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1 **Increased tissue oxygenation explains the attenuation of hyperaemia upon repetitive**
2 **pneumatic compression of the lower leg**

3

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6

7 **Author contribution**

8 AM: conception and design of the experiment, collection, analysis and interpretation of the data, drafting the
9 manuscript

10 GC: collection, analysis and interpretation of the data, drafting of the manuscript

11 WF: design of the experimental set-up, collection, analysis and interpretation of the data

12 DM: design of the experimental set-up ,collection, analysis and interpretation of the data

13 CF: design of the experiment, critical revision of the manuscript

14 SR: conception and design of the experiment and critical revision of the manuscript

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16

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19 **Running Head** Tissue oxygenation modulates compression-induced hyperaemia

20

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26 **ABSTRACT**

27 **Aim**

28 The rapid hyperaemia evoked by muscle compression is short-lived and was recently shown to undergo a
29 rapid decrease even in spite of continuing mechanical stimulation. The present study aims at investigating
30 the mechanisms underlying this attenuation which include local metabolic mechanisms, desensitization of
31 mechano-sensitive pathways, and reduced efficacy of the muscle pump.

32 **Methods**

33 In 10 healthy subjects short sequences of mechanical compressions (n=3-6; 150 mmHg) of the lower leg
34 were delivered at different inter-stimulus intervals (ranging from 20 to 160 s) through a customized
35 pneumatic device. Hemodynamic monitoring included near infrared spectroscopy, detecting tissue
36 oxygenation and blood volume in calf muscles, as well as simultaneous echo-Doppler measurement of
37 arterial (superficial femoral artery) and venous (femoral vein) blood flow.

38 **Results**

39 The results indicate that: i) a long lasting (>100 s) increase in local tissue oxygenation follows the
40 compression-induced hyperaemia ; ii) the compression-induced hyperaemia exhibits different patterns of
41 attenuation depending on the inter-stimulus interval; iii) the amplitude of the hyperaemia is not correlated
42 with the amount of blood volume displaced by the compression; iv) the extent of attenuation negatively
43 correlates with tissue oxygenation ($r=-0,78$, $P<0.05$).

44 **Conclusion**

45 Increased tissue oxygenation appears to be the key factor for the attenuation of hyperaemia upon repetitive
46 compressive stimulation. Tissue oxygenation monitoring is suggested as a useful integration in medical
47 treatments aimed at improving local circulation by repetitive tissue compression.

48

49 **NEW AND NOTEWORTHY**

50 This study shows that i) the hyperaemia induced by muscle compression produces a long-lasting increase in
51 tissue oxygenation; ii) the hyperaemia produced by subsequent muscle compressions exhibits different
52 pattern of attenuation at different inter-stimulus intervals; iii) the extent of attenuation of the compression-
53 induced hyperaemia is proportional to the level of oxygenation achieved in the tissue. The results support the
54 concept that tissue oxygenation is a key variable in blood flow regulation.

55

56 **Keywords:** muscle blood flow, hyperaemia, muscle compression, tissue oxygenation.

57

58 Glossary

59 IPC intermittent pneumatic compression

60 ISI inter-stimulus interval

61 NIRS near-infrared spectroscopy

62 SRS spatially-resolved spectroscopy

63 THI total haemoglobin index

64 TOI tissue oxygenation index

65

66

67 INTRODUCTION

68 Since the seminal work of Mohrman and Sparks (39) several studies have demonstrated that a rapid and
69 transient hyperaemic response can be elicited by a short-lasting muscle compression (10, 30, 38, 56-60).

70 Although the underlying mechanisms have not been fully identified, this phenomenon has been well
71 documented in different experimental models, such as the isolated muscle (39), awake and anesthetized
72 animals (57, 58, 60) and humans (10, 30, 38, 56). In addition a rapid dilatory response to compressive
73 stimuli has also been observed in isolated feed arteries (7). More controversial is the hemodynamic response
74 to repeated compressive stimuli. Kirby et al (30) observed that the response to 5 consecutive compressions
75 was non-significantly attenuated with respect to the response to a single compression. Conversely, Clifford et
76 al (7) using the same pattern of 5 consecutive compressive stimuli on an isolated muscle feed arteries
77 observed a significant increase of the dilatory response as compared to the single compression.

78 In a recent work Turturici and colleagues investigated the blood flow response to a longer lasting sequence
79 of mechanical stimulations (20 compressions, 1 s ON /1 s OFF) reporting that the initial hyperaemic
80 response progressively fades away in spite of continuing stimulation and hypothesized that the
81 mechanosensitive mechanism underlying the response could undergo some kind of transient inactivation
82 (60). In fact, the attenuation of the compression-induced hyperaemia was observed to increase at increasing
83 stimulation frequencies (60). A similar behavior was recently observed also in humans (38).

84 Surprisingly this phenomenon has been poorly described in the several investigations concerning the
85 hyperaemic effect of intermittent pneumatic compressions (IPC) (14, 15, 32-34), and in experimental studies
86 investigating the mechanisms underlying compression and contraction-induced hyperaemia (9, 24, 31, 40,
87 44), with the exception of a short report by Tschakowsky et al (56). In this pioneering investigation the
88 authors observed that repetitive compression of the forearm below heart level exhibited a transient
89 hyperaemia settling to a lower level after 10-20 s from the beginning of the treatment (56). More recently
90 Sheldon et al (47) also reported attenuation of the hyperaemia during IPC treatment, although on a larger

91 time scale (45 vs. 5 min from the beginning) and observed that the effect was dependent on the frequency of
92 stimulation.

93 The issue is relevant because improving limb perfusion is a major aim in the treatment of disorders such as
94 the peripheral arterial disease and is pursued in sport medicine for accelerated recovering from fatigue (1,
95 35). Understanding of the underlying mechanisms is essential for implementing optimal treatments (46).
96 Potential mechanisms underlying attenuation of the hyperaemia during repetitive mechanical stimulation
97 include: 1) inactivation of the mechano-sensitive vasodilatory pathways (60), 2) diminished efficacy of the
98 muscle pump (56), and 3) local regulatory mechanisms that may be activated in response to hyper-perfusion
99 (30, 56). Unfortunately, none of these possibilities is supported by a solid experimental evidence. In
100 particular, 1) mechano-sensitive channels exhibiting inactivation properties have been identified (17, 26), but
101 their actual involvement in the rapid compression-induced dilatation was not ascertained, 2) at high
102 stimulation frequencies incomplete vascular refilling may reduce the contribution of the pump, however, a
103 role for the muscle pump was excluded in a previous animal study (60), and 3) local vasoconstrictory
104 mechanisms are known to act in response to hyper-perfusion but little is known about the actual regulatory
105 variable (O_2 , CO_2 , pH, etc.) and about the strength and timing of this vascular reaction (6, 45). However, in a
106 recent reformulation of the metabolic control of blood flow, a primary role for tissue pO_2 has been postulated
107 (23). According to their model, an excessive rise in O_2 concentration within the tissue would trigger a
108 vasoconstrictory response, mediated by the inhibition of a tonically released vasodilator (23). Along this line,
109 a rise in tissue O_2 occurring during a compression-induced hyperaemia could then trigger a constrictor
110 response and limit further hyperaemic events in response to subsequent mechanical stimuli.

111 On this basis the present study was aimed to test the following hypotheses: 1) the compression-induced
112 hyperaemia elicits a rise in tissue oxygenation, 2) the attenuation of the hyperaemic response to subsequent
113 compressive stimuli is related to the extent of hyper-oxygenation achieved in the tissue, and 3) the other
114 mechanisms, namely, the intrinsic inactivation of mechano-sensitive pathways and the muscle pump would
115 have a minor role in the attenuation of the hyperaemic response upon repetitive compressive stimulation.
116 In order to assess changes in tissue oxygenation, the near infrared spectroscopy (NIRS) was adopted. By
117 locating the NIRS probe under the compressive cuff, continuous monitoring of local oxygenation and blood
118 volume changes from the relevant muscles was achieved. Moreover, in addition to arterial inflow, venous
119 outflow was also monitored as its response to the compression is an indicator of the extent of filling of the
120 venous compartments and thus, of the efficacy of the muscle pump exerted by compressive stimuli.

121

122 **MATERIALS AND METHODS**

123 *Ethical approval*

124 Ten healthy subjects (8 men and 2 women; age: 27.1 ± 3.0 years; weight: 67.9 ± 11.7 kg; height: 176.7 ± 9.7
125 cm) were recruited for the present study. All subjects were normotensive and non-obese.

126 The study conformed to the standards set by the Declaration of Helsinki and was approved by the Local

127 Ethical Committee (Prot. # 60195) and all subjects gave their written informed consent after they were
128 instructed about purpose and procedures of the experiment.

129

130 *Mechanical leg compressions*

131 A previously tested prototype of IPC device was employed in the present study to deliver controlled and
132 repeatable compressions to the leg of the subject (19, 20). Briefly the device exerts a compressive action by
133 inflating five different bladders wrapped around the foot and the calf of the subject, with programmable
134 pressure levels and timing. In the present study all bladders were inflated simultaneously to a supra-systolic
135 pressure of 150 mmHg, with inflation and deflation times of about 3 s each. Two digital pulses are generated
136 by the device to signal the starting time of both inflation and deflation.

137

138 *Near-infrared spectroscopy*

139 Local hemodynamic changes induced by leg compression were measured using a continuous wave NIRS
140 device (NIRO-200NX, Hamamatsu Photonics, Hamamatsu City, Japan), which, besides the classical
141 modified-Lambert-Beer method, supports spatially-resolved spectroscopy (SRS) (16, 52). Since mechano-
142 sensitive vascular reactivity appears to be more prominently expressed by muscular than cutaneous tissues
143 (57) we focused our attention on SRS parameters which, being less affected by cutaneous circulation,
144 provide a more specific monitoring of muscle tissue (2, 36, 37). Since NIRS cannot discriminate between
145 haemoglobin (Hb) and myoglobin (Mb), all measurements always refer to Hb+Mb in the sample volume
146 (51). In particular, TOI (tissue oxygenation index) indicates the ratio $(MbO_2+HbO_2)/(Mbtot+Hbtot)$
147 expressed in percentage, and THI (tissue haemoglobin index) indicates the concentration of (Hb+Mb) in
148 arbitrary units and is therefore an indicator of blood volume changes. Classical Lambert-Beer Parameters
149 (O_2Hb and HHb detecting changes in the concentration of oxygenated and deoxygenated (Hb+Mb),
150 respectively) are only displayed in Fig. 1 and not further considered in the study.

151

152 *Hemodynamic measurements*

153 Measurements of blood velocity in femoral artery and vein were performed simultaneously using two
154 ultrasound systems (MyLab 25 XVision and MyLab 25 Gold, Esaote, Genoa, Italy) equipped with linear
155 arrays (LA 523, Esaote, Genoa, Italy). Superficial femoral artery and femoral vein were insonated distally to
156 the inguinal ligament. Since these instruments could not measure blood velocity and vessel diameter
157 simultaneously, the latter was measured at the beginning and at the end of every stimulation protocol.
158 Doppler measurements were performed by extending the sample volume over the whole vessel size,
159 echographically displayed (transversal approach) in real time. All blood velocity measurements in femoral
160 artery were obtained with insonation angle of about 60° (operating frequency of 6.6 MHz) instead, a higher
161 angle of about 70° (operating frequency of 5 MHz) was used in order to avoid saturation of the recording
162 when assessing the high-speed venous outflow propelled by leg compression. The two probes were placed

163 few centimeters apart with the ultrasound beam of the proximal probe oriented proximally and the one of the
164 distal probe oriented distally, in order to avoid interference between the measurements.

165

166 *Experimental set-up*

167 A schematic representation of the experimental setup is reported in Fig 1 A. All experiments were performed
168 in a quiet room with a constant ambient temperature of about 22-23 °C. The subject sat upright on an
169 adjustable chair with the back supported by a back rest.

170 The NIRS probe was located on the lateral head of gastrocnemius muscle of the right leg (inter-optode
171 distance = 4 cm). The IPC device was wrapped around the lower leg, over the NIRS probe. The two
172 echographic probes were maintained in place by dedicated holders for the whole duration of the protocol.

173

174 *Experimental protocol*

175 After 15 min of rest, an initial series of 3 compressive stimuli with inter-stimulus interval (ISI) of 160 s was
176 delivered to the subject. After other 4 min of rest four series of 6 compressive stimuli were delivered at
177 different frequency (ISI= 20, 40, 60 and 80 s) in randomized order and separated by 4-min resting intervals.
178 Femoral artery and femoral vein diameters were collected at the beginning and at the end of every
179 stimulation protocol. Diameters were measured along a single direction, since both vessels present a circular
180 cross-section in these experimental conditions. Average diameter of the artery was calculated as
181 $(D_s + 2 * D_d) / 3$, D_s being the systolic and D_d the diastolic diameter.

182

183 *Data acquisition and processing*

184 The NIRS signals were digitally acquired along with both Doppler audio signals and the digital synchronism
185 signal from the IPC device by a single acquisition system (CED Micro 1041, Cambridge Electronic Design,
186 Cambridge, UK) and stored on the computer for later analysis with Spike2 software (version 6.10,
187 Cambridge Electronic Design, UK).

188 A specific algorithm was implemented in the Spike2 script language to calculate blood velocity from
189 Doppler audio signals (11, 25). Briefly, power spectra of the audio signals were computed by the Fast
190 Fourier Transform over non-overlapping epochs lasting 25.6 ms. From each spectrum the maximum
191 frequency of the signal (corresponding to maximum blood velocity) was estimated according to D'Alessio
192 (11), then the mean frequency was calculated as the average of all frequencies below the maximum,
193 weighted according to spectral amplitude (25). The mean frequency was then time-averaged over each
194 cardiac cycle and converted into blood velocity, $BV = (MF * C) / (2F * \cos\theta)$, where MF is the mean
195 frequency calculated from Doppler shift, C the averaging speed of ultrasound in soft tissue (1540 m/s), F the
196 operating frequency of the Doppler, and θ the insonation angle). Blood flow, in ml/min, was then calculated

197 as mean blood velocity times cross-sectional area of the vessel ($BF = BV * \pi r^2 * 60$, where BV is the blood
 198 velocity expressed in cm/s, and πr^2 the cross sectional area of the vessel in cm^2).

199 The response to each compressive stimulus was characterized by: pre-compression arterial blood flow,
 200 calculated as the average over the 4 s preceding the compression; pre-compression TOI; pre-compression
 201 THI; peak arterial blood flow, as the hyperaemic peak reached after the compression; Δ TOI, calculated as
 202 the difference between the peak TOI reached after the compression and pre-compression TOI; displaced
 203 blood volume, calculated as the product of the area under the curve of the venous blood velocity response
 204 and the cross-sectional area of vein.

205 In addition, the amplitude of the hyperaemic response was also calculated as the difference between peak
 206 arterial flow and pre-compression flow.

207 In order to assess the extent of attenuation of the response throughout the experimental protocol, changes in
 208 blood flow and blood volume were normalized to the changes observed in response to the first delivered
 209 compressive stimulus.

210

211 **Statistics**

212 To examine the effect of repetitive leg compression performed at different ISI on peak blood flow, displaced
 213 blood volume, pre-compression THI and pre-compression TOI, a two-way repeated-measures ANOVA was
 214 used with factors ISI and repetition (GraphPad Prism v 6.0, GraphPad Software, La Jolla, CA). When
 215 significance was found, a Dunnett's post hoc test was performed to assess significant changes within each
 216 series with respect to the response to the first stimulus. Pearson's coefficient was used to assess the
 217 correlation between different variables. All data are expressed as means \pm standard deviation in the text and
 218 means \pm standard error in diagrams. The level of statistical significance was set at $P < 0.05$.

219

220

221 **RESULTS**

222

223 *Single leg compression*

224 A typical response to a single compressive stimulus is reported in figure 1B. Venous blood velocity exhibits
 225 a prompt and short-lasting increase, peaking 1.7 ± 0.2 s after the beginning and terminating before the end of
 226 the compression. The blood volume displaced by compression was on average 28.3 ± 14.8 ml. The increase
 227 in arterial blood flow starts immediately after deflation and peaks in 4.9 ± 1.4 s passing from a basal value of
 228 74.5 ± 22.7 ml min^{-1} to 260.2 ± 83.3 ml min^{-1} during the peak (peak flow is 3.6 ± 1.0 of baseline). Blood
 229 flow generally returns within 15-25 s The response in tissue oxygenation is further delayed. TOI slowly
 230 increases (from 66.4 ± 5.1 to 78.0 ± 4.0 %) and peaks after 20.6 ± 5.1 s from deflation. Local changes in
 231 blood volume are detected by THI exhibiting a rapid decrease during compression followed by a slower
 232 return to the basal level, in agreement with the changes in venous and arterial blood flow, respectively.

233

234 *Repeated leg compressions*

235 The hemodynamic response to repetitive leg compression at different ISI is summarized in Fig 2, each
236 column representing the response to a single stimulus. The upper two rows show the response in terms of
237 peak arterial blood flow and displaced venous blood volume, both variables exhibiting a significant
238 dependence on ISI ($p < 0.01$) and repetition ($p < 0.01$). It can be observed that when ISI = 160 s the response to
239 subsequent stimuli is unchanged. Unchanged response in terms of peak arterial flow and displaced blood
240 volume is also observed in response to the first compression in each series. Instead, both parameters exhibit a
241 progressive attenuation although with different time course at ISI ranging from 20 to 80 s. In particular, the
242 hyperaemia is consistently reduced starting from the second stimulus in the series, at ISI ranging from 20 to
243 60 s, while displaced blood volume is consistently reduced at ISI = 20 and 40 s, starting from the third
244 stimulus. A peculiar pattern is observed at ISI = 80 s where hyperaemia is only attenuated in response to
245 even and not to odd stimuli, while, at the same time displaced blood volume remains unaffected.

246 NIRS parameters, shown in the lower rows of fig 2, exhibited a significant dependence on repetition
247 ($p < 0.01$) but not on ISI, along with a significant interaction between the two factors. It can be observed that
248 pre-compression THI, which can be considered an indicator of vascular filling, qualitatively parallels the
249 changes in displaced blood volume, remaining unchanged at large ISI and exhibiting the most marked
250 reduction at ISI = 20 s. Pre-compression TOI exhibits instead marked increases at all ISIs lower than 160 s
251 starting from the second stimulus in the sequence. It is interesting to observe that its pattern of change is
252 opposite to peak blood flow: i.e., hyperaemic peak is higher if the pre-compression TOI is lower. Note also
253 that the oscillating pattern previously observed in peak blood flow at ISI = 80 s is also exhibited by pre-
254 compression TOI in an opposite way.

255 In order to provide a better understanding of the interplay between the different parameters in the peculiar
256 response to repetitive compression at ISI = 80 s, original tracings are reported from a representative subject
257 in Fig. 3. As described in Fig. 1, the first stimulus elicits a marked hyperaemia which results in a marked
258 increase in oxygenation. The following compression, which occurs when the tissue oxygenation is still high,
259 now elicits a much smaller hyperaemia, resulting in a proportionally smaller increase in TOI and attenuated
260 vascular refilling in THI. The third compression occurs when the TOI is almost returned to basal levels and
261 the elicited hyperaemia resumes its original size. Although it cannot be fully appreciated with this time scale,
262 the venous blood flow response is comparable in all instances as well as the pre-compression level reached
263 by THI.

264 Another representative recording illustrating the pattern at ISI = 20 s is reported in fig 4. Note the
265 disappearance of the hyperaemic response to the second and subsequent stimuli in spite of the fact that
266 arterial blood flow is returned to basal level. A weak hyperaemia reappears only in response to the last
267 stimulus, when also TOI is almost returned to basal level. Note that THI indicates that blood volume is

268 almost fully returned to basal level after the first stimulus (thanks to the marked hyperaemia) but not
 269 afterwards. Accordingly, the venous response is markedly reduced after the third and subsequent stimuli.

270 In general a good correlation was found between the peak blood flow during hyperaemia and the ensuing
 271 increase in oxygenation as shown in fig 5 A, in which all subjects have been pooled and each dot represents
 272 the response to a single compression. The overall r is 0.76 ($p < 0,05$). When individually computed for the
 273 different subjects r ranged between 0.72 and 0.95 ($p < 0,05$) (average 0.78 ± 0.1).

274 On the contrary the hyperaemic response was not correlated with the amount of displaced blood volume as
 275 shown in Fig. 5B ($r = 0.34$, individual r ranging between -0.4 and $+0.3$).

276 Fig 5C shows the correlation between pre-compression TOI and the peak of the hyperaemic response which
 277 is exhibiting an overall $r = -0,434$ ($p < 0.05$), however a much higher within- subject correlation is observed: -
 278 0.78 ± 0.06 , individual r ranging between 0.7 and 0.9 ($p < 0.05$).

279 In Fig. 5D the amplitude of the hyperaemic response (= peak flow-basal flow) instead of peak flow is plot vs.
 280 pre-compression TOI. While the general pictures resembles that of Fig. 5C, it is here better evidenced that
 281 the hyperaemia can be almost abolished at high TOI levels. Moreover, the slope of the regression lines, m ,
 282 allows to quantify the dependence of the hyperaemic response on tissue oxygenation. On average, $m = -$
 283 0.082 ± 0.026 meaning that the compression-induced hyperaemia is attenuated by 8% per unitary increase of
 284 TOI, with respect to its full amplitude (the one that is evoked in resting conditions).

285

286 *Changes in vessel size*

287 A slight increase in vessel diameter was detected from the comparison of measurements performed at the
 288 beginning and at completion of the experimental protocol in both femoral artery (from 6.0 ± 0.8 to 6.2 ± 0.8
 289 mm, $p < 0.05$) and vein (from 8.3 ± 0.9 to 8.6 ± 1.3 mm, $p < 0.05$)

290

291 **DISCUSSION**

292 For the first time a comprehensive approach has been employed for the investigation of the rapid
 293 compression-induced hyperaemia and its adaptation upon repetitive stimulation, which includes continuous
 294 assessment of NIRS indicators of changes in local tissue oxygenation and blood volume as well as
 295 simultaneous monitoring of arterial inflow and venous outflow. This allowed us to describe the early
 296 hyperaemic changes taking place at the beginning of IPC treatments at different frequencies, and to confirm
 297 our initial hypotheses: i) the compression-induced hyperaemia elicits proportional increases in local tissue
 298 oxygenation; ii) the extent of attenuation of the hyperaemic response to subsequent stimuli is related to the
 299 current level of tissue oxygenation; iii) the extent of attenuation is not strictly dependent on the extent of
 300 vascular filling and on the ISI, therefore the attenuation cannot be attributed to the reduced efficacy of the
 301 muscle pump or to a simple, time-dependent, inactivation mechanism of mechano-sensitive pathways.

302

303 *Compression-induced hyperaemia increases tissue oxygenation*

304 A novel observation of the present study is that muscle compression elicits a prominent increase in local
305 tissue oxygenation. This increase is consequent to the induced hyperaemia but is much longer lasting. This
306 aspect is important because it reveals that the return to “control conditions” is not achieved at the end of the
307 hyperaemia, which normally occurs within 15-25 s and may instead require up to 100 - 200 s. This pattern
308 has never been reported for compression-induced hyperaemia but it is in agreement with what occurs in the
309 rapid-onset hyperaemia induced by short contractions (53).

310 It is generally accepted that an increase in perfusion, with unchanged metabolism, increases tissue
311 oxygenation (3, 12). In the present condition, different factors could contribute to the observed TOI increase
312 in response to compression-induced hyperaemia: 1) depletion of the venous-compartment, which alters the
313 proportion of arterial/venous blood in the sample volume; 2) increased Hb saturation in venous blood due to
314 decreased oxygen extraction, given that the hyperaemia occurs in a condition of constant metabolism; 3)
315 increased saturation of myoglobin. The voiding of venous compartment does not seem to affect the TOI
316 signal considerably, as no relevant changes are observed immediately after the compression, including those
317 associated with large blood volume changes (see original tracings in Figs. 1, 3 and 4). Unfortunately, NIRS
318 cannot discriminate between Mb and Hb saturation nor between arterial and venous compartments, thus no
319 univocal explanation can be provided. Irrespective of the underlying reason, the increase in tissue
320 oxygenation was a very consistent feature of the hemodynamic response to the compression of the resting
321 muscle and exhibited a good correlation with the amplitude of hyperaemia (Fig. 5A).

322

323 *Is compression-induced hyperaemia attenuated by increased tissue oxygenation?*

324 Several lines of evidence from the present study support the finding that elevated tissue oxygenation is the
325 factor responsible for the attenuation of the hyperaemia and for the reduced responsiveness to the mechanical
326 stimulus. By looking at the original tracings of Fig. 3 it can be observed that the response to the second
327 compression is smaller as compared to the first and the third responses, while TOI is higher than baseline.
328 The same is visible in Fig.4: the hyperaemic response almost disappears during the initial high oxygenation
329 phase and only later exhibits a tendency to recover, concomitantly with a decrease in TOI. This dependence
330 of peak hyperaemia on pre-compression TOI is also supported by the histograms of Fig. 2 (see opposite
331 patterns of peak blood flow and pre-compression TOI) and is quantitatively assessed by the correlations in
332 Fig. 5 C and D. Moreover, it appears to be rather linear and rather similar between different subjects.
333 According to these indications, the amplitude of the hyperaemic response is attenuated by 8 ± 2 % per
334 unitary increase of TOI meaning that an increase in TOI by 12.5 points virtually abolishes the response.

335 Notably, the dependence of the active vessel dilatation on tissue oxygenation may explain why the same
336 short sequence of compressive stimuli elicited opposite effects in vitro (7), where tissue hyperoxia does not
337 take place, and in vivo (30).

338 In the several studies investigating hemodynamic effects during IPC treatments this pattern of adaptation of
339 the hyperaemia has not been described, possibly because the attention was focused on medium-long term
340 rather than on early effects. Although different devices and patterns of stimulation have been used in
341 previous investigations, an increase in limb perfusion is generally reported, ranging between 20 and 240 %,
342 and being assessed at 5-60 min from the beginning of the treatment (9, 15, 24, 33, 40, 44, 47), which also
343 appear to be little dependent on the stimulation frequency (47). These results are not readily comparable with
344 the present ones because no steady state was reached in our study. It is reasonable to expected that a certain
345 stable increase in perfusion is obtained with prolonged stimulation, once steady tissue oxygenation is
346 achieved.

347

348 *Underlying mechanisms and implications*

349 As discussed above, the attenuation of the mechano-sensitive dilatatory response to multiple compressions
350 could result as a reaction of the tissue to the hyper-perfusion (generated in response to the first stimulus),
351 which entails the washout of metabolites and alteration of the local milieu in which PO_2 is a most relevant
352 variable (4, 23, 27). It is well known that low oxygenation stimulates vasodilatation and, conversely, that
353 increased oxygenation leads to vasoconstriction, although the effects generally observed in humans exposed
354 to increased levels of inspired PO_2 are rather small (5, 62). In the latter study, increasing arterial PO_2 from
355 100 to 2100 mmHg increased resting vascular conductance only by 20-25% and reduced functional
356 hyperaemia by 20% (5). However it must be observed that tissue PO_2 is differently affected by increased
357 arterial PO_2 and hyper-perfusion. In fact while the hyperbaric hypoxia at 2100 mmHg increases the amount
358 of oxygen carried to the tissue by about 30% (5) a 2-fold increase in perfusion results in a 200% increase in
359 oxygen flow. In early studies reactions to hyper-perfusion were investigated on isolated preparations with
360 externally-controlled blood supply (21). However these studies could not provide a clear indication of the
361 time course of the local tissue response, nor could they discriminate between “metabolic” and myogenic
362 response, given that hyper-perfusion was produced by increased perfusion pressure which also resulted in
363 increased transmural pressure (48). In this respect, the compression-induced hyperaemia offers a peculiar
364 model of (transient) tissue hyper-perfusion, characterized by unchanged tissue metabolism, unchanged
365 arterial PO_2 and most likely unchanged neuro-hormonal drive.

366 The prompt counter-reaction to the compression-induced hyperaemia and the concomitant inactivation of the
367 mechano-sensitive dilatation upon increased tissue oxygenation fits with the “bang-bang” model of blood
368 flow control, recently proposed by Golub & Pittman (23) according to which the feedback signal (O_2^- , whose
369 concentration increases in response to increased O_2 availability) carries the information of excessive

370 perfusion and operates a vasoconstriction by inactivating the tonically released vasodilators (namely, nitric
371 oxide), aim of this regulation being to protect the tissue from hyperoxia and prevent excessive perfusion.

372 Accordingly, the vascular mechano-sensitivity, which is considered to mediate the rapid dilatation and the
373 anticipatory (feed-forward) hyperaemia at the beginning of exercise (8, 30, 43, 60) is promptly abolished if
374 the exercise does not take place, due to the hyper-oxygenation produced by the hyper-perfusion. Instead, in
375 the case of exercise the hyper-oxygenation is quickly reduced even below control levels (18) by increased
376 metabolism and no limitation to vasodilation takes place, which results in the "functional hyperaemia". The
377 same mechanism is likely to explain why both passive movement hyperaemia is attenuated upon repeated
378 stimulation (54, 55) and contraction-induced hyperaemia is attenuated after a sequence of muscle
379 compressions (38).

380 Surprisingly, with one exception (34) no study has ever included NIRS in the characterization of the
381 hyperaemic response to compression and IPC. Although tissue oxygenation can be considered a major
382 outcome of perfusion, in the short term it does not strictly follow arterial blood flow, e.g., in Fig. 2 TOI is
383 maintained at high levels for some time, after the end of hyperaemia. On this basis, it might be more
384 appropriate to monitor TOI rather than blood flow in order to better appreciate the actual effects of the
385 treatment. In addition, adopting NIRS as the monitoring technique gives the possibility to assess the effects
386 specifically on the tissue of interest, as compared to the more global information provided by blood flow in
387 an large supplying artery.

388

389 *Alternative hypothesis 1: Vascular refilling and the muscle pump*

390 The parallelism observed between changes in pre-compression THI and in displaced blood volume (Fig.2),
391 suggests that pre-compression THI is a good indicator of current vascular filling and that its changes mostly
392 reflect volume changes of the venous compartment. By observing its time course after the compressive
393 stimulus we can detect a fast refilling phase, associated to the possible concomitant hyperaemia, and a
394 subsequent slow phase, associated to "resting" blood flow. At high ISI, i.e., 80 and 160 s, a complete
395 vascular refill is granted by both a consistent hyperaemia and a large time interval. Accordingly, the
396 compressive action of the device displaces comparable amount of blood volume at every stimulus. At lower
397 ISI, the lack of hyperaemia and/or insufficient time for the slow phase to yield a significant contribution may
398 result in incomplete vascular refilling and in a reduction of the blood volume displaced by the subsequent
399 compression. This observation is in agreement with the study by Delis and colleagues who reported 3 to 4
400 compressions per minute (i.e., ISI = 20 or 15 s) as the optimum stimulation frequency to maintain low
401 venous pressure in the treated limb (13). Valic et al (61), in the anesthetized dog estimated a refilling time of
402 less than 1 s due to the large contraction-induced hyperaemia. Based on direct foot venous pressure
403 estimation, two human studies reported refilling times of 16 - 40 s after 10 tip-toe movements (42) and
404 pneumatic compression (22). In the present conditions the refill could take place in 10-15 s through the rapid

405 phase in the presence of large hyperaemia but could otherwise require more than one minute when
406 hyperaemia was blunted (Fig. 3).

407 According to the “muscle pump” effect, an increase in intramuscular pressure empties the venous
408 compartments producing a decrease in venous pressure, which in turn increases the artero-venous pressure
409 gradient thus contributing to the ensuing hyperaemia. This mechanism is activated both with active muscle
410 contraction as well as with the compression of the passive muscle and has been often considered to explain
411 the larger hyperaemic responses observed when compressing (10, 56) or contracting (41, 50) limbs muscles
412 below as compared to above heart level. However the issue is still debated (7, 29, 49) due to the conflicting
413 evidence provided by other studies (24, 28, 61). In particular, Jasperse et al. investigated the effect of
414 positional differences on reactive hyperaemia, as a model of hyperaemia dissociated from the muscle pump.
415 They showed that also reactive hyperaemia is larger when evoked below, with respect to above heart level,
416 suggesting that positional effects may be secondary to differences in driving pressure rather than to the
417 muscle pump. The present results support this view through a complementary model, i.e., the muscle pump
418 action dissociated from the hyperaemia. This particular condition was observed in several instances such as
419 the responses to the second compression at ISI ranging from 20 to 80 s (in Fig 2 and in Fig 4), in which
420 maintained vascular filling and compression-displaced blood volume, i.e., an effective muscle pump, was
421 associated with a considerably reduced hyperaemia, as compared to the first compression in the series. This
422 proves that the muscle pump mechanism is not involved in the attenuation of the hyperaemia in response to
423 multiple compressions. Whether the muscle pump plays a role in the hyperaemic response to the first
424 compressive stimulus cannot be ruled out based on the present data. In fact, from scatter plot in Fig 5B we
425 can observe that the largest hyperaemic responses were never associated with low displaced blood volume,
426 which suggests that adequate vascular filling may be a necessary condition to express the full response.
427 Investigating the mechanisms behind compression-induced hyperaemia was not an aim of this study; further
428 investigations will be necessary to elucidate this issue.

429

430 *Alternative hypothesis 2: Desensitization of mechano-sensitive pathways*

431 It was previously observed that the hyperaemic response to the compressive stimulus progressively reduced
432 to 26% of its original amplitude, with decreasing ISI from 4 min to 2 s (60). On this basis the hypothesis was
433 put forward that the attenuation could be due to some transient inactivation (desensitization) of mechano-
434 sensitive dilatory mechanisms. This hypothesis was supported by the observation that desensitization upon
435 repeated activation is a characteristic of certain vascular mechano-sensitive channels (17, 26). A subsequent
436 human study in which similar stimulation protocols were applied to the forearm, qualitatively confirmed the
437 attenuation pattern, although with a less gradual dependence on the ISI (38)

438 The up-and-down pattern exhibited by compression-induced hyperaemia at ISI = 80 s (Fig. 2 and Fig. 3)
439 seems to exclude a simple, frequency-dependent, desensitization mechanism of mechano-sensitive pathways,
440 as previously hypothesized (38, 60). More complex desensitization patterns possibly affecting multiple

441 mechanosensitive pathways cannot be excluded based on the present data. However, in order to explain the
442 peculiar hyperaemic responses observed at ISI=80 s, such desensitization pattern should exhibit an up-and-
443 down time course, as exhibited by TOI, which would appear a quite unlikely coincidence.

444

445 *Limitations*

446 Manual assessment of insonation angles, as required with the transversal approach, is not very accurate and
447 may introduce systematic errors in the calculation of absolute flow values. This is particularly true for
448 assessment of venous blood flow since a wide angle between the vessel axis and the ultrasound beam had to
449 be adopted in order to avoid saturation of the velocity signal (aliasing). However, the analysis was here
450 focused on relative changes, thereby eliminating errors associated with measurement of the insonation angle.

451 Diameter of the femoral vein was not continuously monitored. Possible enlargement of the vessel during the
452 passage of the blood volume displaced by the compression may have resulted in underestimation of venous
453 flow.

454 Diameter of both femoral artery and vein exhibited a small increase throughout the experimental protocol,
455 which was not accounted for. This may also have led to increasing underestimation of blood flow with time.
456 Since the sequence of the series was randomized this aspect should not have affected the results.

457

458 *Conclusions*

459 This study allowed to prove that the attenuation of hyperaemia upon repetitive limb compression is not
460 dependent on vascular filling and the muscle pump nor on a simple ISI-dependent desensitization of
461 mechano-sensitive pathways. In addition, strong evidence is provided, supporting the concept that tissue
462 hyper-oxygenation is the key signal underlying the inactivation of the rapid dilatory response to muscle
463 compression. This evidence is however indirect and other studies are necessary to conclusively prove this
464 assertion.

465 Irrespective of the underlying mechanisms, inactivation of mechano-sensitive pathways may almost
466 completely abolish the compression-induced hyperemia, suggesting a role for this phenomenon in protecting
467 the tissue from hyperperfusion and oxidative stress.

468 The hyperaemic response to muscle compression is proposed as a peculiar model for the investigation of the
469 response to hyper-perfusion characterized by constant arterial pO₂, constant tissue metabolism as well as
470 modest or absent systemic reactions.

471 Finally, tissue oxygenation monitoring is recommended to assess the efficacy of IPC treatments, oriented to
472 improve blood perfusion in limbs.

473

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480 **Disclosures**

481 No competing interest to declare.

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- 621

622

623

624

625 **LEGENDS TO FIGURES**

626

627 **Fig 1**

628 **Experimental setup and typical hemodynamic response to a compressive stimulus.**

629 A) The experimental setup includes: the IPC system for the compression of the lower limb, eco-Doppler
630 monitoring of blood flow from femoral vein and femoral artery, and NIRS monitoring at lateral head of
631 gastrocnemius muscle. B) Typical response to leg compression in a representative subject. From top to
632 bottom: blood velocity in femoral vein (BVfV), blood velocity in femoral artery (BVfA), tissue
633 oxygenation index (TOI), total hemoglobin index (THI), changes in oxygenated hemoglobin (O₂Hb) and in
634 deoxygenated hemoglobin (HHb) and the synchronism signal (Sync.), the thick and thin bars indicating start
635 of inflation and deflation of the cuff, respectively.

636 **Fig 2**

637 **Hemodynamic responses to repetitive compression at different inter-stimulus intervals (ISI).**

638 The ISI is indicated at the bottom of each column of bar- diagrams; each bar refers to the response to a single
639 compressive stimulus. From top to bottom: Peak (arterial) blood flow, displaced (venous) blood volume, Pre-
640 compression THI (indicating local vascular filling reached before the delivery of the compressive stimulus);
641 Pre-compression TOI (indicating local tissue oxygenation before the stimulus). For the first three variables

642 and for each subject, responses have been normalized to the response to the first stimulus in the 160-s series
643 (white bar). * significantly different from the first response in the series ($p < 0.05$)

644 **Fig 3**

645 **Original recordings of the response to repetitive leg compression at inter-stimulus interval = 80 s, from**
646 **a representative subject.**

647 Notations as in Fig.1. Note the pattern of response of arterial blood velocity in relation to tissue oxygenation.
648 The dotted line represents the initial TOI baseline.

649 **Fig 4**

650 **Original recordings of the response to repetitive leg compression at inter-stimulus interval = 20 s, from**
651 **a representative subject.**

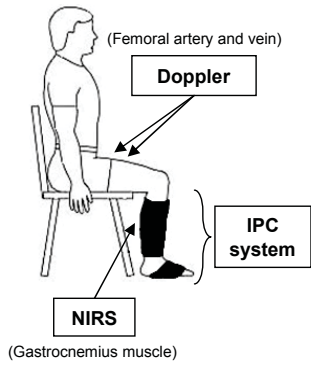
652 Notations as in Fig.1. Note the complete disappearance of the hyperaemic response (BVFA) after the first
653 compressive stimulation, as long as tissue oxygenation (TOI) remains elevated, and the agreement between
654 the displaced blood volume (area under BVFV) and the current vascular filling (THI).

655 **Fig 5**

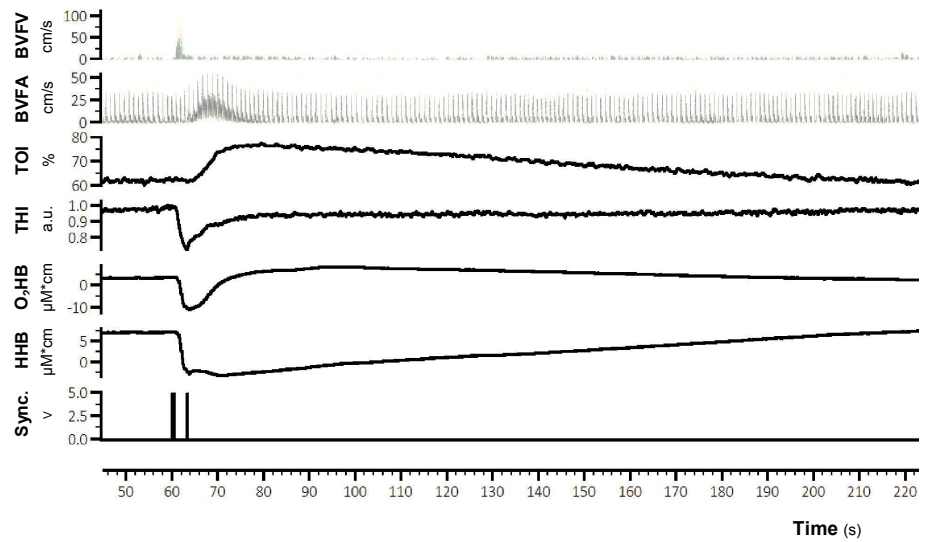
656 **Scatter plots for assessing the correlation between different variables.** Each dot indicates the response to
657 a single compressive stimulus in a single subject. Notations as in Fig. 2. ($n=10$). Straight lines indicate linear
658 regressions for individual subjects. Note that: the increase in tissue oxygenation is related to the peak blood
659 flow (A); Peak blood flow is not related to the displaced blood volume (B) but is inversely related to pre-
660 compression oxygenation level. In D the amplitude of the hyperaemic response (peak-baseline) is plot vs
661 pre-compression TOI to indicate that at high oxygenation levels the hyperaemic response may be almost
662 completely abolished.

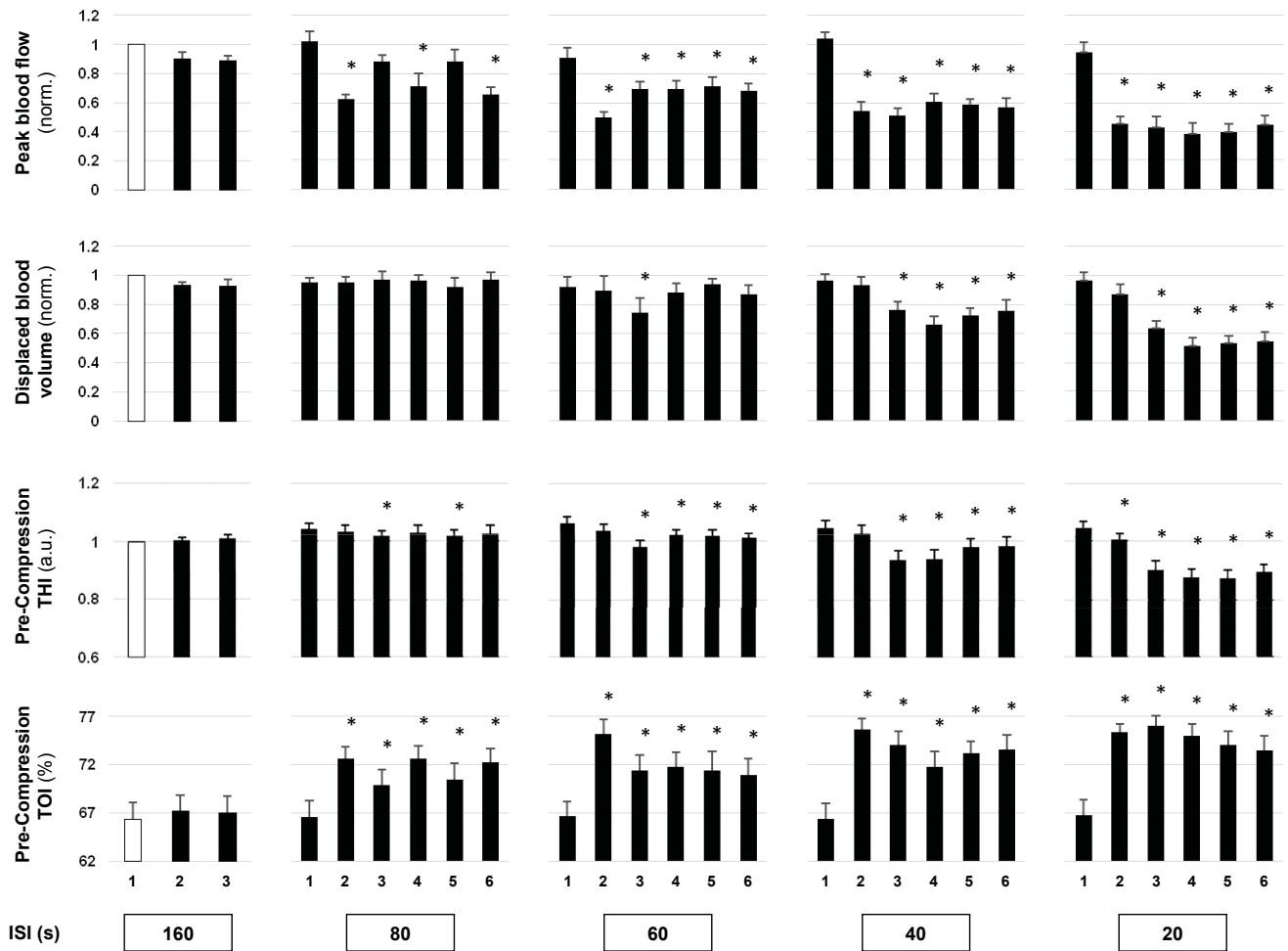
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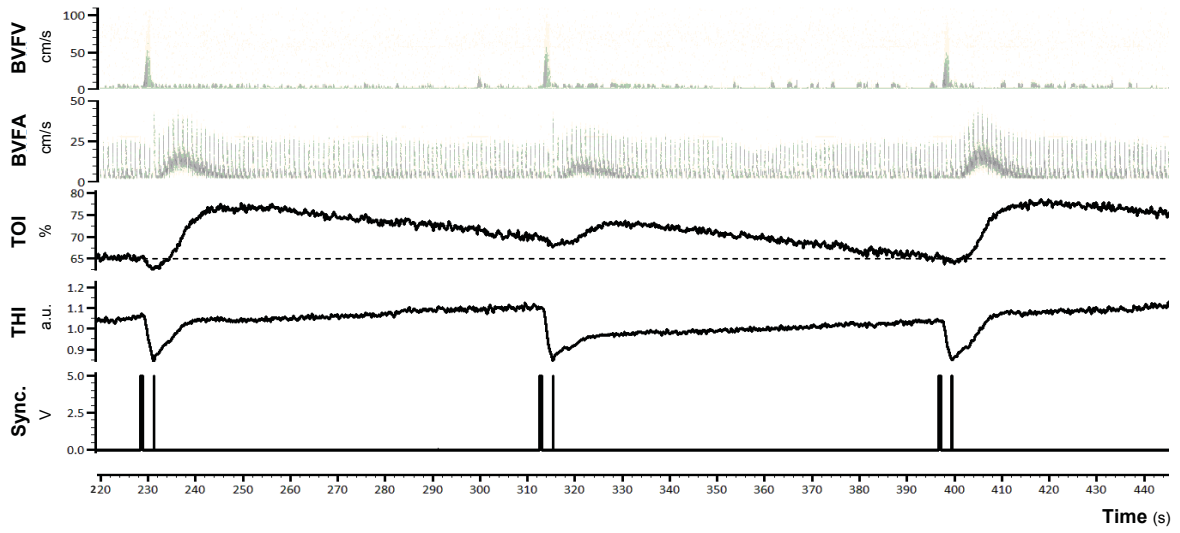
A)

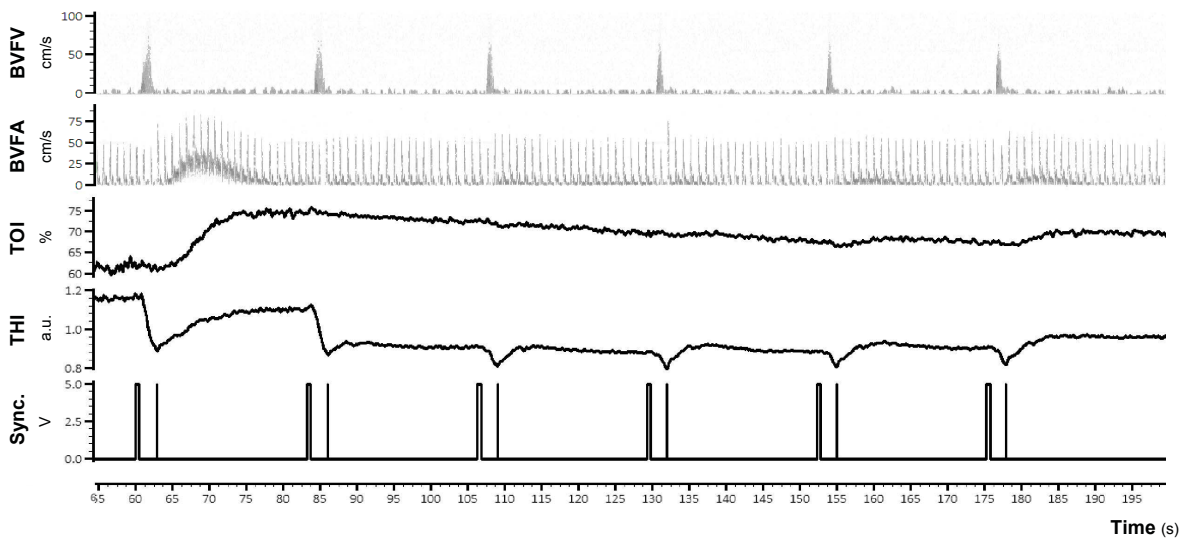


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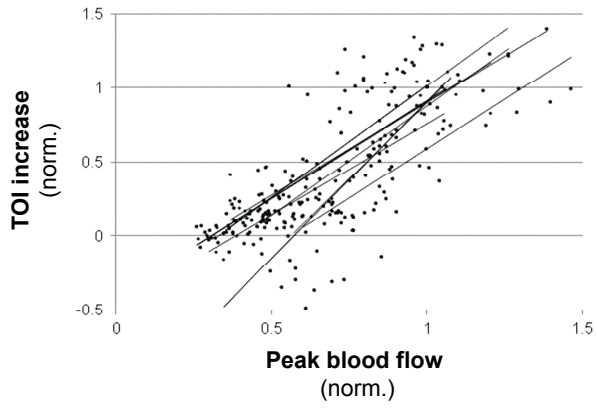




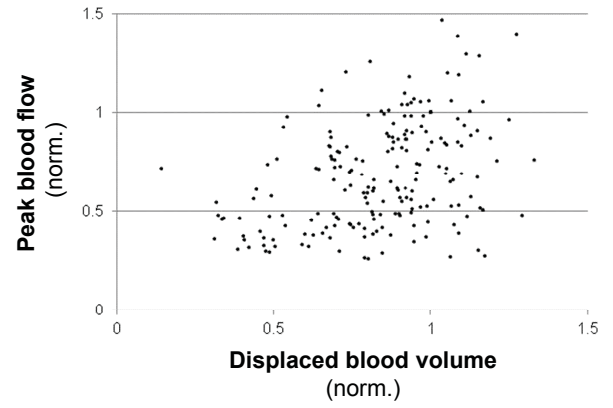




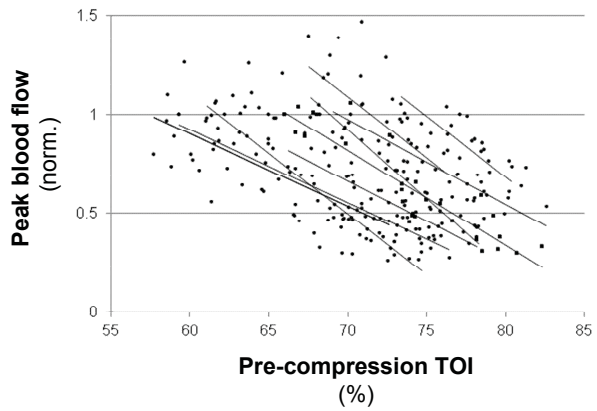
A)



B)



C)



D)

