Arterial Wall Shear Rate Response to Reactive Hyperaemia is Markedly Different between Young and Older Humans

Authors:
Kunihiko Aizawa,[1] Alessandro Ramalli,[2,3] Sara Sbragi,[4] Piero Tortoli,[2] Francesco Casanova,[1] Carmela Morizzo,[4] Clare E Thorn,[1] Angela C Shore,[1] Phillip E Gates,[1] Carlo Palombo.[4]

Affiliations:
[1] Diabetes and Vascular Medicine Research Centre, NIHR Exeter Clinical Research Facility, University of Exeter College of Medicine and Health, Exeter, UK
[2] Department of Information Engineering, University of Florence, Florence, Italy
[3] Laboratory of Cardiovascular Imaging and Dynamics, Department of Cardiovascular Sciences, KU Leuven, Leuven, Belgium
[4] Department of Surgical, Medical, Molecular Pathology and Critical Care Medicine, University of Pisa, Pisa, Italy

Running Head:
Wall shear rate during FMD.

Author Contributions
KA, PT, ACS, PEG and CP contributed to the conception and design of research. KA, SS, FC, CM, CET and PEG performed experiments. KA, AR and PEG analysed data. KA, AR, PT and PEG interpreted results of experiments. KA and AR prepared figures. KA, AR and PEG drafted manuscript. KA, AR, SS, PT, FC, CM, CET, ACS, PEG and CP edited and revised manuscript. KA, AR, SS, PT, FC, CM, CET, ACS, PEG and CP approved final version of manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Corresponding Author:
Kunihiko Aizawa, PhD
Diabetes and Vascular Medicine Research Centre
University of Exeter Medical School
NIHR Exeter Clinical Research Facility
Barrack Road, Exeter
EX2 5AX, UK
+44 1392 403081 (TEL)
+44 1392 403027 (FAX)
k.aizawa@exeter.ac.uk

KEY POINTS:

- The vasodilatory response to reactive hyperaemia is impaired with advancing age, but it is unclear if this is because of an altered wall shear rate (WSR) stimulus or an altered flow-mediated dilatation (FMD) response.
- Using new technology that allows detailed WSR measurement, we assessed the WSR-FMD response in healthy older people.
- Our data show that older people have a markedly altered and diminished WSR response to reactive hyperaemia compared with young people, but reduced WSR alone does not fully explain reduced FMD.
- In young people WSR appears to be coupled to FMD, but by age ~65 years, the arterial vasodilatory response has begun to uncouple from the WSR stimulus.
- These findings point to the importance and utility of comprehensively characterising the WSR-FMD response when using reactive hyperaemia to assess vascular function, as well as giving new insight into the age-related alteration in vascular function.

ABSTRACT:

The vasodilatory response to reactive hyperaemia is impaired with age, but it is unknown if this is because of an altered wall shear rate (WSR) stimulus or an altered flow-mediated dilatation (FMD) response to the WSR stimulus. Inherent difficulties in measuring blood flow velocity close to the arterial wall have prevented detailed assessment of the WSR-FMD response. Using an enhanced multi-gate spectral Doppler ultrasound system [Ultrasound Advanced Open Platform (ULA-OP)], we aimed to produce new data on the WSR-FMD relationship in healthy older adults. Sixty healthy people [28 young (27.5±5.5yrs); 32 older (64.9±3.7yrs)] underwent FMD assessment. Raw data were post-processed using custom-designed software to obtain WSR and diameter parameters. The data revealed that older people have a much altered and diminished WSR response to reactive hyperaemia compared with younger people [e.g. WSR peak: 622 (571-673) vs 443 (396-491) 1/s in young and older respectively; $p<0.05$]. However, reduced WSR alone does not appear to fully explain the reduced FMD response in older people, as associations between WSR and FMD were few and weak. This was in contrast to young adults, where associations were strong. We conclude that WSR during FMD is much altered and diminished in older people, and that

there appears to be an 'uncoupling' of WSR from FMD in older people that may reflect a loss of precision in the reactive hyperaemia stimulus-response relationship. These findings also point to the importance and utility of comprehensively characterising the WSR-FMD response when using reactive hyperaemia to assess vascular function.

KEY WORDS:

Ageing, Brachial artery, Doppler ultrasound, Endothelium, Flow-mediated dilatation, Vasodilatation.

INTRODUCTION:

Blood vessels are important in mammalian ageing because they permeate most tissues and are vital to most cells. In human cardiovascular ageing, impaired function of the vascular endothelium – the interface between tissue and blood - is apparent in the absence of concomitant cardiovascular risk factors (Seals *et al.*, 2011). A widely used physiological marker of arterial endothelial function is brachial artery flow-mediated dilatation (FMD). This is a physiological challenge that provokes an endothelium-dependent vasodilatation (Melkumyants *et al.*, 1989) that is predominantly nitric-oxide mediated (Green *et al.*, 2014). The FMD technique uses reactive hyperaemia to increase arterial wall shear rate (WSR), a powerful mechanical stimulus for endothelial nitric oxide synthesis and subsequent vasodilatation. Evidence to date shows that the vasodilatory response to augmented WSR is impaired with advancing age (Lakatta & Levy, 2003; Donato *et al.*, 2007; Gates *et al.*, 2007), but it is unclear whether this is because of an altered WSR stimulus or an altered FMD response to stimulus (Tortoli *et al.*, 2006; Aizawa *et al.*, 2018).

An altered FMD response to a similar WSR stimulus in older adults is usually interpreted as conduit artery endothelial dysfunction. However, if the WSR stimulus itself is different in older adults, vascular impairment may be more diffuse and not solely attributable to the function of the conduit artery endothelial cells. The limitation here is knowing the WSR stimulus, because of the inherent difficulties of measuring blood flow velocity close to the arterial wall. This is typically attempted using conventional Doppler ultrasound in combination with a series of assumptions about haemodynamic behaviour. However, three different approaches to measuring WSR have shown that these assumptions are not always valid (Silber *et al.*, 2005; Tortoli *et al.*, 2006; Gates *et al.*, 2018). We have recently shown that WSR during reactive hyperaemia is complex and nuanced (Aizawa *et al.*, 2018). To do this, we used an enhanced multi-gate spectral Doppler system (Tortoli *et al.*, 2011; Ramalli *et al.*, 2014; Ramalli *et al.*, 2018) that measures blood velocity close to the vessel wall and from near-to-far wall. This provides a continuous velocity profile across the vessel diameter (Tortoli *et al.*, 2006) and also measures diameter continuously. This allows the complete FMD stimulus-response relationship to be obtained simultaneously and continuously over a long period. In doing so, the system overcomes many of the limitation of using a single pulsed-wave Doppler sample-gate and provides detailed, continuous WSR-diameter data.

Using this method in young people, we found that there was considerable heterogeneity in the blood flow velocity profile during reactive hyperaemia. In particular, we observed the assumed symmetrical parabolic shape of the flow velocity profile to vary greatly, with asymmetric, blunt and M-shaped profiles being common. This variability occurred under different flow-conditions, within the cardiac cycle, and between individuals. This points to the importance of being able to detect flow velocity at different spatial points in the vessel in order to extract local velocities and accurately estimate WSR continuously during reactive hyperaemia. We found that WSR area under the curve until its return to baseline was the strongest predictor of brachial artery FMD, reinforcing the need for continuous measurement (Aizawa *et al.*, 2018). We also identified a number of new WSR magnitude and time-course variables that have not been measured previously and that more comprehensively characterize the WSR-FMD response. However, the WSR-FMD relationship in older people is unknown.

In this study, we aimed to produce new data on the WSR-FMD relationship in a cohort of healthy older adults. First, we wanted to conduct a detailed analysis of the WSR-FMD response in healthy older adults and establish a first point of reference for WSR variables during reactive hyperaemia in this cohort. Second, we wanted to determine whether WSR parameters during reactive hyperaemia are different in young compared with older people. Third, we wanted to know if there are associations between WSR parameters during reactive hyperaemia and the FMD response. Finally, we wanted to determine any contribution of brachial artery stiffness to the WSR-FMD response so that we had a comprehensive assessment of reactive hyperaemia - the influence of local artery stiffness on FMD in healthy, older people is unknown, but in patients with cardiovascular risk, increased brachial artery stiffness affects the magnitude of the FMD response (Witte *et al.*, 2005). Overall, we wanted to comprehensively measure the WSR-FMD response to produce evidence essential to understanding the mechanisms of vascular dysfunction with age.

METHODS:

Ethical Approval

The UK National Research Ethics Service South West Committee (10/H0203/29) and the institutional ethics committee "Comitato Etico di Area Vasta Nord Ovest" (3146/2010) approved all study procedures and written informed consent was obtained from all participants. The study conformed to the standards set by the latest revision of the Declaration of Helsinki, except for registration in a database.

Participants

Sixty people participated in this study. Of these, 28 were young (age≥20, <40 yrs; 16 from Exeter and 12 from Pisa) and 32 were older (defined as age≥60 yrs; all from Exeter). All participants were healthy, without hypertension, type 2 diabetes, dyslipidaemia or overt cardiovascular disease. No one took medications that modify cardiovascular risk factors. Participants arrived in our temperature-controlled laboratories after an overnight fast. They had blood samples drawn for biochemical analysis, consumed a standardized meal and rested for 20 min before initiation of the study protocol.

Experimental Procedures

Brachial artery FMD was assessed as previously described (Gates *et al.*, 2007; Gilchrist *et al.*, 2013; Aizawa *et al.*, 2016; Aizawa *et al.*, 2018) and in accordance with established guidelines (Corretti *et al.*, 2002; Thijssen *et al.*, 2011). Briefly, participants lay supine on an examination bed with the right arm fixed in position and immobilised using a positioning pillow on a sturdy, metal table. A small blood pressure cuff was placed around the proximal part of the forearm. Using the Ultrasound Advanced Open Platform [ULA-OP; Microelectronics Systems Design Laboratory, University of Florence, Italy (Boni *et al.*, 2012)] with a high-frequency linear array transducer (LA523; Esaote SpA, Florence, Italy), B-mode ultrasound images and multi-gate Doppler velocity data from the brachial artery were obtained as previously described (Ramalli *et al.*, 2014; Ramalli *et al.*, 2015; Aizawa *et al.*, 2018). Once the optimal ultrasound image was obtained, the transducer was carefully clamped using a custom-designed transducer holder to prevent movement during the procedure. Baseline brachial artery image and blood velocity were recorded for 60 s. Once obtained, the forearm cuff was rapidly inflated to 250 mmHg to occlude forearm blood flow for 5 min (AI6, Hokanson, Bellevue, WA), and at 5 min, the cuff was rapidly deflated to induce reactive hyperaemia. Recording of brachial artery image and blood velocity was re-started 30 s before cuff

deflation (Aizawa *et al.*, 2016) and continued until 5 min after deflation. A sub-set of participants in Exeter (young, n=14; older, n=29) had endothelium-independent dilatation assessed using sub-lingual glyceryl trinitrate (GTN) spray (0.4 mg) after a 15-min rest. A 60 s recording was started 9 min after administering the spray. We have previously found that measurement between 9 and 10 minutes after GTN administration captures maximal endothelial-independent vasodilation (unpublished observation). All data processing and subsequent data analysis were conducted in a blinded fashion.

Measurements of wall shear rate and diameter

As previously described (Aizawa *et al.*, 2018), a custom-designed signal elaboration system was used to extract detailed WSR and diameter parameters. The WSR magnitude, time-course and kinetics parameters were extracted by one of the investigators (AR) and were those identified by another investigator (PEG) as likely to be the most relevant in the first instance. **Table 1** lists all the parameters for WSR and diameter that were extracted in this study. A schematic outline of the WSR parameters is presented in the upper panel of Figure 1 and an outline of the arterial diameter parameters is presented in the lower panel.

WSR magnitude parameters

Seven WSR *magnitude* parameters were analysed: 1) WSR at baseline, 2) WSR during low-flow, 3) WSR at peak hyperaemia (WSR peak), 4) absolute WSR increase from baseline (WSR Δ), 5) percentage WSR increase from baseline (WSR %Δ), 6) area under the WSR curve until time to peak dilatation (WSR aucttp), and 7) area under the WSR curve (WSR auc), measured between cuff release and the point at which WSR returned to the baseline value.

WSR time-course parameters

Two WSR *time-course* parameters were analysed: 1) time to peak WSR (WSR Tp) and 2) time to return to baseline WSR (WSR Tb).

WSR kinetics parameters

In our previous work, two distinct patterns of WSR response to reactive hyperaemia were identified, a mono-phasic and bi-phasic pattern (Aizawa *et al.*, 2018). In the mono-phasic pattern there is a single steep increase to peak WSR and in the bi-phasic pattern there is an

initial steep increase followed by a gradual increase to peak WSR (see **Figure 1** which shows a bi-phasic pattern). We categorised responses into these two patterns and measured the steepness of the slope of WSR increase during reactive hyperaemia to derive two WSR *kinetics* parameters: 1) first slope of WSR increase during hyperaemia (WSR SL1, an initial steep increase), 2) the second slope of WSR increase during hyperaemia (WSR SL2, the continued, gradual increase in WSR that is sometimes observed after the initial steep increase and, when present, identified subjectively by AR). In line with our previous work (Aizawa *et al.*, 2018), we defined the mono-phasic and bi-phasic patterns of WSR increase as: 1) mono-phasic if the peak WSR value was reached with a single, continuous steep increase (WSR SL1) only; 2) bi-phasic if the peak WSR value was reached with an initial steep increase (WSR SL1) followed by a gradual second increase (WSR SL2). The steepness of the slope was measured as WSR $1/s^2$.

Arterial diameter magnitude parameters

Three diameter *magnitude* parameters were analysed: 1) baseline diameter, 2) absolute diameter increase from baseline, and 3) percentage diameter increase from baseline.

Arterial diameter time-course parameters

Two diameter *time-course* parameters were analysed: 1) time to peak diameter, and 2) time to return to baseline diameter, taken as the time between the start of reactive hyperaemia and the time when diameter returned to its baseline value or reached a plateau that was sustained until the end of data collection.

Measurements of brachial artery stiffness

Brachial artery stiffness parameters - PWV and β-stiffness index - were calculated from diameter, distension and brachial artery blood pressure. PWV was obtained using the distensibility coefficient (DC) (Henry *et al.*, 2003) and was calculated as $PWV=1/(\sqrt{DC}\times\rho)$, where ρ is blood density assumed to be 1060 kg/m^3 and m/s as the unit (van der Heijden-Spek *et al.*, 2000). β-stiffness index was calculated as $\beta=[\ln(SBP/DBP)/(\Delta D/D)]-[\ln(DBP/Pref)]$, where ln is the natural logarithm, SBP is systolic blood pressure, DBP is diastolic blood pressure, ΔD is diameter distension, D is baseline diameter, Pref is a

reference pressure set at 100 mmHg and arbitrary unit as the unit (Hayashi *et al.*, 1980; Spronck *et al.*, 2017). Blood pressure was obtained from the left arm before the procedure started, and brachial artery stiffness parameters were calculated at baseline.

Statistical analysis

Data are presented as means±SD, means (95% confidence intervals) or numbers. A Chi-square test was used to examine the differences in categorical variables. An analysis of covariance (study centre as a covariate) was also used to examine the differences in variables between young and older groups. Pearson correlation and partial correlation analyses between WSR parameters and diameter changes (absolute and percentage) were performed separately for young and older cohorts. The study centre was included as a control variable in the partial correlation analysis. Additionally, mean arterial pressure and heart rate were also included as control variables in the partial correlation analysis between PWV and diameter changes (absolute and percentage). A log-transformation was used for variables with skewed distribution before statistical analysis. All statistical analysis was conducted using IBM SPSS Statistics 24 (IBM, Armonk, NY). Significance was set at $p<0.05$.

RESULTS:

Participants' characteristics

Selected characteristics of the study participants are presented in **Table 2**. Compared with the young cohort, the older cohort had greater body mass index, higher total cholesterol and LDL cholesterol, higher haemoglobin A1c and fasting glucose concentration, higher systolic and diastolic blood pressure, and lower resting heart rate (all $p<0.05$).

WSR parameters during brachial artery FMD: Reference values in older people

Table 3 shows WSR and diameter parameters during brachial artery FMD and GTN-mediated dilatation from the older cohort. During cuff occlusion WSR fell to ~35 % of the baseline value. Following cuff release, peak WSR was about nine-times greater than WSR at baseline and 26-times greater than WSR during cuff-occlusion. Peak WSR was reached after ~12 s and was relatively homogenous between participants. The time taken to reach peak arterial diameter averaged ~68 s, but there was substantial inter-individual variability. There was also a lot of variability in the time taken for WSR and arterial diameter to return to

values measured at baseline. We observed mono-phasic WSR patterns in 19 of the older people and bi-phasic patterns in 13.

WSR parameters during brachial artery FMD: Comparison between young and older people

WSR parameters during brachial artery FMD are shown in **Table 4**. **WSR magnitude parameters:** WSR magnitude parameters at baseline and during low-flow were similar between the young and older cohorts. During reactive hyperaemia, WSR peak was ~40% higher in the young compared with the older cohort ($p<0.05$). The absolute WSR increase from baseline was ~45% greater ($p<0.05$) and total WSR stimulus, expressed as WSR aucttp and WSR auc, was ~40% greater in the young compared with the older cohort (both $p<0.05$). **WSR time-course parameters:** In both cohorts, peak WSR was reached after ~12 s and WSR returned to baseline values after ~110 s. **WSR kinetics parameters:** After cuff-deflation, the initial WSR increase (WSR SL1) was ~40% steeper in the young compared with the older cohort. A bi-phasic WSR pattern was evident in a similar number of young and older participants (14 vs 13 people, 50.0 % vs 40.6% of the cohort, respectively). Where there was a second WSR increase (WSR SL2), the slope was nearly twice as steep in the young people.

Diameter parameters during brachial artery FMD: Comparison between young and older people

Diameter, magnitude and time-course parameters between cohorts during brachial artery FMD assessment are presented in **Table 4**. **Diameter magnitude parameters:** Baseline brachial diameter was similar between cohorts. Both absolute and percentage diameter changes following reactive hyperaemia were significantly greater in the young compared with the older cohort ($p<0.05$). **Diameter time-course parameters:** Time to peak diameter was not different between cohorts. It took ~35s longer for brachial artery diameter to return to baseline in the young compared with the older cohort ($p<0.05$).

WSR and diameter variables during brachial artery glyceryl trinitrate-mediated dilatation: Comparison between young and older people

The results from brachial artery GTN-mediated dilatation are shown in **Table 5**. WSR before and during the assessment was similar between cohorts. Absolute and percentage diameter changes during the assessment were not different between cohorts (**Table 5**).

Associations of WSR parameters with diameter change

The results of partial correlation analyses between WSR parameters and diameter changes during brachial artery FMD are shown in **Table 6**. In the young cohort, WSR peak, WSR Δ, WSR aucttp, WSR auc and WSR SL1 were significantly associated with both absolute and percentage diameter changes (all $p<0.05$). In the older cohort, only WSR %Δ showed an association with percentage diameter change ($p<0.05$); no other variables showed any associations with diameter changes in this cohort.

Associations between baseline brachial artery diameter and WSR-FMD

The results of partial correlation analysis between baseline brachial artery diameter and FMD parameters (diameter and WSR) are shown in **Table 7**. In the young cohort, WSR peak, WSR Δ, WSR aucttp and WSR auc showed significant, inverse associations with baseline brachial diameter (all $p<0.05$). In the older cohort, WSR Δ, WSR %Δ, WSR SL1 and WSR SL2 showed significant, inverse associations with baseline brachial diameter (all $p<0.05$). Percentage diameter change was significantly and inversely associated with baseline brachial diameter in both cohorts (both $p<0.05$).

Brachial artery stiffness and its association with diameter changes

Young and older cohorts had similar values for PWV [9.6 (8.7-10.4) vs 9.5 (8.7-10.2) m/s, $p=0.834$] and β-stiffness index [17.9 (14.7-21.1) vs 17.2 (14.3-20.2) au, $p=0.779$]. **Table 8** shows the results of partial correlation analysis between brachial artery stiffness parameters and diameter changes during brachial artery FMD and GTN-mediated dilatation assessments in each cohort. In the young cohort, no association was observed between brachial artery stiffness parameters and diameter change obtained during FMD and GTN-mediated dilatation assessments. Similarly, there was no association in the older cohort, although there was a trend for an association between PWV and percentage diameter change during GTN-mediated dilatation assessment ($p=0.08$).

DISCUSSION:

The main finding of this study is that older people have an altered and much diminished WSR response to reactive hyperaemia during FMD. However, reduced WSR alone does not appear to be sufficient to fully explain the reduced FMD response seen in older people. In our cohort of older people, there were few associations between WSR parameters and the FMD response, and those that did exist were weak. This was in contrast to younger adults, where associations between the WSR stimulus and the FMD response were strong. Our data indicate that in young people WSR is coupled to FMD, but by age ~65 years, the arterial vasodilatory response has begun to uncouple from the WSR stimulus during hyperaemia.

Diminished WSR during reactive hyperaemia in older people

Age diminishes hyperaemic WSR magnitude and kinetics. There were no differences between young and older people in baseline or low-flow WSR, but all five WSR magnitude parameters during reactive hyperaemia were substantially lower in the older cohort. The increase in WSR following cuff-release was also more gradual and the slope less steep (WSR SL1, and WSR SL2 when present) in older people. Although the time taken to reach peak WSR was not different from the young cohort, we note that in the same period of time (~12s), older people were unable to reach a similar magnitude of WSR. Similarly, the time for WSR to return to baseline was similar between younger and older people. Overall, our data show that WSR magnitude and rate of change during hyperaemia is markedly reduced in older people.

Association between diminished hyperaemic WSR magnitude and reduced FMD. Consistent with many previous studies [for review, see: (Seals *et al.*, 2006; Seals *et al.*, 2011)], we found that brachial artery diameter change was lower in older people in response to hyperaemia. In this study, we were able to show that WSR during hyperaemia was also diminished and, consequently, the stimulus for brachial artery dilatation was reduced. The function of vasodilatation during reactive hyperaemia is to normalise WSR and thereby protect the vessel wall. It might be, therefore, that the lower vasodilatory response in older people is appropriate for the diminished WSR stimulus. That is, diminished WSR during reactive hyperaemia means that the reduced FMD response observed in older people is the

appropriate response to the (reduced) WSR stimulus, rather than a consequence of a dysfunctional brachial artery vasodilatory response.

Reduced WSR during FMD means that there is less stimulus for vasodilation, but further analysis of our data reveal that the explanation for reduced FMD may be more complex than a diminished WSR stimulus alone. In younger people, the WSR response was strongly associated with brachial artery dilatation, suggesting an intact stimulus-response relationship. However, in the older cohort, associations between the hyperaemic WSR parameters and FMD were absent or very weak. The strongest predictors of FMD in young people were not associated with FMD in the older cohort, and the only significant association was between WSR percentage change and percentage diameter change. Although our data show that in older people the brachial artery responds to an increased WSR, the stimulus-response relationship appears to be, to an extent, uncoupled.

Uncoupling of the WSR stimulus from the FMD response suggests that the vascular endothelium has lost the ability to produce a precise normalisation of WSR. Many factors could exert an influence on the ability of an artery to dilate when stimulated, including local arterial stiffness and the sequence of events from mechano-transduction of the WSR stimulus to endothelium-derived nitric oxide synthesis, hyperpolarisation, and the overall bioavailability of endothelial-derived relaxing and contracting factors to smooth muscle cells.

Brachial artery stiffness does not influence age-related differences in WSR-FMD. Increased brachial artery stiffness in older people could alter the haemodynamic and vasodilatory response to reactive hyperaemia. Stiffening of the large elastic arteries (Avolio *et al.*, 1983) and an overall increase in systemic vascular impedance (Mazzaro *et al.*, 2005) is a prominent feature of vascular ageing and is influenced by health, nutrition and lifestyle factors (Gates *et al.*, 2004; Gates *et al.*, 2005; Seals & Gates, 2005; Gates & Seals, 2006). If the brachial artery also becomes stiff with age, it could, conceptually (Witte *et al.*, 2005), mechanically resist vasodilatation and reduce the FMD response. In this study, the integrated ultrasound system allowed us to use continuous diameter tracking of the brachial artery to obtain distention data, and from this, calculate PWV and β-stiffness index during the baseline data

acquisition. We found that brachial artery stiffness, whether expressed as local PWV (blood pressure-dependent) or β-stiffness index (blood pressure-independent), was similar between the younger and older cohorts. Furthermore, neither cohort demonstrated any associations between brachial artery stiffness and the magnitude of FMD response, nor between brachial artery stiffness and the GTN-mediated vasodilatory response. These observations suggest that brachial artery stiffness did not play a major role in modulating the vasodilatory response following either haemodynamic or exogenous vasodilatory stimulation in this study. Importantly, brachial artery stiffness does not appear to explain the age-related reduction in the FMD response observed in our cohort of older people.

Mechano-transduction and endothelial-derived relaxing- and contracting- factors. One explanation of our data is that in older adults the WSR stimulus is not transduced as precisely as it is in younger people. Recent evidence obtained using intravital microscopy and electron microscopy (Machin *et al.*, 2017; Machin *et al.*, 2018) has shown that the endothelial glycocalyx is diminished in older people and old mice. The glycocalyx plays an important role in transducing WSR to the endothelial cell and a diminished glycocalyx in older people could, to an extent, 'uncouple' WSR from the vascular endothelium during reactive hyperaemia.

Likewise, age-associated intra-cellular changes may alter the cellular response to WSR. For example, reduced nitric oxide bioavailability resulting from increased oxidative stress (Donato *et al.*, 2007), a shift in balance between nitric oxide and endothelin-1 (Donato *et al.*, 2009) and a reduction in the bioavailability of the enzyme co-factor tetrahydrobiopterin (Eskurza *et al.*, 2005) have all been reported in aged endothelium. Preliminary data from mouse primary endothelial cells and from commercial human endothelial cells indicate that ageing causes a shift away from glycolytic metabolism to a greater reliance on mitochondria (Gogulamudi *et al.*, 2017). This raises the possibility of a mitochondrial source of superoxide that increases oxidative stress in older endothelial cells (Gogulamudi *et al.*, 2017) and reduces nitric oxide bioavailability. Alterations in the ability of endothelial cells to hyperpolarise smooth muscle cells and changes in vasoconstrictor activity (for review, see: (Vanhoutte *et al.*, 2017) may also be important components that could reduce the precision of the FMD response to an increase in WSR during reactive hyperaemia. All of these

mechanisms lie downstream of the WSR stimulus and may contribute to the diminished strength of association between WSR and FMD in older people. Alternative explanations need to be considered, but our data demonstrate the importance of knowing the WSR stimulus in order to investigate the role of downstream mechanisms of vasodilatation to reactive hyperaemia and to more accurately understand vascular dysfunction measured using FMD.

Causes of reduced WSR during reactive hyperaemia in older adults. Altered WSR during reactive hyperaemia also conjures a conundrum: if this represents a failure of the endothelium to adequately normalise the sudden increase in WSR, we might expect that WSR would be higher in older people, not lower. An explanation could be that older people do not augment WSR during reactive hyperaemia to the extent that young people do. Reactive hyperaemia following cuff release is mostly the result of microvascular dilatation in response to ischaemia during cuff-occlusion. The extent of this dilatation and subsequent reperfusion influences blood flow and, therefore, WSR (Mitchell *et al.*, 2004). If the microvascular response to cuff-occlusion is altered and/or diminished in older people, this could alter brachial artery WSR during reperfusion. We have previously shown distinct differences in the autoregulatory response to reperfusion by microvessels, and these differences temporally altered perfusion and oxygenation of tissue. (Adingupu *et al.*, 2015). Structural alterations in the microcirculation (Schiffrin, 2004) can also influence its ability to respond to ischemia-reperfusion, which might influence the upstream WSR stimulus. Age-related changes to the microvascular endothelium (Gates *et al.*, 2009; Strain *et al.*, 2010) and diminished microvascular glycocalyx and mechano-transduction (Machin *et al.*, 2018) could also combine to alter the pattern and amplitude of the WSR stimulus in older people. Consolidating these ideas, an abnormal microvascular response during cuff-occlusion may result in a diminished WSR during hyperaemia with a correspondingly low FMD; further, the FMD response is less precise in older people, perhaps because of altered mechano-transduction and age-associated changes in endothelial cell function.

Novel WSR data in older people

Novel WSR parameters in healthy older people. In this study we have produced new data that give a first point of reference for WSR-FMD in older people. As well as demonstrating the need to know WSR during hyperaemia, our data show that the mono-phasic and bi-phasic patterns of WSR seen in younger people (Aizawa et al., 2018) are also evident in older people, although the significance and utility of these patterns is uncertain. Mono- (n=19) and bi-phasic (n=13) WSR patterns did not appear to influence FMD in older people after controlling for hyperaemic WSR, but these different patterns are of interest because they may be indicators of different physiological responses to reperfusion.

WSR and brachial artery diameter during low-flow in older people. During cuff-occlusion WSR was reduced in 28 out of 32 people and of these, five reduced brachial artery diameter and the remainder increased it. In four people who exhibited an increased WSR during cuff-occlusion, one exhibited reduced- and three people increased- brachial artery diameter. In keeping with our previous observations (Aizawa *et al.*, 2018), these findings suggest that the brachial artery response to low-flow might be independent of WSR in older people.

WSR response and vasodilatory response to glyceryl trinitrate are preserved with ageing. In young people, WSR was slightly reduced at the time of peak diameter after GTN administration, indicating that GTN-mediated vasodilatation is WSR-independent (Aizawa *et al.*, 2018). Our data from older adults are similar, suggesting that GTN administration overrides any effect of altered WSR in older people and that the sensitivity of vascular smooth muscle to an exogenous nitric oxide donor is preserved.

Implication and applications

WSR-FMD as a biomarker. Our findings reveal for the first time that older people have a diminished WSR response during WSR-FMD compared with young people. This has important implications for the accurate interpretation of FMD in general and also for our understanding of vascular ageing. The differences between our two cohorts also suggest that the measurement of WSR-FMD could produce a specific ‘signature’ of vascular function using the forearm model and adds to our work to develop novel biomarkers to provide intermediate predictors of cardiovascular events (Shore *et al.*, 2018). The measurement of

WSR provides detailed and nuanced data that has the potential to be applied to different diseases, interventions or physiological manipulations. This study also demonstrates the utility of multi-gate Doppler and the challenge now is for this to become more widely available.

Normalising FMD to WSR in older people. Thijssen et al. (Thijssen *et al.*, 2009) have cautioned against normalising FMD to WSR in older adults. Our data contribute evidence consistent with this assertion and we suggest that the WSR stimulus and FMD responses should be reported together but without normalising FMD to WSR. It may be possible to develop population-specific statistical approaches to normalise FMD to WSR, but the problem of normalising artery dilatation to a complex, non-linear or two-dimensional (area under the curve) WSR stimulus should be resolved first. Given that three separate methods – MRI (Silber *et al.*, 2005), multi-gate Doppler (Tortoli *et al.*, 2006) and echo particle image velocimetry (Gurung *et al.*, 2017; Gates *et al.*, 2018) – indicate the assumptions used to calculate WSR from centre-line Doppler velocity are not valid, and given the complexity of the WSR response demonstrated in this study, caution should be exercised using this method to interpret FMD data.

Association of baseline brachial artery diameter with WSR and the FMD response. The association of baseline brachial artery diameter with FMD has been well reported and creates problems if baseline brachial artery diameter is different between cohorts. In our cohorts, brachial artery diameter was similar. Analysis of our data revealed for the first time that baseline brachial artery diameter was also associated with WSR during FMD and that this association tended to be stronger in young people. Understanding if this is mechanical and/or physiological is beyond the scope of the present study, but these new finding warrant attention. It will also be necessary to determine how to statistically analyse and scale baseline diameter to WSR responses so that groups with different baseline diameters can be compared. For example, in our cohorts, females and males were included in both groups in a balanced design. However, had the aim of our study been to determine sex-specific differences in WSR-FMD, amongst other things, the design would need to account for any differences in arterial diameter between females and males. We also note here that the vasodilatory control by endothelial-derived relaxing- and contracting- factors may differ

between females and males (Vanhoutte *et al.*, 2017) and that there may be an interaction with age. Thus, our study suggests the need to determine sex differences in WSR during FMD, the influence on downstream signalling mechanisms and the interaction with ageing.

Conclusion

This study demonstrates that WSR during FMD is much diminished in older people. There also appears to be an 'uncoupling' of WSR from FMD in older people that may reflect a loss of precision in the stimulus-response relationship. We speculate that diminished WSR during reactive hyperaemia is due to a dysfunctional microvascular response during ischaemia and/or reperfusion and the loss of precision in the stimulus-response relationship is caused by a loss of mechano-transduction of the WSR stimulus and/or endothelial cell dysfunction.

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TRANSLATIONAL PERSPECTIVE:

Brachial artery flow-mediated dilatation (FMD), a widely used marker of arterial endothelial cell function, uses reactive hyperaemia to increase arterial wall shear rate (WSR), a powerful mechanical stimulus for arterial vasodilatation. In this study, we show that older people have an altered and much diminished WSR during FMD and that the FMD response has begun to 'uncouple' from the WSR stimulus. This suggests that diminished FMD is partly due to an altered WSR, rather than brachial artery endothelial dysfunction alone. This provides new insight into the age-related decline in vascular function, which is a precursor to cardiovascular diseases and likely complicates diseases with significant vascular involvement. We also found that WSR during FMD is complex, detailed and nuanced. Differences between older and younger people suggest that WSR measurement could provide a specific 'signature' of vascular function that might be specific to different diseases and different stages of a disease. In this case, it may give clues about the underlying disease and facilitate clinical management. Our findings also indicate the need for careful interpretation of studies that use FMD as a clinical outcome. For example, arterial FMD is

diminished in patients with cardiovascular disease, but, based upon our findings, it is possible that WSR is diminished during hyperaemia in these patients, explaining some of the impaired FMD. In this case, the microvasculature might be the site responsible for reduced WSR and an important site of vascular dysfunction in patients with poor FMD. Clinical studies are needed to test these speculative ideas.

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COMPETING INTERESTS:

Nothing to disclose.

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FIGURE LEGENDS:

Figure 1. A schematic description of WSR parameters obtained from brachial artery FMD assessment using continuous multi-gate Doppler and simultaneous diameter. In the upper panel, traces in light blue and red represent the peak and mean values of WSR, respectively. In the lower panel, traces in light blue and red represent the variations in diameter (due to cardiac cycle) and mean diameter of the brachial artery, respectively. WSR, wall shear rate; SL1, first slope of wall shear rate increase; SL2, second slope of wall shear rate increase; aucttp, area under the curve until time to peak dilation (area shaded with turquoise); auc, area under the curve until its return to baseline level (area shaded both with turquoise and grey); Tp, time to peak value; Tb, time to return to baseline value; Δ, changes.

Table 1. Description of WSR magnitude, WSR time-course, WSR kinetics, diameter magnitude and diameter time-course parameters.

	Description
WSR magnitude parameters	
WSR Baseline, 1/s	WSR at baseline
WSR Low-flow, 1/s	WSR during low-flow
WSR Peak, 1/s	WSR at peak hyperaemia
WSR Δ, 1/s	Absolute WSR increase from baseline
WSR %Δ, %	percentage WSR increase from baseline
WSR aucttp, au	Area under the WSR curve until time to peak dilatation
WSR auc, au	Area under the WSR curve measured between cuff-release and the point at which WSR returned to the baseline value
WSR time-course parameters	
WSR Tp, s	Time to peak WSR
WSR Tb, s	Time to return to baseline WSR
WSR kinetics parameters	
WSR SL1, $1/s^2$	First slope of WSR increase during hyperaemia
WSR SL2, $1/s^2$	Second slope of WSR increase during hyperaemia
Diameter magnitude parameters	
Diameter Baseline, mm	Baseline diameter
Diameter Peak Δ, mm	Absolute diameter increase from baseline
Diameter Peak %Δ, %	Percentage diameter increase from baseline
Diameter time-course parameters	
Diameter Tp, s	Time to peak diameter
Diameter Tb, s	time to return to baseline diameter

Table 2. Selected characteristics of the study participants

	Young (n=28)	Older (n=32)	*p*
Age, yrs	27.5±5.5	64.9±3.7	<0.001
Female, n	17	16	0.340
BMI, kg/m^2	22.4±2.4	24.6±2.5	0.001
Total CHOL, mmol/l	4.4±0.7	6.0±1.4	<0.001
LDL CHOL, mmol/l	2.2±0.7	3.7±0.9	<0.001
HDL CHOL, mmol/l	1.8±0.4	1.7±0.5	0.747
Glucose, mmol/l	4.9±0.4	5.3±0.5	0.026
HbA1c, mmol/mol	36.3±3.2	40.0±3.6	0.002
Systolic BP, mmHg	114±9	124±13	0.002
Diastolic BP, mmHg	69±7	73±7	0.047
Heart Rate, beats/min	65±10	60±8	0.037

Data are shown as means±SD or numbers. BMI, body mass index; CHOL, cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HbA1c, haemoglobin A1c; BP, blood pressure.

Table 3. Parameters of wall shear rate and arterial diameter during brachial artery flow-mediated assessment in older people

	Values	Ranges
WSR magnitude parameters		
WSR Baseline, 1/s	50.5±34.8	0.5 – 147.0
WSR Low-flow, 1/s	17.8±12.2	-12.5 – 44.3
WSR Peak, 1/s	463.8±106.3	286.7 – 751.9
WSR Δ, 1/s	413.3±96.6	279.8 – 695.5
WSR %Δ, %	1023 (695 – 1545)	117 – 10616
WSR aucttp, au	11198±4482	4356 – 25433
WSR auc, au	14344±4812	6418 – 24603
WSR time-course parameters		
WSR Tp, s	11.8±2.4	8.0 – 15.7
WSR Tb, s	109.1±37.0	32.4 – 189.4
WSR kinetics parameters		
WSR SL1, $1/s^2$	67.8±18.1	33.5 – 95.7
WSR SL2, $1/s^2$*	9.7±5.0	1.7 – 17.7
Diameter magnitude parameters		
Diameter Baseline, mm	3.60±0.67	2.05 – 5.28
Diameter Peak Δ, mm	0.16±0.10	0.00 – 0.35
Diameter Peak %Δ, %	4.87±3.19	0.09 – 14.0
Diameter time-course parameters		
Diameter Tp, s	68.8±53.0	17.4 – 156.2
Diameter Tb, s	96.9±35.2	54.5 – 156.2

Data are shown as means±SD for variables with normal distribution and median (interquatile range) for variables with skewed districution. *Obtained from 14 young and 13 older participants who showed the bi-phasic WSR increase response. WSR, wall shear rate; Δ, changes; aucttp, area under the curve until time to peak dilatation; auc, area under the curve until its return to baseline value; Tp, time to peak value; Tb, time to return to baseline value; SL1, first slope of wall shear rate increase; SL2, second slope of wall shear rate increase.

Table 4. Parameters of wall shear rate and diameter during brachial artery flow-mediated dilatation assessment between young and older cohorts.

	Young (n=28)	Older (n=32)	*p*
WSR magnitude parameters			
WSR Baseline, 1/s	78.8 (62.1 – 95.5)	64.1 (48.7 – 79.5)	0.234
WSR Low-flow, 1/s	21.8 (15.7 – 27.8)	25.3 (19.7 – 30.9)	0.438
WSR Peak, 1/s	622 (571 – 673)	443 (396 – 491)	<0.001
WSR Δ, 1/s	543 (498 – 587)	379 (338 – 420)	<0.001
WSR %Δ, %	829 (604 – 1138)	811 (606 – 1087)	0.926
WSR aucttp, au	15373 (13195 - 17552)	11258 (9243 - 13274)	0.013
WSR auc, au	18514 (16151 - 20876)	13570 (11384 - 15755)	0.006
WSR time-course parameters			
WSR Tp, s	12.1 (11.1 - 13.2)	12.0 (11.0 – 13.0)	0.849
WSR Tb, s	105.9 (90.5 – 121.3)	108.2 (93.9 - 122.4)	0.841
WSR kinetics parameters			
WSR SL1, $1/s^2$	85.7 (77.9 – 93.5)	62.5 (55.3 – 69.7)	<0.001
WSR SL2, $1/s^2$*	18.6 (14.4 – 22.9)	9.5 (5.1 – 14.0)	0.009
Diameter magnitude parameters			
Diameter Baseline, mm	3.28 (3.05 - 3.52)	3.54 (3.33 - 3.76)	0.138
Diameter Peak Δ, mm	0.23 (0.19 - 0.27)	0.16 (0.12 - 0.20)	0.027
Diameter Peak %Δ, %	7.11 (5.70 - 8.52)	4.88 (3.57 - 6.19)	0.036
Diameter time-course parameters			
Diameter Tp, s	55.0 (36.3 – 73.8)	68.7 (51.3 - 86.0)	0.326
Diameter Tb, s	122.8 (102.5 – 143.2)	87.8 (62.5 – 113.2)	0.047

Data are shown as means (95% confidence intervals). Tp, time to peak value; Tb, time to return to baseline value. *Obtained from 14 young and 13 older participants who showed the bi-phasic WSR increase response. WSR, wall shear rate; Δ, changes; aucttp, area under the curve until time to peak dilatation; auc, area under the curve until its return to baseline value; Tp, time to peak value; Tb, time to return to baseline value; SL1, first slope of wall shear rate increase; SL2, second slope of wall shear rate increase.

Table 5. Parameters of wall shear rate and diameter during brachial artery glyceryl trinitrate-mediated dilatation between the young and older people

	Young (n=14)	Older (n=29)	*p*
WSR magnitude parameters			
WSR Baseline, 1/s	57.9 (39.5 – 76.3)	53.3 (40.5 – 66.1)	0.680
WSR GTN, 1/s	42.3 (25.6 – 59.0)	38.4 (26.8 – 50.0)	0.695
Diameter magnitude parameters			
Diameter Baseline, mm	3.42 (3.09 - 3.76)	3.75 (3.52 – 3.98)	0.112
Diameter GTN Δ, mm	0.76 (0.64 - 0.89)	0.69 (0.61 - 0.78)	0.325
Diameter GTN %Δ, %	22.5 (18.6 - 26.5)	19.4 (16.6 - 22.2)	0.197

Data are shown as means (95% confidence intervals). WSR, wall shear rate; GTN, glyceryl trinitrate; Δ, changes.

Table 6. Partial correlation analysis between parameters of WSR and diameter changes during brachial artery flow-mediated dilatation assessment between the young and older cohorts.

	Young (n=28)		Older (n=32)	
	Diameter Δ	**Diameter % Δ**	**Diameter Δ**	**Diameter % Δ**
WSR magnitude parameters				
WSR Baseline	*r*=0.20, *p*=0.322	*r*=0.17, *p*=0.408	*r*=-0.26, *p*=0.146	*r*=-0.30, *p*=0.092
WSR Low-flow	*r*=0.22, *p*=0.279	*r*=0.22, *p*=0.271	*r*=0.03, *p*=0.864	*r*=-0.03, *p*=0.852
WSR peak	*r*=0.41, *p*=0.035	*r*=0.48, *p*=0.012	*r*=-0.12, *p*=0.531	*r*=-0.03, *p*=0.887
WSR Δ	*r*=0.41, *p*=0.034	*r*=0.50 *p*=0.008	*r*=-0.03, *p*=0.864	*r*=0.08, *p*=0.663
WSR %Δ	*r*=-0.11, *p*=0.592	*r*=-0.05, *p*=0.811	*r*=0.30, *p*=0.100	*r*=0.35, *p*=0.049
WSR aucttp	*r*=0.45, *p*=0.019	*r*=0.55, *p*=0.003	*r*=0.16, *p*=0.372	*r*=0.16, *p*=0.384
WSR auc	*r*=0.44, *p*=0.023	*r*=0.54, *p*=0.004	*r*=0.20, *p*=0.284	*r*=0.18, *p*=0.313
WSR time-course parameters				
WSR Tp	*r*=0.29, *p*=0.142	*r*=0.28, *p*=0.160	*r*=0.09, *p*=0.634	*r*=-0.01, *p*=0.945
WSR Tb	*r*=0.21, *p*=0.300	*r*=0.29, *p*=0.136	*r*=0.05, *p*=0.797	*r*=0.02, *p*=0.923
WSR kinetics parameters				
WSR SL1	*r*=0.49, *p*=0.010	*r*=0.52, *p*=0.005	*r*=-0.07, *p*=0.713	*r*=0.03, *p*=0.876
WSR SL2*	*r*=-0.09, *p*=0.777	*r*=-0.03, *p*=0.923	*r*=0.03, *p*=0.927	*r*=0.21, *p*=0.496

*Obtained from 14 young and 13 older participants who showed the bi-phasic WSR increase response. WSR, wall shear rate; Δ, changes; aucttp, area under the curve until time to peak dilatation; auc, area under the curve until its return to baseline value; Tp, time to peak value; Tb, time to return to baseline value; SL1, first slope of wall shear rate increase; SL2, second slope of wall shear rate increase.

Table 7. Partial correlation analysis showing the associations between brachial artery diameter at baseline and WSR and diameter parameters during flow-mediated dilatation in the young and older cohorts.

	Young (n=28)	Older (n=32)
	Diameter Baseline	**Diameter Baseline**
WSR magnitude parameters		
WSR Baseline	*r*=-0.04, *p*=0.856	*r*=0.17, *p*=0.361
WSR Low-flow	*r*=-0.14, *p*=0.474	*r*=0.22, *p*=0.229
WSR peak	*r*=-0.41, *p*=0.032	*r*=-0.31, *p*=0.083
WSR Δ	*r*=-0.48, *p*=0.011	*r*=-0.40, *p*=0.022
WSR %Δ	*r*=-0.14, *p*=0.480	*r*=-0.36, *p*=0.043
WSR aucttp	*r*=-0.53, *p*=0.004	*r*=-0.14, *p*=0.456
WSR auc	*r*=-0.51, *p*=0.006	*r*=-0.17, *p*=0.341
WSR time-course parameters		
WSR Tp	*r*=-0.18, *p*=0.370	*r*=0.16, *p*=0.387
WSR Tb	*r*=-0.37, *p*=0.057	*r*=-0.01, *p*=0.982
WSR kinetics parameters		
WSR SL1	*r*=-0.28, *p*=0.161	*r*=-0.40, *p*=0.025
WSR SL2*	*r*=-0.16, *p*=0.601	*r*=-0.64, *p*=0.020
Diameter magnitude parameters		
Diameter Peak Δ	*r*=-0.18, *p*=0.365	*r*=-0.24, *p*=0.192
Diameter Peak Δ%	*r*=-0.42, *p*=0.032	*r*=-0.52, *p*=0.002

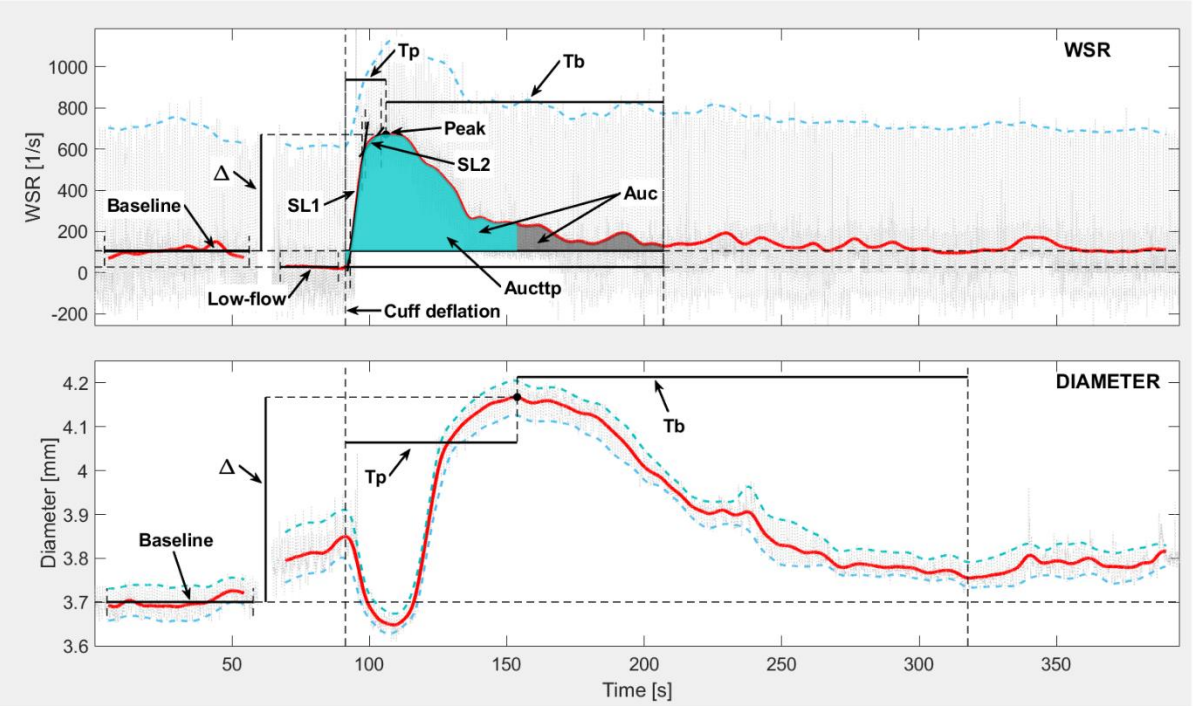
*Obtained from 14 young and 13 older participants who showed the bi-phasic WSR increase response. WSR, wall shear rate; Δ, changes; aucttp, area under the curve until time to peak dilatation; auc, area under the curve until its return to baseline value; Tp, time to peak value; Tb, time to return to baseline value; SL1, first slope of wall shear rate increase; SL2, second slope of wall shear rate increase.

Table 8. Partial correlation analysis between parameters of brachial artery stiffness and diameter changes during brachial artery flow-mediated and glyceryl trinitrate-mediated dilatation assessments between the young and older cohorts.

	Young		Older	
	Diameter Δ	**Diameter % Δ**	**Diameter Δ**	**Diameter % Δ**
Flow-Mediated Dilatation (Young, n=28; Older, n=32)				
PWV, m/s	*r*=0.18, *p*=0.393	*r*=0.15, *p*=0.465	*r*=-0.08, *p*=0.660	*r*=-0.28, *p*=0.130
β-stiffness index, au	*r*=0.32, *p*=0.105	*r*=0.24, *p*=0.226	*r*=-0.05, *p*=0.783	*r*=-0.23, *p*=0.217
Glyceryl Trinitrate-Mediated Dilatation (Young, n=14; Older, n=29)				
PWV, m/s	*r*=0.13, *p*=0.684	*r*=-0.14, *p*=0.673	*r*=-0.16, *p*=0.416	*r*=-0.34, *p*=0.081
β-stiffness index, au	*r*=-0.06, *p*=0.846	*r*=-0.36, *p*=0.204	*r*=-0.13, *p*=0.496	*r*=-0.29, *p*=0.123

Centre was included as a controlling variable. In case of PWV, mean arterial pressure and heart rate were additionally included as controlling variables. PWV, pulse wave velocity.

Figure 1.


WSR
WSR [1/s]
1000
800
600
400
200
0
-200
Tp
Tb
Peak
SL2
Δ
Baseline
SL1
Auc
Aucttp
Low-flow
Cuff deflation
DIAMETER
Diameter [mm]
4.2
4.1
4
3.9
3.8
3.7
3.6
Tb
Δ
Tp
Baseline
50
100
150
200
250
300
350
Time [s]