an improvement in AIx, was found after acute aerobic exercise [49]. The same authors found that acute resistance training might result in a transient reduction in arterial compliance [49] and these changes persisted in some [11], but not all chronic resistance training studies [3]. Furthermore, despite the purported inter-relationship between endothelial function and arterial stiffness [44], these measures are not always measured concurrently. In studies where the aforementioned are

measured in a test battery, improvements in all measures are not always observed [11,21]. These discrepancies demonstrate a clear benefit of being able to measure both endothelial function and arterial stiffness in a single testing session, i.e. as a 'test battery'. However, to be clinically useful, multimodal vascular function assessment needs to be repeatable, yet there is a paucity of data with regards to the reliability of multiple indices of vascular function taken in a single session [25,68].

Woodman, Kingwell, Beilin, et al. [68] reported the repeatability of a combination of common techniques used to assess peripheral and central arterial stiffness taken one week apart; however some methods exhibited increased variability when taken in combination rather than when taken independently. For instance, the coefficient of variations (CVs) for the stiffness index (SI) derived from digital volume pulse (DVP) are typically less than 10% in healthy individuals [38,39], but were higher (20.7%) when taken together with other measures [68]. This study highlights the importance of assessing the repeatability of multiple measurements taken in a single testing session. In this instance, the assessment of arterial stiffness measures are calibrated by peripheral blood pressure (BP) and are influenced by heart rate that would likely change depending on the testing duration [35]. In addition, commonly used methods to assess endothelial function such as laser Doppler imaging with iontophoresis (LDI) and flow mediated dilation (FMD) are reliant on nitric oxide (NO) release [14,64] and NO might alter arterial stiffness [58]. Therefore, measurements of endothelial function and arterial stiffness taken in a single testing session, which is becoming increasingly common [29,46,52], might exhibit different levels of repeatability than those taken independently. In this context, the aim of this study was to evaluate the reliability of a battery of non-invasive vascular function measures taken within- (repeatability) and between- (reproducibility) sessions.

METHODS

Participants and study design

A convenience sample of twenty one healthy, normotensive, non-smoking males aged 21-46 years (Mean ± SD; stature: 180 ± 6.5 cm; mass: 79.9 ± 15.1 kg; BMI: 24.7 ± 4.0 kg/m^2) took part in this study. The study was ratified by the Northumbria University Research Ethics Committee and was conducted in line with the journal's ethical standards [19]. Participants were required to visit the laboratory on two separate occasions. For all testing visits they were required to arrive fasted (≥ 10h), avoid alcohol, caffeine and strenuous exercise, any medication or nutritional supplements, 24 h before each testing day. Vascular function was measured by a battery of tests, as described below, which were taken twice in the same session (intra-session; repeatability [*n* = 20]) and again a week later (inter-session; reproducibility [*n* = 21]) to determine the short-term reliability. The order of measurement was; automated BP, LDI, DVP, PWV, AIx measured by pulse wave analysis (PWA) and FMD, which took approximately 1.5 h to measure. To reduce the potential for variation, participants were required to wear loose fitting garments; whilst testing was performed in the same sequence on each participant, by the same researcher on each occasion. As LDI and FMD are both reliant on the release of NO, these measures were done at the beginning and end of the sequence, in contralateral arms. All vascular measures were taken at the same time of day (8 am ± 1 h) in ambient temperature (22 ± 2°C) with the participants in the supine position following a minimum of 5 minute acclimation period at the beginning of each test battery.

Blood pressure

Blood pressure was measured using a validated, non-invasive, automated vital signs monitor (Carescape V100; Dinamap) in accordance with the British Hypertension Society Guidelines [51]. Supine brachial BP measurements were taken with the arm supported at the level of the heart. Based on expert consensus for diagnosing hypertension, there might be differences taken in different arms, therefore initially blood pressure was measured in both arms and the arm with the highest reading used for subsequent measurement [36,42]. Two subsequent BP measurements were taken in the respective arm, each separated by 1 min, and the mean systolic BP (SBP) and diastolic BP (DBP) was used for analysis [48].

Laser Doppler imaging with iontophoresis

All subjects had an acclimation period of at least 15 min before the measurements were taken. Two Perspex chambers (ION6, Moor Instruments Limited, UK) with an internal platinum wire electrode were attached to the skin using adhesive discs (MIC-1AD; Moor Instruments Limited, UK) on the ventral aspect of the left forearm and connected to the iontophoresis controller (MIC2, Moor Instruments Limited, UK). A 2.5 ml volume of 1% acetylcholine chloride (ACh) dissolved in 0.5% NaCl solution was placed in the anodal chamber and the same volume of 1% sodium nitroprusside (SNP) in 0.5% NaCl solution was placed in the cathodal chamber (all reagents acquired from Sigma-Aldrich, UK). Circular glass coverslips (MIC-ION6-CAP; Moor Instruments Limited, UK) were placed over each chamber to prevent loss of solutions. Current delivery was controlled by a laser Doppler imager Windows software v.6.1 (Moor Instruments Limited, UK). Measurement of skin perfusion was carried out using

a moor LDI2-IR laser Doppler imager (Moor Instruments Limited, UK). The scanner head was positioned 30 cm above the chambers. The laser beam was directed by a moving mirror in a raster fashion over both chambers. A total of twenty repeat scans were taken; the first set with no current to act as a control, then four scans at 5 µA, four at 10 µA, four at 15 µA and two at 20 µA, the final five scans were measured with no current. The back scattered light (flux) is measured in arbitrary perfusion units (PU) and area under the median flux PU vs. time curve over the twenty scans was calculated (using the trapezoidal rule) as a measure of microvascular response to ACh (endothelium-dependent vasodilation) and SNP (endothelium-independent vasodilation), respectively.

Digital volume pulse

A PulseTrace PCA 2 with a photoplethysmograph transducer transmitting infrared light at a wavelength of 940 nm (MicroMedical, Kent, UK) was placed on the index finger of the right hand and used to calculate the DVP stiffness index (DVP-SI) and DVP reflection index (DVP-RI). The DVP records the systolic and diastolic waveforms of the pulse by measuring infrared-light transmission through the finger. The DVP-SI (in m/s) is defined as the height of the subject divided by the time between the peaks of the first and the second wave, and it is correlated with the stiffness of large arteries [40,68]. The DVP-RI is the relative height of the second peak compared with the first and is associated vascular tone of small arteries [39].

Pulse wave velocity

The PWV was determined between carotid and femoral sites. A pencil-like pressure tonometer (SphygmoCor CPV system, ScanMed Medical, UK) was held at the base of the neck over the carotid artery and at the inguinal crease over the femoral artery on the right side of the body. The distance between carotid and femoral sites was measured and electrocardiogram gating permitted the time lapse between pulse waves at the carotid and femoral sites to be calculated. The PWV was calculated as the ratio of the distance between the two sites and the pulse transit time (PTT). Recordings were taken when a consistent signal was obtained with a high amplitude excursion. A minimum of two acceptable readings were obtained for PWV (PTT variation ≤ 10%) and the average used for analysis.

Pulse wave analysis

The PWA was recorded at the radial artery using the same pencil-like pressure tonometer and software (SphygmoCor CPV system, ScanMed Medical, UK). Peripheral pulse waveforms were recorded for a minimum 11 s and the aortic artery waveform determined using a generalised transfer function [47]. The AIx was calculated as the: $\frac{augmentaion\ pressure}{pulse\ pressure} \times 100$; where augmentation pressure is the difference between the "shoulder" of the wave and "peak" systolic pressure. Since AIx is influenced by heart rate [67], AIx normalised for a standard heart rate of 75 bpm (AIx@75) is also reported. The AIx@75 is only calculated when a participants heart rate is between 40 and 110 bpm, outside this range it is not computed by the software [57]. A minimum of two acceptable readings were obtained for PWA (Quality Index

≥ 80%; pulse height ≥ 80 units; pulse height variation ≤ 5%; and diastolic variation ≤ 5%) and the average used for analysis.

Flow mediated dilation

Flow mediated dilation of the brachial artery was determined according to previously established guidelines [12,60] using an ultrasound (HDI-5000 SONO CT ultrasound machine; Philips Medical System) and semi-automated computer software (Brachial Analyzer; Medical Imaging Applications). Briefly, a blood pressure cuff placed around the forearm (approximately 55 mm below the antecubital fossa), using a 7.5 MHz linear-array transducer, baseline images of the brachial artery were recorded for 60 s while the cuff remained deflated. The cuff was then inflated to 50 mmHg above systolic BP and following 5 min of occlusion, the pressure was rapidly released to allow reactive hyperaemia. Images were recorded for the last minute of occlusion and continuously for 3 min after release. Peak diameter was defined as the maximum diameter obtained post-occlusion. Average baseline diameter (60 s pre-occlusion) and the peak diameter were used to calculate the absolute change (peak diameter − baseline diameter) and percentage FMD: $\left(\frac{absolute\ change}{baseline\ dimater}\right) \times 100$.

Statistical analysis

All measures are expressed as mean ± standard deviation (SD) unless otherwise stated. Variables were tested for normality (Shapiro-Wilks test) and Log transformed where appropriate. For intra-session repeatability and inter-session reproducibility; relative

consistency of all vascular measures was assessed using intra-class correlation coefficient ($ICC_{3,1}$[22]), where an ICC >90, 0.75-90, 0.50–0.75 and <0.50 indicate excellent, good, moderate and poor reliability, respectively [30]. Typical error (TE) was calculated as the between-subject SD of the measurement pairs divided by $\sqrt{2}$ to represent absolute index of repeatability that encapsulates both the random and systematic error associated with each measurement [6]. For non-negative values [7], the within-subject variability was also assessed using coefficients of variation (CV) for each pair of measurements, determined by using the following equation: $(\frac{SD}{mean}) \times 100$. A CV ≤ 10% was considered good, 10–25% moderate and ≥ 25% poor reproducibility [59]. Paired samples *t*-tests or Wilcoxon's rank test were analysed for systematic error [5]. The LDI measures produced significant level of variability so these were further explored by Kruskal-Wallis test and post-hoc Wilcoxon's rank tests for perfusion response over the 20 scans. Regression analysis was also conducted for LDI measures because of evidence of unexplained systematic bias. All data were analysed using IBM SPSS statistics (v 24.0 for Windows; SPSS, Chicago, IL).

RESULTS

Intra-session repeatability

Results for repeatability of all measures are presented in Table 1. Intra-session SBP and DBP showed a moderate-good level of repeatability (ICCs ≥ 0.73; CV ≤ 3.5%). Repeatability of LDI perfusion response to SNP taken within-session was poor (ICC: 0.34; CV = 28.4%). The AUC for ACh displayed a good level of consistency (ICC: 0.84) however CV was high. There was also evidence of systematic error for intra-session LDI-ACh. The perfusion response for

ACh during the first measure was significantly higher for scans 7-17 compared to the second measure taken within-session (Figure 1). There was a significant positive correlation between measure 1 and measure 2 ($Y = 0.659x + 255.3$; $R^2 = 0.754$; $P < 0.0001$). The response to SNP did not differ over the 20 scans, nor was the correlation significant.

Repeatability of DVP-SI, DVP-RI, PWV, AIx and AIx@75 were moderate-excellent within-sessions (ICC ≥ 0.64; CV $\leq 9.5\%$). However, a consistent pattern of pulse waveforms could not be established in one participant, therefore all AIx data presented is for 19 participants. Additionally, three participants heart rates fell below 40 bpm and AIx normalised to 75 bpm was not calculated. Pulse wave velocity was highly repeatable within-session (ICC ≥ 0.89). For FMD two participants data was excluded from analysis, one due to substantial movement of the arm during deflation and the other because clear images of the artery could not be obtained ($n = 18$). Baseline diameter had excellent repeatability (ICC: 0.90; CV = 3.1%) and both FMD absolute and percentage FMD had a moderate level of repeatability (ICC ≥ 0.53).

Inter-session reproducibility

Inter-session reproducibility data is presented in Table 1. Both SBP and DBP displayed a similar level of reproducibility between-sessions (ICC ≥ 0.73; CV $\leq 3.4\%$). Both LDI measures (ACh and SNP) displayed poor reproducibility inter-session (ICC ≤ 0.40; CV $\geq 32.7\%$). The DVP-SI was highly reproducible, whereas DVP-RI had poor consistency between measures (ICC: 0.17) despite only a moderate level of within-subject variability (CV = 15.1%). The PWV and AIx was highly reproducible (ICC ≥ 0.83), however paired samples *t*-test suggested systematic bias between inter-session AIx measures ($P = 0.030$). No systematic error was found

for AIx@75, which remained highly reproducible between-sessions (ICC = 0.93). Absolute

FMD and percentage FMD displayed a moderate-good level of reproducibility (ICC ≥ 0.71)

and baseline diameter (ICC = 0.90; CV = 2.7%).

Table 1. Intra-session repeatability and inter-session reproducibility

	Measure 1 (mean ± SD)	**Intra-session**					**Inter-session**				
		Measure 2 (Mean ± SD)	TE (raw units)	CV (%)	ICC	*P* value	Measure 3 (Mean ± SD)	TE (raw units)	CV (%)	ICC	*P* value
Systolic BP (mmHg)	117 ± 6	116 ± 7	3.3	2.2	0.733	0.741	117 ± 6	3.0	1.9	0.753	0.385
Diastolic BP (mmHg)	65 ± 7	66 ± 7	2.9	3.5	0.812	0.113	64 ± 5	3.0	3.4	0.734	0.256
LDI-ACh (AUC; PU)	2125 ± 1494	1664 ± 1147	542	29.4	0.837	0.007	2459 ± 1543	1265	36.7	0.306	0.566
LDI-SNP (AUC; PU)	2262 ± 1271	1985 ± 1026	946	28.4	0.339	0.433	2488 ± 1150	943	32.7	0.395	0.274
DVP-SI (m/s)	5.5 ± 0.6	5.6 ± 0.5	0.2	2.7	0.876	1.000	5.5 ± 0.5	0.3	3.3	0.763	0.927
DVP-RI (%)	59.6 ± 13.3	61.0 ± 14.9	8.6	9.5	0.635	0.839	63.3 ± 13.1	11.9	15.1	0.173	0.337
PWV (m/s)	6.0 ± 0.9	6.0 ± 0.7	0.3	3.1	0.894	0.319	5.8 ± 1.1	0.4	6.2	0.825	0.149
AIx[†] (%)	-0.4 ± 11.3	-1.2 ± 12.0	3.2	−	0.926	0.156	-2.2 ± 11.3	2.5	−	0.955	0.030
AIx @75[†] (%)	-13.0 ± 11.9	-12.9 ± 12.5	3.4	−	0.920	0.483	-13.7 ± 11.6	3.1	−	0.932	0.541
FMD Abs (mm)	0.36 ± 0.09	0.39 ± 0.15	0.1	18.1	0.625	0.288	0.37 ± 0.11	0.1	12.3	0.745	0.539
FMD (%)	8.2 ± 2.2	8.8 ± 2.9	1.8	16.6	0.528	0.391	8.6 ± 2.8	1.3	14.2	0.714	0.408

[†]CV is not reported for AIx or AIx@75 as these produce both negative and positive values.

Blood pressure (BP); laser Doppler imaging with iontophoresis (LDI); acetylcholine chloride (ACh); sodium nitroprusside (SNP); perfusion units (PU); area under the curve (AUC); digital volume pulse (DVP); stiffness index (SI); reflection index (RI); pulse wave velocity (PWV); augmentation index (AIx); flow mediated dilation (FMD); absolute (Abs); typical error (TE); coefficient of varation (CV); intraclass correlaction (ICC)

For intra-session SBP, DBP, LDI-ACh and LDI-SNP ($n = 20$), DVP- SI, DVP-RI and AIx ($n = 19$); PWV, FMD abs and percentage FMD ($n = 18$); and AIx@75 ($n = 16$)

For inter-session SBP, DBP, LDI-ACh, LDI-SNP, DVP-SI and DVP-RI ($n = 21$); PWV and AIx ($n = 20$); FMD abs and percentage FMD ($n = 19$); and AIx@75 ($n = 17$)

Reasons for excluding data from analyses include: technical problems with equipment (DVP), no consistent pattern of waveforms (PWV and AIx), substantial arm movement (FMD) and heart rate <40 bpm (AIx@75)
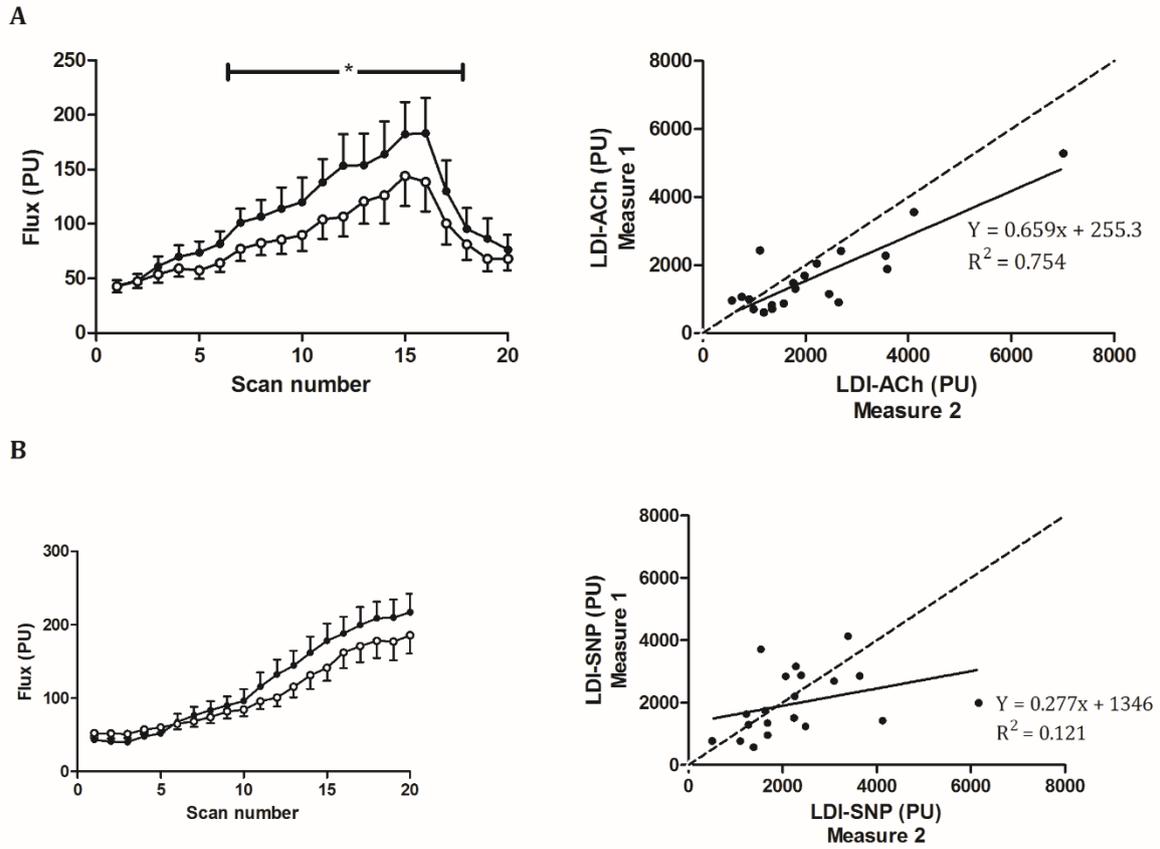
Figure 1. Differences between intra-session LDI perfusion response for individual scans (left; Mean ± SEM ●= Measure 1 and ○ = Measure 2) and regression analysis for area under the curve data (right) for; (A) ACh and (B) SNP, respectively (n = 20). Dashed line represents line of agreement. * denotes significant differences between measurement pairs (P < 0.05).

DISCUSSION

The aim of the current study was to comprehensively determine the repeatability and reproducibility of a battery of vascular function tests. The main finding of this study was that taken together, there is a considerable range of variability regarding the repeatability and reproducibility (poor-excellent; Table 1) of non-invasive measures of endothelial function and arterial stiffness, which holds important information for research designs. Before comparing the reliability of a vascular function test battery to measures taken independently, there is a number of important factors relating to the technical and biological variability that need to be considered. Firstly, there are extensive differences between the degree of skill needed to perform the measures reported in the current study [2], and therefore to reduce the amount of variability several trained researchers might be needed to carry out each measurement, but this is an unlikely scenario in a single site research study. Secondly, the testing duration to do multiple methods can be much longer than a single measurement, which increases participant burden and the potential for additional stress that might influence vascular function variables [50]. Lastly, the close interplay between the BP, endothelial function and arterial stiffness could also contribute to sources of variability. With this in mind, the automated BP was found to be measured with an adequate level of repeatability and reproducibility (Table 1). The between-day reproducibility appears to be consistent with previous reports of resting SBP and DBP [56]. However, in this scenario non-invasive measurements of arterial stiffness were more reliable (within- and between-sessions) than methods used to assess endothelial function in the test battery.

Endothelial dysfunction is a hallmark of the early development of atherosclerosis and CVD [9,16]. Moreover, because of the systemic nature of endothelial function, dysregulation of microvascular endothelial function is thought to be a surrogate marker of coronary endothelial

function [18]. As such, the micro-vascular endothelial function, as measured by LDI and DVP-RI, and macro-vascular endothelial function measured by FMD, are important therapeutic targets [1]. With regards to micro-vascular endothelial function, laser Doppler methods are increasingly popular, because they are relatively easy to perform and do not require extensive operator experience [53]. That said, there are a number of extraneous factors that introduce variability into these measures, especially when used in conjunction with iontophoresis [32]. Moreover, despite the putative clinical utility of these methods, there is currently no standardisation of methods and/or data analysis [53,64]. In the current study an iontophoresis protocol that had previously been reported to be reliable was adopted [26] and is presented as AUC (Table 1) as this is commonly used in randomised controlled trials , [27,29,34]. In contrast to our findings, Jadhav *et al.* [26] reported good reproducibility of the AUC for both ACh and SNP taken 8 weeks apart in females with cardiac syndromes, which might be attributable to the longer rest period before the measure, correction for skin resistance and the different populations between studies. Nonetheless, the findings in the current study are in line with others, in that, laser Doppler methods in conjunction with iontophoresis have been reported to produce high day-to-day CVs [31,41], low ICCs [61] and SNP perfusion response can be less reproducible compared to ACh [26,31,61].

It is particularly noteworthy that a second measurement taken within the same testing session produced a lower endothelial-dependent (ACh) perfusion response; despite identical placement of the Perspex chambers, limiting variations and spatial differences in capillary density. Although the reason for this remains unknown, it might be related to redistribution of endothelium derived hyperpolarizing and/or other vasodilators factor as a result of the FMD taken in the same session [8,43]. Further analysis demonstrated that during the protocol, iontophoresis ≥ 10 µA and peak perfusion was lower between measurements intra-session (Figure 1a). The fact that both peak perfusion and AUC might be different within-session,

despite demonstrating a good level of consistency and significantly related, needs consideration and attention in future studies; namely because the acute effects of an intervention might be masked. Other methods to assess microvascular endothelial function could be more repeatable and reproducible [32], but this was outside of the remits of the current study.

The DVP method is quick-to-perform, operator-independent and the DVP-RI is strongly related to the vascular tone of small arteries [39] but there are limited published data concerning the reproducibility of DVP-RI. Notwithstanding, in agreement with others, the current study found that RI had higher intra-individual variability and poorer reliability within and between-sessions than SI [39]. The current study also found better intra-session reliability for DVP-RI than inter-session, which has been reported elsewhere [39,55]. The within-subject variability (15.1%) is also similar to those reported by Millasseau and colleagues [39] who found inter-session CVs of 13.8% in a very small cohort of 8 healthy males. However, in the current study the low ICC demonstrated poor reproducibility between-sessions. On the other hand, macro-vascular endothelial function was assessed using FMD which showed moderate repeatability and reproducibility, despite requiring more operator skill than LDI and DVP-RI. Moreover, FMD produced a comparable level of technical and biological variation as previous studies (median CV of 17.5% [66] and TE ranges from 0.4-4.8 in healthy individuals [15]).

Arterial stiffness, which is an independent predictor of CVD [37], was measured in the current test battery by several different methods due to an absence of a true 'gold standard' [68]. Firstly, we measured DVP-SI, which has been associated with stiffness of the large arteries [38], vascular aging [39] and risk of CVD [17,65]. The current study demonstrated that the intra- and inter-session DVP-SI can be measured consistently with very little variability (ICC ≥ 0.76; CV $\leq 3.3\%$), which is supported by the literature [38,39]. In the current study, arterial stiffness was also measured by PWV and PWA, which were both highly reproducible (ICC ≥ 0.83) within- and between-sessions, and compare favourably with previous research [63,68].

Notably, there was also evidence of systematic bias between-days for AIx, although all other statistical tests supported excellent repeatability and reproducibility. Conversely, AIx normalised for heart rate (75 bpm) was not different between-session, suggesting HR might have contributed to the observed systematic bias, however it should be acknowledged that fewer individuals contributed to AIx@75 analysis ($n = 17$). This is because some participants HR fell below 40 in the supine position, which might pose a problem and minimises the utility of this measure in athletic or bradycardic populations.

This study has various strengths such as the use of well-established, commonly used methodologies, but it is conceivable that other methods used to assess endothelial function might have been more reproducible. There are several other limitations that warrant discussion, firstly, the population in the current study were healthy, non-smoking young males, which has limited application to wider populations, but nonetheless was importantly not confounded by the presence of disease or medications and hence represent a relatively stable population to examine vascular function. Moreover, different populations might produce more reliable results; specifically AIx has been shown to be less reliable in young healthy individuals due to the different waveforms [10,24,68]. Secondly, as recommended, we used a minimum of 5 minutes acclimation period [45,48]; however it should be acknowledged that a longer acclimation period might have provided a more stabilised BP reading [54] and perhaps more reproducible results. Nonetheless, this would have increased the testing period and participant burden. Lastly, although our re-test is after a longer period than previous studies [31,39] this research represents short-term repeatability, therefore future studies might want to consider the use of a battery of non-invasive cardiovascular measures taken longitudinally.

The current research demonstrates considerable inconsistencies in the repeatability and reproducibility of non-invasive measures of structural and functional vascular health. Here we report that BP, indices of arterial stiffness and macro-vascular endothelial function can be taken

in a battery with adequate reliability in healthy males. However, measurements of microvascular endothelial function (LDI and DVP-RI) taken in conjunction with the other measures demonstrates poor reproducibility between-sessions. This study highlights the importance of testing the reliability of multiple measures taken in a single session, which needs careful consideration in research designs.

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