

1 **Brachial artery vasodilatory response and wall shear rate determined by**
2 **multi-gate Doppler in a healthy young cohort**

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15 **Running Head:**

16 Brachial vasodilation and wall shear rate in the young

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18 **Author Contributions**

19 Conception and design of research: KA, PT, AS, PG, CP. Performed experiments: KA, SS, FC,
20 CM, CT, PG. Analyzed data: KA, AR, PG. Interpreted results of experiments: KA, PT, AS, PG,
21 CP. Prepared figures: KA, AR. Drafted manuscript: KA, AR, PG. Edited and revised
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35 **ABSTRACT:**

36 Wall shear rate (WSR) is an important stimulus for the brachial artery flow-mediated
37 dilation (FMD) response. However, WSR estimation near the arterial wall by conventional
38 Doppler is inherently difficult. To overcome this limitation, we utilised multi-gate Doppler to
39 accurately determine the WSR stimulus near the vessel wall simultaneously with the FMD
40 response using an integrated FMD system [Ultrasound Advanced Open Platform (ULA-OP)].
41 Using the system, we aimed to perform a detailed analysis of WSR-FMD response and
42 establish novel WSR parameters in a healthy young population. Data from 33 young healthy
43 individuals (27.5 ± 4.9 yrs, 19F) were analysed. FMD was assessed with reactive hyperemia
44 using ULA-OP. All acquired raw data were post-processed using custom-designed software
45 to obtain WSR and diameter parameters. The acquired velocity data revealed that non-
46 parabolic flow-profiles within the cardiac cycle and under different flow-states, with
47 heterogeneity between participants. We also identified seven WSR magnitude and four WSR
48 time-course parameters. Among them, WSR area under the curve until its return to
49 baseline was the strongest predictor of the absolute ($R^2=0.25$) and percentage ($R^2=0.31$)
50 diameter changes in response to reactive hyperemia. For the first time, we identified mono-
51 and biphasic WSR stimulus patterns within our cohort that produced different magnitudes
52 of FMD response [absolute diameter change: 0.24 ± 0.10 mm (monophasic) vs 0.17 ± 0.09 mm
53 (biphasic), $p < 0.05$]. We concluded that accurate and detailed measurement of the WSR
54 stimulus is important to comprehensively understand the FMD response and that this
55 advance in current FMD technology could be important to better understand vascular
56 physiology and pathology.

57

58

59 ***Key Words***

60 Endothelial function; Reactive hyperemia; Ultrasound; Vasodilation; Wall shear stress.

61

62 ***New & Noteworthy***

63 An estimation of wall shear rate (WSR) near the arterial wall by conventional Doppler

64 ultrasound is inherently difficult. Using a recently-developed integrated FMD ultrasound

65 system, we were able to accurately estimate WSR near the wall, and identified a number of

66 novel WSR variables that may prove to be useful in the measurement of endothelial

67 function, an important biomarker of vascular physiology and disease.

68 **INTRODUCTION:**

69 Brachial artery flow mediated dilation (FMD) has been used extensively to assess the
70 function and health of the vascular endothelium since its first use by Celermajer et al (5).
71 However, a persistent problem with this method is the inability to accurately measure the
72 wall shear stress *stimulus* that produces the measured FMD *response*. This is a perplexing
73 problem that has resulted in the extensive use of Doppler ultrasound peak velocity to
74 estimate wall shear stress, or its surrogate, wall shear rate (WSR). This is currently the only
75 practical solution available when measuring FMD, but this method underestimates WSR (22)
76 and is a blunt instrument to dissect the complex and dynamic WSR events occurring during
77 FMD.

78

79 In the absence of WSR data, the accurate interpretation of the FMD response may be
80 confounded and this has likely limited its full potential as a scientific and clinical tool. For
81 example, the mechanisms of diminished FMD could involve impaired brachial artery
82 endothelial function or alternatively could result from altered microvascular function
83 blunting the hyperaemic response and, in turn, WSR stimulus. Knowledge of the WSR
84 stimulus may also help to resolve and exploit issues relating to differences in baseline
85 diameter and 'low-flow' vasoconstriction occurring during cuff-occlusion (2, 11, 26),
86 providing a more comprehensive characterization of the underlying vascular physiology.

87

88 For physiological studies, the optimal tool for FMD needs to combine continuous and
89 simultaneous measurement of WSR and vessel diameter in order to comprehensively
90 characterise the WSR-FMD stimulus-response relationship. We have previously reported a
91 method to simultaneously measure WSR and vessel diameter during FMD (28). Unlike the

92 estimation of WSR from Doppler ultrasound, this system uses multi-gate spectral Doppler to
93 acquire velocity data at different sites across the vessel diameter and close to the vessel
94 wall, producing a continuous velocity profile from near to far wall (27). These data can be
95 used to generate a detailed spectral Doppler profile across the vessel diameter and close to
96 the vessel wall to accurately determine WSR (27). This overcomes the limitation of a single
97 pulsed-wave Doppler sample-gate and the need to assume a perfect parabolic velocity
98 profile (12).

99

100 Using this method we were able to show that estimation of WSR by conventional Doppler
101 ultrasound is inaccurate (28), at least partly because the presumed parabolic velocity profile
102 (15) is often asymmetric during hyperaemia and varies considerably through the cardiac
103 cycle (12, 23, 28). Because of this, the extant literature presents a well characterised arterial
104 diameter response to reactive hyperaemia, but lacks a well characterised WSR stimulus.

105

106 We have recently enhanced and refined this system in a collaborative effort between
107 engineers, clinicians and physiologists with extensive experience of using ultrasound, to
108 provide a sophisticated tool that can advance current FMD technology. This novel tool has
109 an additional acquisition system capable of storing the large amount of raw data generated
110 during the FMD procedure (18, 19). This has vastly expanded data collection capacity so that
111 it can continuously measure WSR and vessel diameter over an extended period of time to
112 provide accurate, detailed and simultaneous WSR-FMD stimulus-response data.

113

114 Here we report, for the first time, the use of this method. Our overall aim was to establish a
115 new benchmark in WSR-FMD measurement. First, we wanted to establish the relevant

116 variables derived from the measurement system. Second, because the WSR-FMD response
117 in humans is currently unknown, we wanted to establish a 'normal reference' by conducting
118 a detailed analysis of the WSR-FMD response in a cohort of healthy young adults. Third, we
119 wanted to determine which WSR variables were the best predictors of a normal FMD
120 response. Fourth, we wanted to investigate the patterns of WSR during hyperaemia and
121 determine whether these influenced the FMD response. We had noticed two distinct WSR
122 patterns during our pilot work: A 'monophasic' pattern, where WSR increases sharply
123 reaching its peak in one go during hyperaemia; and a 'biphasic' pattern, where WSR
124 increases sharply followed by a slow increase before reaching its peak during hyperaemia.
125 These two distinct WSR patterns cannot readily be seen with conventional Doppler and we
126 wanted to know if the biphasic WSR pattern resulted in a greater magnitude of hyperaemic
127 WSR than the monophasic WSR pattern, and if so, whether this greater WSR produced a
128 greater brachial artery vasodilatory response than the monophasic WSR pattern.

129

130 **METHODS:**

131 ***Participants***

132 Thirty-three young individuals (27.5±4.9 yrs, 19 females) participated in this study. Of these,
133 15 participants were studied in Exeter, UK and 18 in Pisa, Italy. All were healthy, without
134 hypertension, type 2 diabetes, dyslipidaemia, or overt cardiovascular disease. UK National
135 Research Ethics Service South West Committee and the institutional ethics committee at
136 University of Pisa approved all study procedures and written informed consent was
137 obtained from all participants.

138

139 ***Experimental procedures***

140 Participants arrived in our temperature-controlled laboratories after an overnight fast, had
141 blood samples drawn for biochemical analysis, consumed a standardized meal and rested
142 for 20 min before initiation of the study protocol.

143

144 Brachial artery FMD was assessed non-invasively following established guidelines (6, 10, 24)
145 and as previously described by us elsewhere (2, 8, 9). Briefly, participants lay supine on an
146 examination bed with the right arm fixed in position and immobilised using a positioning
147 pillow on a metal table. A small blood pressure cuff was placed around the proximal part of
148 the forearm. A complete and open research system for ultrasound imaging and acquisition,
149 the Ultrasound Advanced Open Platform (ULA-OP; Microelectronics Systems Design
150 Laboratory, University of Florence, Italy) was connected to a high-frequency linear array
151 transducer LA523 (Esaote SpA, Florence, Italy) and used to obtain both B-mode and multi-
152 gate velocity data from the brachial artery (4). Once the optimal ultrasound image was
153 obtained, the transducer was carefully clamped to prevent movement during the procedure
154 using a custom-designed transducer holder. If necessary, a micro-adjuster was used to
155 obtain a precisely aligned image.

156

157 Baseline brachial artery image and blood velocity were recorded for 60 s. Reactive
158 hyperemia was then induced by rapidly inflating the forearm cuff (AI6, Hokanson, Bellevue,
159 WA) to 250 mmHg to occlude forearm blood flow for 5 min. At 5 min, the cuff was rapidly
160 deflated. Recording was restarted 30 s before deflation and continued until 5 min following
161 deflation.

162

163 In a sub-set of participants (n=13), baseline brachial artery diameter measurements were
164 repeated after a 15 min rest period, and endothelium-independent dilation was assessed
165 using sub-lingual nitroglycerin spray (0.4 mg). A 60 s recording was started 9 min after
166 administering the spray. We have previously found that measurement between 9 and 10
167 minutes after nitroglycerin administration captures maximal endothelial-independent
168 vasodilation (unpublished observation).

169

170 ***Integrated FMD evaluation system***

171 An integrated system capable of estimating both stimulus (WSR) and response (diameter)
172 during the FMD assessment was developed by suitably modifying the existing hardware and
173 software of the ULA-OP as well as by developing a post-processing software platform (19,
174 28). In particular, the ULA-OP offers much wider experimental possibilities than
175 commercially-available Doppler ultrasound systems as it can be re-configured at run time
176 and enables complete access to imaging data in every stage of the processing chain. The
177 ULA-OP system also provides real-time imaging visualization functions in connection to a
178 host personal computer. For the FMD data acquisition, an additional dedicated acquisition
179 board was developed to store all raw (quadrature demodulated) echo data over a long time
180 interval (up to 15 min). Furthermore, the ULA-OP system was recently upgraded to include
181 the real-time measurement of arterial diameter (19).

182

183 In addition, a post-processing software platform was developed in order to evaluate
184 endothelial function (19). It is based on Matlab® (The Mathwork Inc, Natick, MA) and
185 consists of several processing blocks that, starting from the baseband acquired data,
186 compute B-mode images and multi-gate spectral Doppler profiles. B-mode processing

187 organizes the data in lines and frames, interpolates them, applies 2D spatial filters, and
188 finally applies a logarithmic scale compression. Multi-gate spectral Doppler processing
189 reconstructs the information about the distribution of blood velocities at different depths
190 (256 up to 512 gates) through 256-point fast Fourier transforms (FFTs), hence the profiles
191 are low-pass filtered in order to remove the low-frequency spectral components through a
192 frequency domain mask. In cascade to the latter blocks, other specific processing blocks
193 extract wall positions of the artery and its diameter through a first order absolute central
194 moment (FOAM) algorithm (7), as well as WSR by a method that employs the direct
195 measurement of the whole velocity profile (20). Next, diameter and WSR time trends are
196 saved in output files which are finally loaded by a further Matlab® interface that extracts the
197 main parameters needed to investigate endothelial function.

198

199 ***Measurements of wall shear rate and diameter***

200 The signal elaboration system extracts detailed WSR parameters for describing both
201 magnitude and time-course of WSR changes. Seven *WSR magnitude* parameters were
202 analysed: 1) WSR at baseline, 2) WSR during low-flow, 3) WSR at peak hyperemia, 4)
203 absolute WSR increase from baseline, 5) percentage WSR increase from baseline, 6) area
204 under the WSR curve until time to peak dilation (WSR auc_{ttp}), and 7) area under the WSR
205 curve (WSR auc), measured between cuff release and the point at which WSR returned to
206 the baseline value. Four *WSR time-course* parameters were analysed: 1) first slope of WSR
207 increase during hyperemia (WSR SL1, an initial steep increase), 2) the second slope of WSR
208 increase during hyperemia (WSR SL2, a gradual increase after the initial steep increase; the
209 biphasic pattern only), 3) time to peak WSR (WSR T_p), and 4) time to return to baseline WSR
210 (WSR T_b). The monophasic and biphasic patterns of WSR increase were defined as: 1)

211 Monophasic - the peak WSR value was reached with a single, continuous steep increase
212 only; 2) Biphasic - the peak WSR value was reached with an initial steep increase followed by
213 a gradual second increase.

214

215 The signal elaboration system also extracts detailed diameter parameters. Three diameter
216 *magnitude* parameters were analysed: 1) baseline diameter, 2) absolute diameter increase
217 from baseline, and 3) percentage diameter increase from baseline. Two diameter *time-*
218 *course* parameters were analysed: 1) time to peak diameter, and 2) time to return to
219 baseline diameter, taken as the point at which diameter returns to its baseline values or
220 plateaus.

221

222 ***Statistical analysis***

223 Data are presented as means \pm SD for variables with a normal distribution, and median
224 (interquartile range) for variables with a skewed distribution. A partial correlation analysis
225 was performed between WSR parameters and diameter changes (absolute and percentage)
226 controlling for the study centre in the whole cohort. A stepwise multivariate regression
227 analysis was also performed in the whole cohort to determine the strongest predictor(s) of
228 WSR parameters that were significantly associated with both absolute and percentage
229 diameter changes in the partial correlation analysis (WSR at peak hyperaemia, WSR auc and
230 absolute WSR increase from baseline). The study centre was also included as an
231 independent variable in the model. Analysis of covariance (study centre as a covariate) was
232 used to examine the differences in variables between monophasic and biphasic groups. A
233 log-transformation was used for variables with skewed distribution before statistical

234 analysis. All statistical analysis was conducted using IBM SPSS Statistics 22 (IBM, Armonk,
235 NY). Significance was set at $p<0.05$.

236

237 **RESULTS:**

238 Selected baseline characteristics of the study participants are shown in Table 1. Body mass
239 index (24.1 ± 3.0 vs 21.7 ± 2.7 kg/m²) and systolic blood pressure (121.5 ± 7.5 vs 108.6 ± 8.8
240 mmHg) were higher in male participants than female participants (both $p<0.05$). Other
241 participants' characteristics were similar between males and females.

242

243 ***Establishment of relevant WSR variables and their 'normal reference' values***

244 Figure 1 exhibits examples of multi-gate Doppler spectral profiles obtained at different time
245 points in the cardiac cycle at baseline and peak hyperemia, to illustrate the variability and
246 complexity of the WSR events occurring during FMD. A schematic description of detailed
247 WSR parameters extracted from the integrated FMD system is also presented in Figure 2. In
248 addition, parameters of WSR and diameter during brachial artery FMD and nitroglycerin-
249 mediated dilation in a healthy young cohort are shown in Table 2.

250

251 ***WSR variables that best predict a normal FMD response***

252 The results of partial correlation analysis between WSR parameters and diameter changes
253 during brachial artery FMD in the whole cohort are shown in Table 3. WSR at peak
254 hyperemia, WSR auc and absolute WSR increase were significantly associated with both
255 absolute and percentage diameter changes (all $p<0.05$). WSR SL1 and WSR aucttp were
256 significantly associated with percentage diameter changes (both $p<0.05$). We then
257 performed a stepwise multivariate regression analysis to determine the strongest WSR

258 predictor(s) of diameter changes during brachial artery FMD in the whole cohort. When
259 WSR at peak hyperemia, WSR auc, absolute WSR increase and the study centre were all
260 included in the same model at the same time, WSR auc was the best predictor of absolute
261 brachial artery diameter change ($\beta=0.503$, $R^2=0.25$) and percentage diameter change
262 ($\beta=0.560$, $R^2=0.31$). Collinearity statistics (tolerance and variance inflation factor) did not
263 indicate a collinearity problem in this model. These associations remained significant when
264 including WSR at baseline or baseline brachial artery diameter in the model above.

265

266 ***Influence of the WSR patterns during hyperemia on the FMD response***

267 A schematic description of the monophasic and biphasic patterns of WSR increase is
268 presented in Figure 3. Table 4 shows the parameters of WSR and diameter during brachial
269 artery FMD and nitroglycerin-mediated dilation stratified by monophasic and biphasic WSR
270 increase patterns. During reactive hyperemia, we observed the monophasic pattern of WSR
271 increase in 15 participants (9 females) and the biphasic pattern of WSR increase in 18
272 participants (10 females). Individuals with the biphasic pattern showed a significantly
273 greater WSR SL1 than those with the monophasic pattern ($p<0.05$). The parameters that
274 were associated with diameter changes (WSR at peak hyperemia, WSR auc and absolute
275 WSR increase from baseline) were significantly greater in the biphasic pattern than the
276 monophasic pattern (Figure 4A-C). Similarly, individuals with the biphasic pattern showed a
277 significantly greater WSR baseline and WSR aucttp than those with the monophasic pattern
278 (all $p<0.05$). WSR Tp took longer in individuals with the biphasic pattern than in those with
279 the monophasic pattern ($p<0.05$). The absolute diameter increase following reactive
280 hyperemia was significantly greater in individuals with the biphasic pattern than in
281 individuals with the monophasic pattern (0.24 ± 0.10 mm vs 0.17 ± 0.09 mm, $p<0.05$, Figure

282 5A). Percentage diameter increase tended to be greater in individuals with the biphasic
283 pattern than individuals with the monophasic pattern ($7.6\pm 3.3\%$ vs $5.3\pm 3.5\%$, $p=0.08$,
284 Figure 5B). However, to determine if WSR auc stimulus (the strongest predictor of FMD)
285 explained the difference in FMD response between the two groups, we used an ANCOVA
286 model that included WSR auc. This analysis showed that there were no differences in
287 absolute or percentage brachial artery diameter change during FMD between the
288 monophasic and biphasic groups when the WSR auc stimulus was taken into account (data
289 not shown). Following nitroglycerin spray, there were no differences in WSR or change in
290 brachial artery diameter between the mono- and biphasic groups (Table 4).

291

292 **DISCUSSION:**

293 Our main findings are that, using multi-gate spectral Doppler, we were able to acquire and
294 extract seven WSR magnitude and four WSR time-course parameters over a long time-
295 period to more comprehensively characterize the FMD response. We were also able to
296 derive the first WSR-FMD stimulus-response data in humans using this method to provide a
297 first point of reference in healthy, young adults. Furthermore, we were able to show that in
298 this cohort, the WSR area under the curve until its return to baseline was the strongest
299 predictor of the brachial artery diameter change in response to hyperemia. For the first
300 time, we were able to identify mono- and biphasic WSR stimulus patterns within our cohort
301 that produced different magnitude of FMD response. However, these responses were not
302 different when the strongest WSR predictor (WSR auc) was statistically taken into account,
303 illustrating the importance of knowing the WSR stimulus in order to correctly interpret the
304 response.

305

306 ***Novel WSR variables, observations and comments on their values***

307 We identified seven WSR magnitude variables and four WSR time-course variables that add
308 novel measurements to the WSR-FMD response. These measurements took into account
309 key phases of the FMD procedure and in combination with more traditional measurements,
310 provide a comprehensive characterization of FMD-WSR response. Our data show that WSR
311 is reduced during cuff-occlusion to about one-third that of baseline and that during reactive
312 hyperaemia, peak WSR is almost six-times greater compared with baseline and is over 18-
313 times greater compared with WSR during cuff-occlusion.

314

315 Our data also show that peak WSR was reached quickly (~12s) and preceded the time of
316 peak dilation by an average of ~43s. We also note that the time taken to reach peak WSR
317 was relatively homogenous between subjects, whereas there was considerable inter-
318 individual variability in the time for diameter to reach peak diameter. The time taken for
319 WSR to return to baseline (~104s) preceded the time for diameter to return to baseline in 15
320 participants but in the remaining 18, arterial diameter returned to baseline before WSR. The
321 physiological mechanisms underlying this variability are unknown and were beyond the
322 scope of this study, but putatively provide an opportunity to better understand the arterial
323 response to the dynamic WSR changes during reactive hyperemia.

324

325 ***Multi-gate Doppler WSR auc is a good predictor of FMD***

326 A key aim of the current study was to determine which WSR stimulus variable was the best
327 predictor of the FMD response in healthy young adults. In a regression model that included
328 the three WSR variables that were significantly associated with both absolute and relative
329 diameter change (WSR peak, WSR auc and WSR absolute increase), we found that WSR auc

330 was the only predictor of both absolute ($R^2=0.25$) and relative ($R^2=0.31$) diameter changes,
331 and this was the case irrespective of whether baseline diameter was included in the model.
332 Given that WSR auc explains 25-31% of the variance in the regression model, it seems
333 reasonable to conclude that this is an important predictor of the FMD response, whilst
334 acknowledging the limitations of the R^2 statistics. Other factors influencing diameter change
335 likely include the stiffness of the brachial artery, mechano-transduction of WSR to the
336 endothelium, cell signalling in response to the transduced stimulus and the regulation of
337 smooth muscle cell tone.

338

339 The association between WSR and the brachial artery FMD response has previously been
340 reported in a cohort of young adults (14, 16, 17, 25). Using a similar cross-sectional study
341 design as ours, Thijssen and colleagues found that WSR aucttp explained 14% of the
342 variance in the FMD response in young healthy adults (25) and we found that WSR auc using
343 our method explained 25-31% of the variance in the FMD response. This suggests that the
344 WSR stimulus is an important contributor to the FMD response, but whether FMD should be
345 'normalized' to WSR is a contentious issue. Whereas some have suggested 'correcting (or
346 normalizing)' data by dividing FMD by WSR (17), recent guidelines (24) recommend
347 reporting WSR and FMD together without corrections. Consistent with this, where WSR has
348 been measured with multi-gate Doppler, we suggest that the 'normal' stimulus-response
349 characteristics should be reported as WSR auc and FMD together, and that the association
350 between WSR auc and FMD are determined using statistical models. It was not the purpose
351 of this study to determine this relationship in characteristically different populations or
352 diseased populations; instead, the data presented here, together with the novel method of
353 data acquisition, provide a platform for future studies of this nature.

354

355 ***Mono- and biphasic WSR responses in healthy young adults***

356 An advantage of being able to continuously measure WSR was that we were able to
357 observe, for the first time to our knowledge, two distinct patterns of WSR increase during
358 reactive hyperemia: A 'monophasic' and a 'biphasic' WSR pattern that occurred between
359 cuff release and peak WSR. Compared to the monophasic pattern, the biphasic pattern was
360 associated with a greater magnitude as well as faster kinetics (steeper increase) of WSR
361 increase during hyperemia. The biphasic pattern was also associated with a greater brachial
362 artery vasodilatory response. However, when WSR auc stimulus was statistically taken into
363 account, the vasodilatory response was no longer different between mono- and biphasic
364 groups; that is, the dilation was matched to the WSR stimulus. This finding shows that
365 within an otherwise characteristically similar cohort, there are two distinct vasodilatory
366 responses to reactive hyperemia but that vasodilation is, ultimately, matched to the WSR
367 stimulus.

368

369 The two different WSR responses may allude to underlying physiological differences
370 between the cohorts, although their significance is unknown. It is possible that different
371 mechanisms regulating blood flow during hyperemia produce the two different stimulus-
372 response relationships. There are two candidate sites for the regulation of FMD: First, the
373 arterial endothelium above the cuff that is experiencing an increase in WSR during
374 hyperemia; second, the downstream microvasculature below the cuff that has dilated
375 during cuff-occlusion and experiences a sudden increase in blood flow. During hyperemia,
376 the endothelium of the brachial artery stimulates dilation in order to normalise the
377 increased WSR brought about by hyperaemic flow. Differences in mechano-transduction of

378 WSR or differences in the paracrine response to the change in WSR could cause differences
379 in the temporal pattern of WSR and magnitude of dilation. Microvascular function
380 downstream of the cuff-occlusion site could also explain different WSR patterns (3, 13)
381 because flow during reperfusion is influenced by downstream microvascular dilation (6).
382 Microvessels also respond to reperfusion, including a vasoconstrictor response that likely
383 influences upstream blood flow and, therefore, WSR. Consistent with this, we have
384 previously shown distinct differences in the autoregulatory response to reperfusion by
385 microvessels that temporally altered perfusion and oxygenation of tissue (1). Structural
386 alterations in the microcirculation (21) can also influence its ability to respond to ischemia-
387 reperfusion, which might influence the upstream WSR stimulus and explain differences
388 between individuals. This is a first observation of two distinct WSR increase patterns and we
389 did not explore control mechanisms in the first instance. Nor do we know if these WSR
390 increase patterns are observed in characteristically different populations or diseased
391 populations. Future studies will shed light on these issues and will ultimately determine
392 whether there is value in determining two different WSR responses during the FMD
393 assessment. But the ability to measure different hyperemic responses itself may be useful
394 for future physiological studies.

395

396 ***The flow velocity profile is not always parabolic and symmetrical***

397 One advantage of using multi-gate Doppler is that the flow velocity profile (normally
398 assumed to be parabolic and symmetric, but which cannot be seen with conventional
399 Doppler) can be seen in real time during data acquisition. One revelation from the current
400 study was the heterogeneity in this flow-profile within the cardiac cycle, under different
401 flow-states, and between subjects. During hyperemia, the shape of the parabola was seen

402 to be blunt, M-shaped, asymmetric and symmetric (see examples in Figure 1) and the shape
403 of the parabola typically varied within the same cardiac cycle. Heterogeneity was also
404 observed between subjects and was apparent at baseline, low-flow and during reactive
405 hyperemia. These observations point to the importance of being able to detect flow velocity
406 at different spatial points in the vessel in order to accurately measure WSR by extracting
407 these local velocities.

408

409 ***'Low-flow' WSR and diameter during cuff-occlusion***

410 We also found that all participants reduced WSR during cuff-occlusion but only 17 exhibited
411 reduced brachial artery diameter (1 remained similar to baseline and 15 exhibited increased
412 brachial artery diameter). This finding suggests that the brachial artery response to low-flow
413 might be independent of WSR, at least in young, healthy adults.

414

415 ***Reduced vessel diameter immediately after cuff-release and its influence on WSR increase*** 416 ***and diameter response***

417 We noticed that immediately after cuff-release, 24 participants showed a reduced brachial
418 artery diameter and nine did not. To determine whether this reduction of diameter
419 immediately after cuff-release influenced the initial increase in WSR as well as the
420 subsequent FMD response, we performed a sub-group analysis of participants stratified by
421 the presence or absence of reduced brachial diameter. We found that absolute WSR
422 increase (496.1 ± 128.4 vs 477.6 ± 134.4 $1/s$, $p=0.733$) and WSR SL1 (80.3 ± 24.5 vs 78.7 ± 25.5
423 $1/s^2$, $p=0.874$) were both similar between those with reduced brachial diameter and those
424 without. Similarly, absolute diameter change (0.20 ± 0.10 vs 0.22 ± 0.12 mm, $p=0.707$) and
425 relative diameter change (6.5 ± 3.9 vs 6.8 ± 3.9 %, $p=0.852$) were not different between those

426 with reduced diameter and those without. These observations indicate that the reduced
427 brachial artery diameter immediately after cuff-release does not play a major role in the
428 initial increase in WSR as well as the subsequent FMD response, at least in our population of
429 young, healthy adults. The reduction in diameter may be due to an acute pressure drop, a
430 brief period of turbulent flow, and alteration of smooth muscle cell tone or some
431 combination.

432

433 ***WSR response during nitroglycerin-mediated vasodilation***

434 There is a paucity of WSR data during the assessment of nitroglycerin-mediated
435 vasodilation. It has been used as an endothelium-independent control test to ensure the
436 validity of the FMD assessment. The application of nitroglycerin, thought to be an
437 exogenous nitric oxide donor, reduces smooth muscle cell tone and induces vasodilation. As
438 such, this has been considered WSR-independent and, thus, that there is no clear benefit for
439 acquiring WSR data during the assessment. Our observations support the position that
440 nitroglycerin-mediated vasodilation is WSR-independent, because WSR was slightly reduced
441 (Table 2) at the time that peak diameter occurred following administration of nitroglycerin.
442 Reduced WSR is likely a result of increased brachial artery diameter at the time of
443 measurement and suggests that nitroglycerin administration over-rides any effect of altered
444 WSR.

445

446 ***Implications from this study***

447 Our study highlights the importance of being able to measure the WSR stimulus as well as
448 the vasodilatory response to hyperemia. An accurate estimation of WSR close to the arterial
449 wall by conventional Doppler ultrasound, especially during reactive hyperemia, is inherently

450 difficult, further exacerbated by the uncertainties associated with the assumptions used to
451 estimate WSR (especially that blood flow maintains a parabolic profile). We have shown that
452 multi-gate Doppler overcomes these limitations by measuring blood flow velocity from
453 near-to-far wall and by directly estimating WSR close to the arterial wall. By integrating
454 these measurements with continuous and simultaneous measurement of arterial diameter,
455 we were able to generate detailed information about the WSR stimulus and FMD response
456 that has not been seen previously. For example, the system enabled us to reveal the
457 monophasic and biphasic WSR patterns and allowed us to determine the most important
458 WSR predictor of FMD. We are also able to characterise the 'normal WSR-FMD relationship
459 in our cohort of healthy young adults, establishing a reference for these measurements. As
460 such, we have demonstrated the usefulness of multi-gate Doppler as a modality for
461 measuring arterial WSR, in this instance integrated into the ULA-OP system.

462

463 Overall, our study represents a technical advance that enables comprehensive WSR-FMD
464 stimulus-response measurement within an integrated ultrasound system. The need to
465 measure WSR continues to cause researchers to rely heavily on imprecise WSR data derived
466 from vessel center-line peak velocity, creating uncertainties for the accurate interpretation
467 of the FMD response. In a clinical setting, measurement of WSR has found little utility. Our
468 broader aim is to provide a better tool for researchers and clinicians to augment the
469 accuracy and usefulness of WSR-FMD measurement. This has the potential to expand
470 current understanding of vascular physiology and pathophysiology, vascular ageing and the
471 vascular response to interventions. In a clinical setting, this has the potential to improve
472 clinical evaluation and management of patients with many diseases that involve blood
473 vessels.

474

475 **Limitations**

476 We did not assess brachial artery stiffness which has been reported to affect the magnitude
477 of FMD response (29) and may have contributed to the differences seen here. In addition,
478 due to the cross-sectional nature of this study, we cannot infer any causation from our
479 results. Finally, because multi-gate spectral Doppler is an extension of pulsed-wave (PW)
480 Doppler, it has the same limitations of PW Doppler; for example, analysis limited to the axial
481 velocity component (28) or possible velocity detection difficulties in the presence of high-
482 level clutter.

483

484 **CONCLUSION:**

485 Overall, our results demonstrate the importance of being able to accurately determine a
486 simultaneous WSR-FMD measurement, provide a reference for the 'normal' WSR-FMD
487 response, and present a number of novel variables that may enable better understanding of
488 vascular physiology and pathology.

489

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493

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502

503 **DISCLOSURE:**

504 Nothing to disclose.

505

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589 dilatation of the brachial artery is affected by local elastic vessel wall properties in high-risk patients.
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591 **FIGURE LEGENDS:**

592 **Figure 1.** Examples of multi-gate spectral Doppler profiles obtained from the ULA-OP
 593 system, in red-to-white color scale, and the related mean frequency, which is overlaid in
 594 blue. The first row shows multi-gate spectral Doppler profiles at baseline at different time
 595 points in the cardiac cycle from the same individual (1A, early systole; 1B, peak systole; 1C,
 596 early diastole). The second row shows multi-gate spectral Doppler profiles during peak
 597 hyperemia at the same time points in the cardiac cycle as Fig 1A-C from the same
 598 participant (1D, early systole; 1E, peak systole; 1F, early diastole). The third row shows
 599 multi-gate spectral Doppler profiles during peak hyperemia at the same time points as Fig
 600 1D-F in the cardiac cycle but from a different participant (1G, early systole; 1H, peak systole;
 601 1J, early diastole). Note the asymmetry in velocity in some profiles (e.g. 1E, 1H and 1J), blunt
 602 profile (1F) and M-shaped profile (1G). Also note the differences in spectral profile during
 603 cardiac cycle as well as between participants. Sub 1, subject 1; Sub 2, subject 2.

604

605 **Figure 2.** A schematic description of WSR parameters obtained from brachial artery FMD
 606 assessment using continuous multi-gate Doppler and simultaneous diameter. In the upper
 607 panel, traces in light blue and red represent the peak and mean values of WSR, respectively.
 608 In the lower panel, traces in light blue and red represent the variations in diameter (due to
 609 cardiac cycle) and mean diameter of the brachial artery, respectively. WSR, wall shear rate;
 610 SL1, first slope of wall shear rate increase; SL2, second slope of wall shear rate increase;
 611 aucttp, area under the curve until time to peak dilation (area shaded with turquoise); auc,
 612 area under the curve until its return to baseline level (area shaded both with turquoise and
 613 grey); Tp, time to peak value; Tb, time to return to baseline value; Δ , changes.

614

615 **Figure 3.** Representative examples of monophasic (upper panel) and biphasic (lower panel)
616 patterns of WSR increase and diameter changes during brachial artery FMD assessment.
617 Traces in red represent a mean value of WSR or brachial artery diameter.

618

619 **Figure 4.** WSR at peak hyperemia (A), WSR auc (B) and absolute WSR increase from baseline
620 (C) between monophasic (n=15) and biphasic (n=18) WSR increase patterns. Data are shown
621 as means±SE. *Significantly different from the monophasic group ($p<0.05$).

622

623 **Figure 5.** Absolute diameter changes (A) and percentage diameter changes (B) between
624 monophasic (n=15) and biphasic (n=18) WSR increase patterns. Data are shown as
625 means±SE. *Significantly different from the monophasic group ($p<0.05$).

626 **Table 1. Selected characteristics of the study participants**

627

	Values
Participants, n	33
Age, yrs	27.5±4.9
Sex, m/f	14/19
BMI, kg/m²	22.7±3.1
Systolic BP, mmHg	114.1±10.4
Diastolic BP, mmHg	69.2±6.6
Heart Rate, bpm	64.2±9.5

628

629 Data are means±SD or numbers. BMI, body mass index; BP, blood pressure.

630 **Table 2.** Parameters of wall shear rate and diameter during brachial artery flow-mediated
 631 dilation and nitroglycerin-mediated dilation assessments in a healthy young cohort
 632

	Values	Ranges
<i>Flow-mediated dilation (n=33)</i>		
<i>WSR magnitude parameters</i>		
WSR baseline, 1/s	103.9±54.9	17.5 - 222.6
WSR low-flow, 1/s	32.6±23.4	-1.8 - 94.9
WSR peak, 1/s	594.9±158.2	269.1 - 924.6
WSR Δ, 1/s	491.0±160.7	188.4 - 845.0
WSR %Δ, %	564 (267-994)	155.2 - 3270.5
WSR aucttp, au	13414±5629	3515 - 30540
WSR auc, au	17337±6724	4064 - 39473
<i>WSR time-course parameters</i>		
WSR SL1, 1/s ²	79.9±27.3	24.3 - 126.3
WSR SL2, 1/s ² *	16.4±8.8	0.33 - 35.4
WSR Tp, s	12.2±2.6	7.3 - 20.6
WSR Tb, s	104.1±36.6	57.5 - 182.5
<i>Diameter magnitude parameters</i>		
Diameter baseline, mm	3.29±0.45	2.57 - 4.24
Diameter Δ, mm	0.21±0.10	0.03 - 0.47
Diameter %Δ, %	6.5±3.5	0.95 - 14.8
<i>Diameter time-course parameters</i>		
Diameter Tp, s	55.3±31.2	26.8 - 197.9
Diameter Tb, s	113.6±59.8	10.5 - 249.5
<i>Nitroglycerin-mediated dilation (n=13)</i>		
<i>WSR magnitude parameters</i>		
WSR baseline, 1/s	58.6±20.9	33.1 - 101.2
WSR peak dilation, 1/s	44.7±24.6	17.9 - 87.8
<i>Diameter magnitude parameters</i>		
Baseline diameter, mm	3.51±0.56	2.48 - 4.30
Diameter Δ, mm	0.77±0.15	0.56 - 1.10
Diameter %Δ, %	22.5±4.8	13.8 - 28.4

633
 634 Data are means±SD for variables with normal distribution, median (interquartile range) for
 635 variables with skewed distribution, and range of each variable (minimum to maximum).
 636 *Obtained from 18 participants who showed the biphasic WSR increase response. WSR, wall
 637 shear rate; SL1, first slope of wall shear rate increase; SL2, second slope of wall shear rate
 638 increase; aucttp, area under the curve until time to peak dilation; auc, area under the curve
 639 until its return to baseline value; Tp, time to peak value; Tb, time to return to baseline value;
 640 Δ, changes.

641 **Table 3.** Partial correlation analysis between parameters of WSR and diameter changes in
 642 the whole cohort.
 643

	Diameter Δ	Diameter % Δ
WSR baseline	<i>r</i> =0.18, <i>p</i> =0.327	<i>r</i> =0.13, <i>p</i> =0.470
WSR low-flow	<i>r</i> =0.32, <i>p</i> =0.072	<i>r</i> =0.32, <i>p</i> =0.077
WSR SL1	<i>r</i> =0.35, <i>p</i> =0.053	<i>r</i> =0.36, <i>p</i> =0.041
WSR SL2*	<i>r</i> =0.17, <i>p</i> =0.524	<i>r</i> =0.24, <i>p</i> =0.353
WSR peak	<i>r</i> =0.41, <i>p</i> =0.020	<i>r</i> =0.47, <i>p</i> =0.007
WSR Δ	<i>r</i> =0.41, <i>p</i> =0.021	<i>r</i> =0.49, <i>p</i> =0.004
WSR %Δ	<i>r</i> =0.04, <i>p</i> =0.843	<i>r</i> =0.11, <i>p</i> =0.558
WSR aucttp	<i>r</i> =0.34, <i>p</i> =0.061	<i>r</i> =0.42, <i>p</i> =0.017
WSR auc	<i>r</i> =0.46, <i>p</i> =0.008	<i>r</i> =0.56, <i>p</i> =0.001
WSR Tp	<i>r</i> =0.32, <i>p</i> =0.072	<i>r</i> =0.32, <i>p</i> =0.079
WSR Tb	<i>r</i> =0.04, <i>p</i> =0.109	<i>r</i> =0.35, <i>p</i> =0.052

644
 645 *Obtained from 18 participants who showed the biphasic WSR increase response. WSR, wall
 646 shear rate; SL1, first slope of wall shear rate increase; SL2, second slope of wall shear rate
 647 increase; aucttp, area under the curve until time to peak dilation; auc, area under the curve
 648 until its return to baseline value; Tp, time to peak value; Tb, time to return to baseline value;
 649 Δ , changes.

650 **Table 4.** Parameters of wall shear rate and diameter during brachial artery flow-mediated
 651 dilation and nitroglycerin-mediated dilation assessments stratified by monophasic and
 652 biphasic patterns of wall shear rate increase.
 653

	Monophasic (n=15)	Biphasic (n=18)
Flow-mediated dilation		
WSR magnitude parameters		
WSR baseline, 1/s	89.6±54.6	115.9±53.8*
WSR low-flow, 1/s	32.7±23.8	32.5±23.7
WSR %Δ, %	596(181-1374)	552(286-735)
WSR aucttp, au	10784±4961	15607±5309*
WSR time-course parameters		
WSR SL1, 1/s ²	68.8±30.0	89.1±21.5*
WSR SL2, 1/s ²	-	16.4±8.8
WSR Tp, s	11.1±3.2	13.2±1.6*
WSR Tb, s	92.1±31.9	114.1±38.1
Diameter magnitude parameters		
Diameter baseline, mm	3.29±0.44	3.28±0.47
Diameter time-course parameters		
Diameter Tp, s	56.6±43.8	54.2±15.8
Diameter Tb, s	110.8±62.3	116.0±59.3
Nitroglycerin-mediated dilation (5 monophasic and 8 biphasic)		
WSR magnitude parameters		
WSR baseline, 1/s	43.0±10.2	68.4±20.1*
WSR peak dilation, 1/s	38.9±21.5	48.3±27.0
Diameter magnitude parameters		
Baseline diameter, mm	3.38±0.60	3.59±0.56
Diameter Δ, mm	0.83±0.10	0.74±0.17
Diameter %Δ, %	25.0±3.9	21.0±4.8

654

655 Data are means±SD for variables with normal distribution, and median (interquartile range)
 656 for variables with skewed distribution. *significantly different from the monophasic group
 657 ($p<0.05$). WSR, wall shear rate; SL1, first slope of wall shear rate increase; SL2, second slope
 658 of wall shear rate increase; aucttp, area under the curve until time to peak dilation; auc,
 659 area under the curve until its return to baseline value; Tp, time to peak value; Tb, time to
 660 return to baseline value; Δ, changes.

Figure 1.

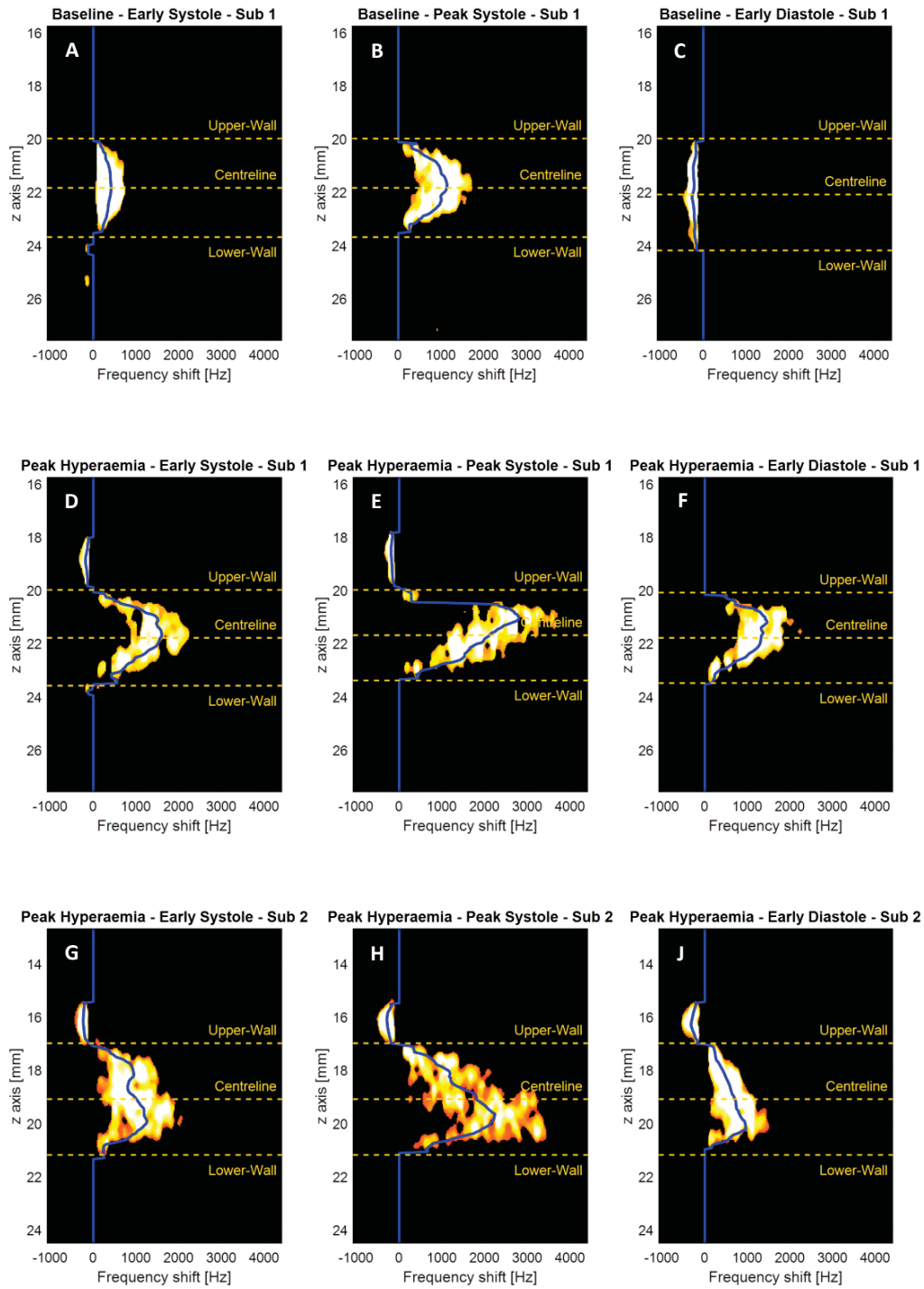


Figure 2.

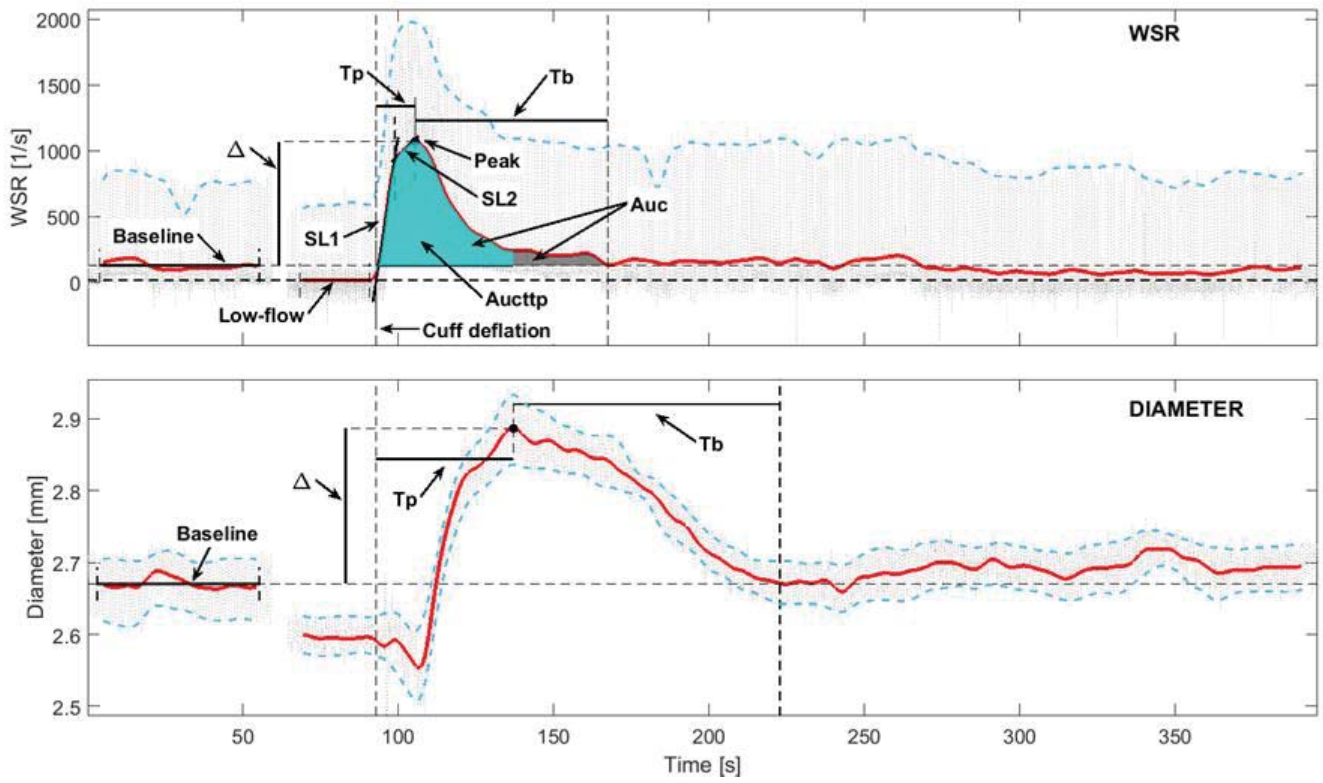


Figure 3.

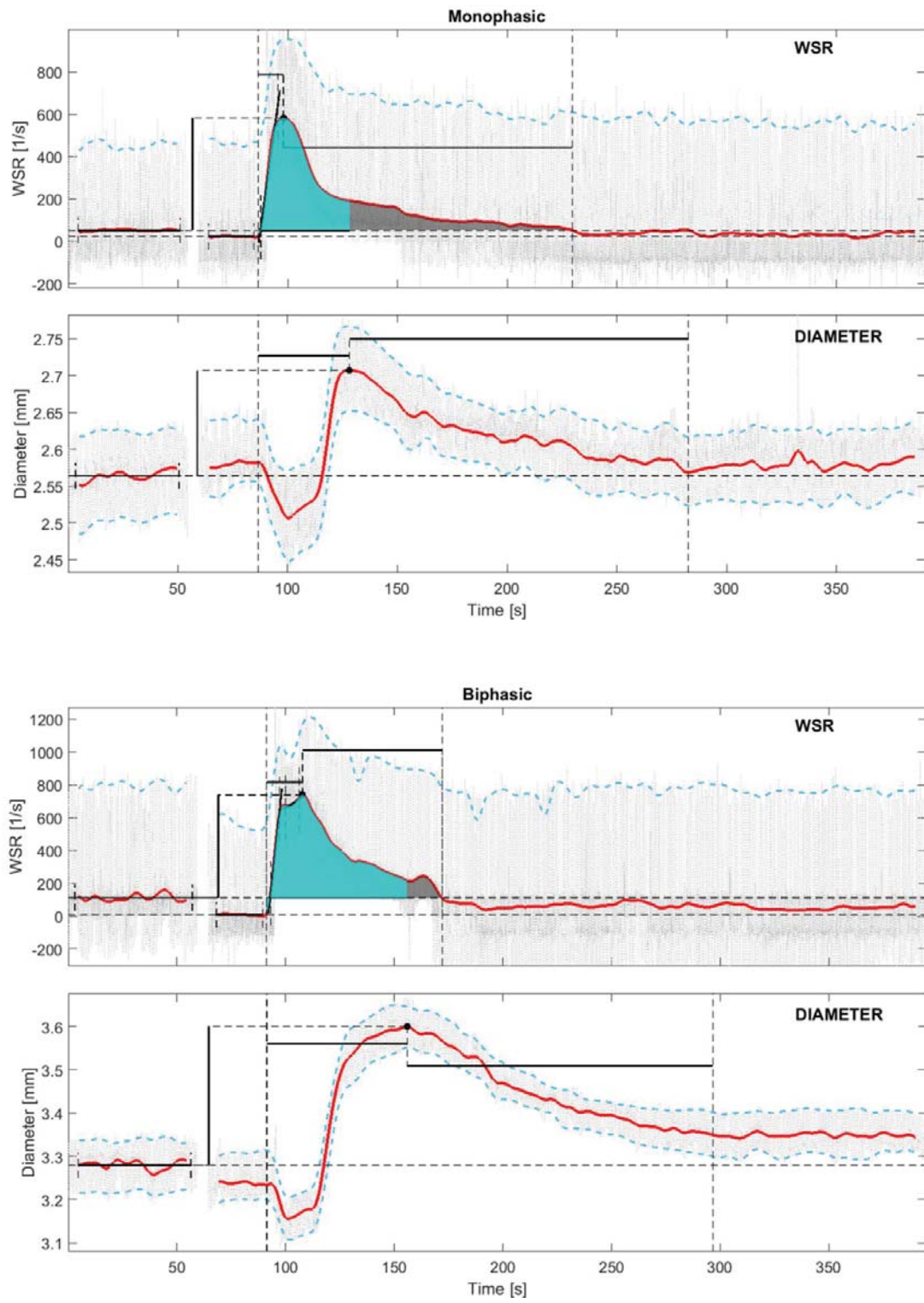
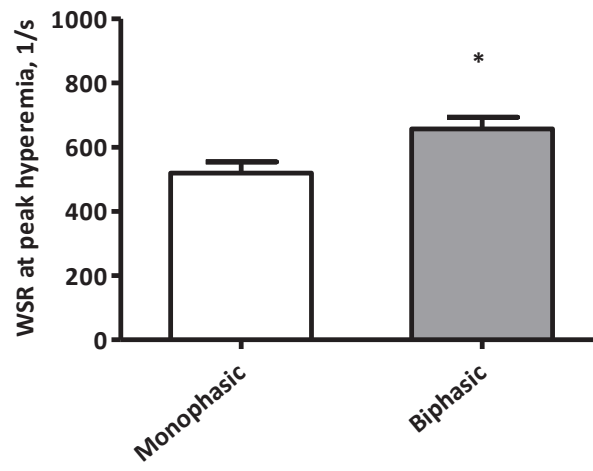
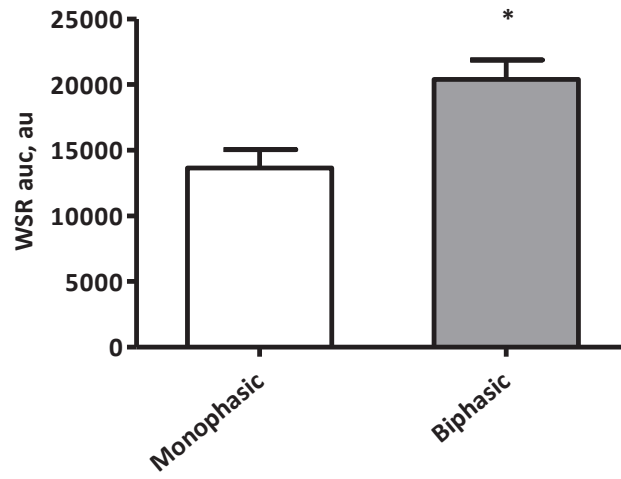


Figure 4.

A



B



C

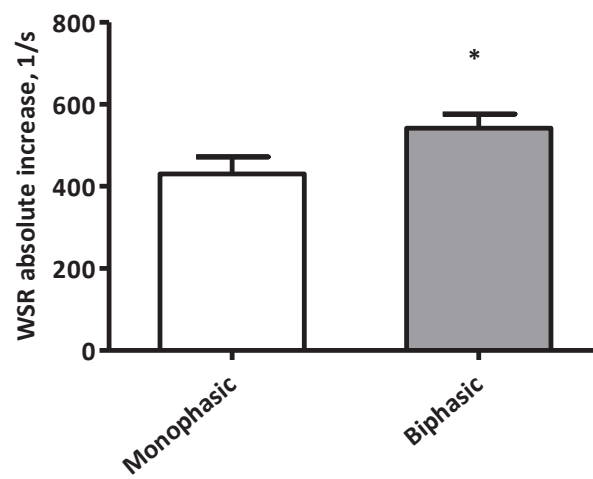


Figure 5.

