

Evidence for improved systemic and local vascular function after long-term passive static stretching training of the musculoskeletal system

A. V. Bisconti^{1,3,4}, E. Cè^{1,2} , S. Longo¹ , M. Venturelli^{3,5} , G. Coratella¹ , E. Limonta¹ , C. Doria¹, S. Rampichini¹  and F. Esposito^{1,2} 

¹Department of Biomedical Sciences for Health (SCIBIS), University of Milan, Milan, Italy

²IRCCS Galeazzi Orthopaedic Institute, Via Riccardo Galeazzi, 4, Milan 20161, Italy

³Department of Internal Medicine, The University of Utah, Salt Lake City, UT, USA

⁴Geriatric Research, Education, and Clinical Centre, Veterans Affairs Medical Centre, Salt Lake City, UT, USA

⁵Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona, Verona, Italy

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Key points

- Vascular function and arterial stiffness are important markers of cardiovascular health and cardiovascular co-morbidity.
- Transitional phases of hypoemia and hypermia, with consequent fluctuations in shear rate, occurring during repetitive passive stretching administration (passive stretching training) may constitute an effective stimulus to induce an amelioration in vascular function, arterial stiffness and vascular remodelling by improving central and local blood flow control mechanisms.
- Vascular function, arterial stiffness and vascular remodelling were evaluated before and after 12 weeks of passive stretching training and after 6 weeks from training cessation, in the femoral, popliteal (treated with stretching), and brachial arteries (untreated) of both sides.
- After passive stretching training, vascular function and arterial remodelling improved, and arterial stiffness decreased in all the arteries, suggesting modifications of both central and local blood flow control mechanisms. Passive stretching-induced improvements related to central mechanisms seemed to have a short duration, as they returned to pre-training baseline within 6 weeks from training cessation, whereas those more related to a local mechanism persisted in the follow-up.

Angela Valentina Bisconti received a BSc in exercise science at the Università Cattolica del Sacro Cuore (Italy), an MSc in Exercise Science for Healthy Life, and the PhD in integrative biomedical research from the Università degli Studi di Milano (Italy). Currently, she is Postdoctoral Fellow at the University of Utah (US). **Emiliano Cè** received a degree in sport sciences and a PhD in morphological sciences (2007) at the Università degli Studi di Milano (Italy). Currently, he is Associate Professor at the Department of Biomedical Sciences for Health of the Università degli Studi di Milano (Italy). Their common research interests cover the acute and long-term effects of different types of exercise and training on oxygen uptake, oxygen delivery and utilization, vascular function, and neuromuscular activation in both healthy subjects and athletes.



A. V. Bisconti and E. Cè contributed equally to the work.

Abstract Acute passive stretching (PS) effects on blood flow (\dot{Q}), shear rate (\dot{Y}), and vascular function in the feeding arteries of the stretched muscle have been extensively investigated; however, few data are available on vascular adjustments induced by long-term PS training. We investigated the effects of PS training on vascular function and stiffness of the involved (femoral and popliteal) and uninvolved (brachial) arteries. Our hypothesis was that PS-induced changes in \dot{Q} and \dot{Y} would improve central and local mechanisms of \dot{Q} control. Thirty-nine participants were randomly assigned to bilateral PS ($n = 14$), monolateral PS ($n = 13$) or no PS training ($n = 12$). Vascular function was measured before and after 12 weeks of knee extensor and plantar flexor muscles' PS training by single passive limb movement and flow-mediated dilatation (FMD). Central (carotid-femoral artery PWV, PWV_{CF}) and peripheral (carotid-radial artery PWV, PWV_{CR}) arterial stiffness was measured by pulse-wave velocity (PWV), together with systolic (SBP) and diastolic (DBP) blood pressure. After PS training, increases of 30%, 25% and 8% ($P < 0.05$) in femoral $\Delta\dot{Q}$, popliteal and brachial artery FMD%, respectively, occurred in both PS training groups. A decrease in PWV_{CF} , PWV_{CR} , SBP and DBP (-25% , -17% , -4% and -8% , respectively; $P < 0.05$) was noted. No changes occurred in controls. Vascular function improved and arterial stiffness reduced in the arteries involved and uninvolved with PS training, suggesting modifications in both central and local \dot{Q} control mechanisms. PS-induced improvements had a short duration in some of vascular function parameters, as they returned to baseline within 6 weeks of PS training cessation.

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Corresponding author Emiliano Cè: Department of Biomedical Sciences for Health (SCIBIS), University of Milan, Via Colombo 71, 20133 Milan, Italy. Email: emiliano.ce@unimi.it

Introduction

Vascular function (Hotta *et al.* 2013; Nishiwaki *et al.* 2015), the ability of an artery to dilate and constrict, is an important marker of cardiovascular health and cardiovascular co-morbidity (Qureshi *et al.* 2007). Alterations in vascular function often precede an increase in arterial stiffness, which is inversely related to cardiovascular health (Kruse & Scheuermann, 2017). Improving and/or maintaining vascular function is crucial for the prevention of cardiovascular disease (Green *et al.* 2017b).

Blood flow (\dot{Q}) distribution throughout body vasculature is strongly influenced by the balance of the sympathetic activity (Sandoo *et al.* 2010; Thijssen *et al.* 2014; Venturelli *et al.* 2019) and localized vasodilator mechanisms (Widlansky *et al.* 2003; Wilson *et al.* 2016). Recent studies have reported that acute passive static stretching (PS), a well-established practice in rehabilitation and sport to increase joint range of motion (ROM) (Esposito *et al.* 2011; Kay & Blazevich, 2012; Behm *et al.* 2015), may have a positive effect on vascular function, arterial stiffness and arterial structure (Cortez-Cooper *et al.* 2008; Kato *et al.* 2017; Shinno *et al.* 2017).

Acute PS administration elicits two opposite responses: vasoconstriction with reduced \dot{Q} in the feeding artery of the stretched muscle (Venturelli *et al.* 2019), which is triggered by a systemic increase in sympathetic neural tone (Cui *et al.* 2006) due to PS-induced stress on muscle mechano- and metabo-receptors (Venturelli *et al.* 2017a); and vasodilatation with subsequent increase in \dot{Q} in the

feeding artery of the stretched muscle potentially due to the release of endogenous vasoactive substances, as a result of the stretch-induced stress on the vessel's wall that overwhelms systemic sympathetic vasoconstriction (Venturelli *et al.* 2017a, 2019). During multiple stretch-relaxation cycles, the first acute hyperaemic response to stretching seems to progressively attenuate until it disappears during subsequent stretching bouts, possibly due to the depletion of vasoactive substances (Venturelli *et al.* 2017a, 2019). Moreover, the relaxation phase in between two subsequent stretches is always characterized by hyperaemia due to a reduction in peripheral vascular resistance after stretch-induced deformation of the vessel (Venturelli *et al.* 2019). While the effects of acute PS on \dot{Q} , and vascular function and structure in the feeding arteries of the stretched muscle have been already extensively investigated, some data are also available on vascular function and arterial stiffness ameliorations induced by long-term PS training (Cortez-Cooper *et al.* 2008; Kato *et al.* 2017; Shinno *et al.* 2017). However, the indirect approach used in the previous studies to assess vascular function changes after PS training did not permit evaluation of possible PS training-induced mechanical remodulations of the arterial wall in the vessels directly involved in PS training. A plausible explanation for these phenomena involves the shear rate (\dot{Y}), which is the frictional or drag force acting on the vessel's inner lumen that can trigger a chain of reactions, leading to higher endothelial NO synthase activity (Niebauer & Cooke,

1996). Continuous, repetitive increases in \dot{Y} induced by PS training may indeed act as a sort of ‘vascular training’ leading to endothelial remodelling (i.e. changes in vascular stiffness and structure), thus improving vascular function, in a manner similar to skeletal muscle training (Green *et al.* 2017a,b; Bisconti *et al.* 2019). Noticeably, changes in arterial stiffness may be also attributable, at least in part, to changes in the structural properties of the connective tissue. A biomarker to determine changes in human vascular structure *in vivo* is the maximal arterial dilatation capacity (Naylor *et al.* 2005), which can be induced by hyperaemia in response to ischaemic exercise (Naylor *et al.* 2005).

Acutely, during a bout of PS, a reduction in \dot{Q} in the contralateral, unstretched limb was found to promptly recover during the relaxation phase (Venturelli *et al.* 2019). The authors suggested that recovery was induced by systemic sympathetic-mediated vasoconstriction activated by the stretch-induced mechanoreflex (Venturelli *et al.* 2019). When PS is applied chronically over a long period, repeated stimulations of the vessel wall (alternating vasoconstriction and vasodilatation) may induce changes in the systemic autonomic control of \dot{Q} distribution, possibly leading to an increase in vascular function. It is still debated, however, whether PS training may also affect the vascular function in the feeding artery of the contralateral muscle, which is not directly involved in stretching. Together with changes in local control mechanisms, possible PS training-induced changes in the systemic autonomic control of \dot{Q} distribution have also been reported (i.e. reduced blood pressure and aortic wave reflection magnitude (Wong & Figueroa, 2014)), although its effectiveness remains controversial (Farinatti *et al.* 2011; Hotta *et al.* 2013; Williams *et al.* 2013).

Vascular training, i.e. possible PS training-induced changes in local and systemic mechanisms underlying vascular function, may have practical implications for maintaining or even improving cardiovascular health in people with limited mobility and/or while bedridden.

With this in mind, we investigated the effects of PS training on vascular function and stiffness of the arteries directly involved (i.e. femoral and popliteal arteries) and not directly involved (i.e. contralateral femoral and popliteal arteries and brachial artery) in manoeuvres applied to the plantar flexor, knee extensor and hip flexor muscles. To do this, we measured vascular function and arterial stiffness before and after 12 weeks of PS training. Our hypothesis was that repetitive fluctuations in \dot{Q} and \dot{Y} during PS bouts would provide effective stimulus to affect the central and local \dot{Q} control mechanisms, thus improving vascular function and arterial stiffness not only in the feeding arteries of the stretched muscle, but also in those districts not directly involved in PS training.

Methods

Ethical approval

All participants provided written, informed consent after being informed about the aims of the study and the potential risks derived from tests and methods. The Institutional Review Board of the Università degli Studi di Milano approved the study (CE 27/17). The study was registered at ClinicalTrials.gov (ID: NCT04271241) and performed in accordance with the principles of the Helsinki Declaration. The researchers who analysed the data were blinded to group allocation.

Participant recruitment

Figure 1 illustrates the study flow chart. A total of 54 healthy adults volunteered to participate in the study. Exclusion criteria were: neurological, vascular and musculoskeletal disorders of the lower and upper limbs; being on pharmacological therapy related to either neural and/or vascular response, including hormonal contraceptives and oral supplements; being a current or former smoker; having an irregular menstrual cycle (26 to 35 days) up to 3 months before the beginning of the study; contraindications to joint mobilization; regular involvement in a PS training programme. Based on previous research (Hotta *et al.* 2013; Nishiwaki *et al.* 2015; Venturelli *et al.* 2017a), for this study we used changes in blood flow as main outcomes, a two-way analysis of variance (ANOVA; within-group factor: time; between-groups factor: intervention) in the statistical approach, with an α level of 0.05 and a required power ($1-\beta$) of 0.80; the desired sample size, computed using statistical software (G-Power 3.1, Dusseldorf, Germany) resulted in 36 participants. Accordingly, the study sample was 39 participants (19 females and 20 males, age 23 ± 2 years, body mass 69 ± 3 kg, stature 1.68 ± 0.11 m, mean \pm standard deviation (SD)) were enrolled and randomly assigned to one of three groups: bilateral PS (PS_{Bil}; $n = 14$ (7F/7M), age 23 ± 2 years, body mass 68 ± 4 kg, stature 1.68 ± 0.09 m, ankle ROM 25 ± 3 deg, knee ROM 145 ± 9 deg); mono-lateral PS (PS_{MonO}; $n = 13$ (6F/7M), age 22 ± 2 years, body mass 70 ± 3 kg, stature 1.71 ± 0.11 m, ankle ROM 24 ± 4 deg, knee ROM 145 ± 12 deg), and control group (no stretching, Ctr; $n = 12$ (6F/6M), age 23 ± 2 years, body mass 69 ± 3 kg, stature 1.70 ± 0.08 m, ankle ROM 26 ± 6 deg, knee ROM 146 ± 12 deg).

Study design

Before testing, a preliminary session was conducted for familiarizing the participants with the procedures to identify maximum isometric voluntary contraction (MVC) of the knee extensor and plantar flexor muscles of both limbs.

Measurements were taken bilaterally: pulse wave analysis (PWA) and pulse wave velocity (PWV) were measured at the femoral and the radial artery by photo-plethysmography as an indirect marker of arterial stiffness (Doupis *et al.* 2016); single passive limb movement (sPLM), flow-mediated dilatation (FMD), and hypoxic exercise (HEx) tests were performed on the femoral, the popliteal and the brachial arteries, respectively. Knee and ankle flexion ROM were measured.

On completion of measurements, the ultrasound probe position was marked on a transparency sheet, together with skin landmarks (moles, scars, angiomas, etc). Results

of the familiarization and the pre-training experimental sessions were used to calculate inter-session reliability. All measurements were taken at the beginning (Pre), at 6 weeks (week 6), 12 weeks of PS training (week 12), and then again at 6 weeks post-intervention (follow-up). The participants were tested at the same time of the day in a climate-controlled laboratory (temperature $20 \pm 1^\circ\text{C}$ and relative humidity $50 \pm 5\%$) to minimize confounders due to circadian rhythms. For the women, all the tests were conducted on the same day of the menstrual cycle (early follicular phase days 3 ± 3). The female participants recorded their menstrual cycle in a personal

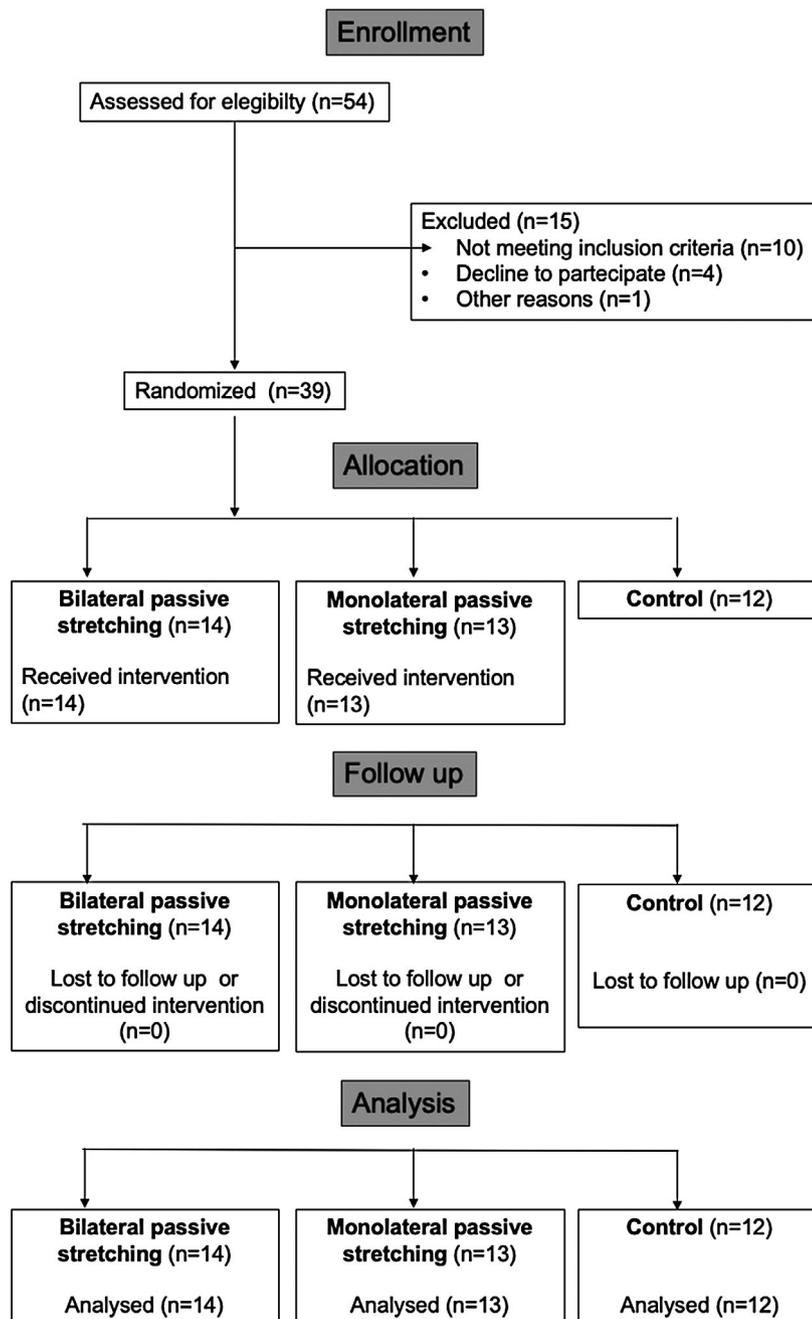


Figure 1. Study flow chart

diary throughout the study, which was used to assess the early follicular phase and allowed the women to be tested on the same menstrual day. On the test days, the participants came to the laboratory after having fasted overnight and refrained from caffeine and other similar substances for at least 12 h and from intensive exercise for at least 48 h prior to the tests. The two stretching groups (PS_{Bil} and PS_{Mono}) underwent 12 weeks of PS training, 5 sessions per week (60 sessions in total). Each PS_{Bil} session lasted 40 min and included two exercises for the knee extensor and the plantar flexor muscles: 45 s elongation and 15 s recovery in the resting position; the set was repeated five times (Venturelli *et al.* 2019). In the PS_{Mono} sessions the exercises were performed using only the right limb and lasted 20 min. The Ctrl group received no PS training (Fig. 2 gives an example of the PS training exercises). To enhance compliance, daily classes were held at different times of the day (morning and afternoon) at the University Sports Centre gym. Each class was led by an expert supervisor who monitored attendance, correct exercise execution and intensity (80% of the point of discomfort) (Cè *et al.* 2020). Participants failing to attend at least 80% of classes were excluded from the study, and a new participant was recruited to substitute the drop out.

Since the increase in \dot{Y} occurring during PS has been advocated as the mechanism possibly triggering the improvements in vascular function, a subsample of 20 participants (10F/10M), age 22 ± 1 years, body mass 69 ± 4 kg, stature $1.71 \pm .0.12$ m, ankle ROM 23 ± 4 deg, knee ROM 143 ± 13 deg) underwent a third session during which the femoral and popliteal artery \dot{Y} were calculated during a stretching bout involving the knee extensor and plantar flexor muscles.

Measurements and data analysis

Measurements were performed bilaterally in all groups. Data for the PS_{Bil} group are presented as the average between the two limbs, while the data for the PS_{Mono} group are presented separately for the stretched (PS_{Mono}SL) and the contralateral limb (PS_{Mono}CL); this was done to detect any possible crossover effect. The data for the upper limbs were calculated as the average of the two limbs.

Range of motion (ROM). To monitor the changes in knee and ankle joint ROM, a bi-axial electrogoniometer (TSD 130A, Biopac System, Goleta, CA, USA) was utilized. To measure knee ROM, the electrogoniometer was placed with one axis on the external condyles of the knee joint and the other on the external face of the fibula; to measure ankle joint ROM, the instrument was positioned on the external face of the fibula and on the calcaneum.

Maximum isometric voluntary contraction (MVC). MVC of the knee extensor muscles was measured with the participant supine on an ergometer and the knee flexed at 90 deg and firmly secured at the ankle with a Velcro strap (Velcro Industries Inc., Manchester, NH, USA) to a load cell (SM-2000N operating linearly between 0 and 2000 N; Interface, Crowthorne, UK) for force signal detection. The MVC of the plantar flexor muscle was measured with the participant prone on the ergometer, with the foot fixed by a Velcro strap to a mobile metal plate instrumented with a load cell (SM-2000 N, Interface). The hips and shoulders were firmly secured to the ergometer. After a standardized warm-up (10×2 -s contractions at 50% MVC determined during familiarization), three MVC trials were performed interspersed by at least 3 min of recovery. The participants

Passive stretching training

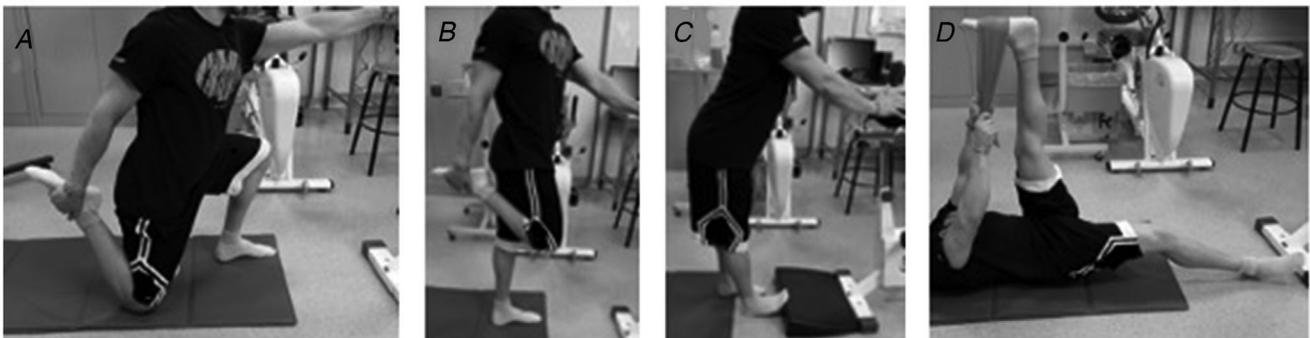


Figure 2. Photographs showing the passive stretching exercises

Each exercise comprises a set of 5 stretches of 45 s with 15 s of rest in between. The exercises were repeated bilaterally in the PS_{Bil} group and unilaterally (right limb) in the PS_{Mono} group. A, hip extension + knee flexion; B, hip extension + knee flexion in orthostatic position; C, ankle dorsiflexion in orthostatic position; D, hip flexion + ankle dorsiflexion with straight leg in supine position.

were instructed to push as fast and hard as possible for 3 s. The force signal was transmitted to an A/D converter (UM 150, Biopic, Biopac Systems Inc., Goleta, CA, USA), sampled at a fixed sampling rate of 1000 Hz, and stored on a personal computer. The maximum force recorded during the three trials was defined the MVC and entered in the data analysis.

Pulse wave analysis (PWA) and velocity (PWV). Blood pressure wave analysis and arterial stiffness were measured by determining the PWA and PWV using an applanation tonometry technique (Doupis *et al.* 2016). PWA and PWV were measured using a SphygmoCor PX (Atcor Medical Blood Pressure Analysis System, Sydney, Australia) after the participant had rested for at least 20 min in supine position.

Blood pressure wave parameters, i.e. systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP), aortic augmentation (AA), augmentation index (AIx), AIx corrected for heart rate of 75 beats \cdot min⁻¹ (AIx75), and tension time index referred to the systolic tension time index (TTI) were measured by means of applanation tonometry of the radial artery, as described elsewhere (O'Rourke, 1999). Radial artery pressure wave amplitude was recorded non-invasively with a pencil-type probe (tonometer) placed over the right radial artery of the wrist. After a reproducible signal was obtained, 20 sequential waveforms covering a complete respiratory cycle were acquired from the system and used by the software to generate an average peripheral and a corresponding central waveform, which was then underwent further analysis (O'Rourke, 1999). The systolic part of the wave form was characterized by two pressure peaks of the central waveform. The first peak results from left cardiac ventricle ejection and the second one from wave reflections from the periphery. The difference between the two peaks is the degree of central arterial pressure augmentation due to wave reflection (i.e. the AA). The AIx calculated by the software SphygmoCor (Version 9.0) was the proportion of the central aortic PP that is attributed to the reflected pulse wave. Moreover, due to its dependency on heart rate, the AIx was corrected for a heart rate of 75 beats min⁻¹ (AIx75). The TTI was defined as an indirect marker of myocardial oxygen demand (Chemla *et al.* 2008). The amplitude and timing of the reflected wave depend on the stiffness of small vessels and large arteries and are measurements of systemic arterial stiffness (O'Rourke, 1999; Pauca *et al.* 2001; Doupis *et al.* 2016).

PWV was measured between the carotid and the radial (PWV_{CR}) and between the carotid and the femoral artery (PWV_{CF}) with the participant lying still in supine position. Pulse measurements were performed non-invasively using a SphygmoCor probe over the carotid and the femoral or the radial artery while ECG was performed simultaneously (Pauca *et al.* 2001; Qureshi *et al.* 2007; Doupis *et al.* 2016).

To ensure a stable, artefact-free ECG, the skin was properly prepared (hair removed at the electrode site and the skin cleaned with an alcohol wipe). A minimum of 20 s of signal was recorded after a strong, accurate and reproducible pulse wave signal was obtained. The distance from the carotid to the femoral or the radial artery was measured directly between each artery and the suprasternal notch. The measurements were entered into the SphygmoCor software database. PWV was calculated by measuring the time delay between two characteristic time points (i.e. carotid-femoral arteries, PWV_{CF}, or carotid and radial arteries, PWV_{CR}) on two pressure waveforms at a known distance apart. The SphygmoCor method uses the foot of the waveform as an onset point for calculating the time differences between the R wave of the ECG and the pulse waveforms at each site. PWV was automatically calculated by the SphygmoCor software as PWV_{CF} or PWV_{CR} distance divided by the wave travelling time between the two measurement sites (Pauca *et al.* 2001; Qureshi *et al.* 2007; Doupis *et al.* 2016). PWV_{CF} and PWV_{CR} were used as central and peripheral arterial stiffness indexes, respectively. PWA and PWV measurements were taken at least 3 times, obtaining an operator index \geq 95%, and are expressed as the average.

Single passive limb movement (sPLM). In sPLM, the blood flow hyperaemic response is determined by a single knee flex-extension and not a continuous movement as in the traditional PLM. In sPLM, central haemodynamic responses are minimized, but the manoeuvre still facilitates the assessment of peripheral vascular function. Indeed, sPLM in comparison to PLM, did not evoke a measurable increase in either heart rate or cardiac output, probably as a consequence of the minimal afferent feedback due to the brevity of the sPLM manoeuvre (Venturelli *et al.* 2017b). sPLM was performed in accordance with recommended procedures (Trinity *et al.* 2012; Venturelli *et al.* 2017b; Bisconti *et al.* 2019). Participants rested in an upright-seated position for 10 min before data collection started and remained in this position until the end of the test. The sPLM protocol consisted of 30 s of baseline peripheral haemodynamic data collection, followed by a single passive knee flexion and extension of 1 s, after which the leg was maintained fully extended for the remaining 59 s for post-movement data collection. sPLM was performed by a member of the research team, who moved the participant's leg through a 90 deg ROM at 1 Hz. Arterial blood velocity (V_{mean}) and vessel diameter (D) were measured at the common femoral artery of the passively moved leg, distal to the inguinal ligament and proximal to the deep and superficial femoral bifurcation by Doppler ultrasound (Logiq-7, General Electric Medical Systems, Milwaukee, WI, USA). Mean blood velocity was measured with a 9 MHz linear array transducer positioned an insonation angle of 60 deg. The sample volume was

centred and size-maximized according to vessel diameter (Trinity *et al.* 2012). Femoral \dot{Q} (\dot{Q}_{fem}) was calculated at baseline and at peak after single passive knee flexion and extension by calculating the D and mean blood velocity as:

$$Q_{\text{fem}} (\text{ml} \times \text{min}^{-1}) = V_{\text{mean}} \times \pi \times (\text{vessel } D/2)^2 \times 60.$$

The cumulative \dot{Q}_{fem} was integrated (area under the curve, AUC) using the trapezoidal rule and then calculated.

Flow-mediated dilatation (FMD). FMD was measured at the popliteal and the brachial artery according to recommended procedures (Harris *et al.* 2010; Bisconti *et al.* 2018, 2019). Before FMD, the participants lay supine for approximately 20 min to restore baseline cardiovascular values. An arterial pressure cuff was placed around the calf muscles (popliteal artery) and on the forearm immediately distal to the olecranon process (brachial artery) to generate an ischaemic stimulus when inflated. Following baseline assessment, the blood pressure cuff was inflated to 280 mm Hg for the popliteal and 250 mm Hg for the brachial artery for 5 min. Arterial D and V_{mean} recordings resumed at baseline, 30 s prior to cuff deflation and continued for 2 min post-deflation, as described elsewhere (Corretti *et al.* 2002; Harris *et al.* 2010; Wray *et al.* 2013). A 9-MHz linear array and a 15-MHz linear array transducer attached to a high-resolution ultrasound machine were used to image the popliteal artery in the distal third of the leg and the brachial artery in the distal third of the upper arm. When an optimal image was obtained, the probe was held stable and longitudinal in B-mode, and images of the lumen-arterial wall interface were acquired. Continuous Doppler velocity was measured, and the data were collected using the lowest possible insonation angle (<60 deg). The FMD data were exported in AVI format and analysed using commercially available software (Brachial Artery Analyzer for Research, Medical Imaging Applications, LLC, Coralville, IA, USA), which is largely independent of investigator bias. FMD was quantified as the maximal change in artery diameter after cuff release, expressed as a percentage increase above baseline:

$$(D_{\text{peak}} - D_{\text{bas}}) / D_{\text{bas}} \times 100.$$

Popliteal (\dot{Q}_{pop}) and brachial artery blood flow (\dot{Q}_{brac}) was calculated as described for sPLM measurement. Popliteal and brachial artery \dot{Y} was calculated post-cuff release with the equation:

$$\text{shear rate } (s^{-1}) = 8V_{\text{mean}}/D$$

The cumulative \dot{Y} , corresponding to the reactive hyperaemia post-cuff release (total \dot{Y} from cuff release to time-to-peak, \dot{Y} AUC) was calculated using the trapezoidal rule.

The \dot{Y} AUC reflects the amount of mechanical stimulus applied to the endothelium during cuff release hyperaemic response until time-to-peak. Given that FMD is primarily dependent on endothelial response to mechanical stimuli, the FMD was divided by the \dot{Y} AUC (FMD/ \dot{Y}) (Pyke & Tschakovsky, 2005; Padilla *et al.* 2008).

Artery dilatation response to ischaemic exercise (IEx).

In order to detect possible arterial structural changes, popliteal and brachial artery dilatation after IEx was tested by applying a protocol described elsewhere (Naylor *et al.* 2005). IEx was performed after a further 30 min of rest and consisted in 5 min of ischaemia, during which a voluntary isometric contraction of the plantar flexor muscles (popliteal artery) or the handgrip muscles (brachial artery) was over-impressed. The force output was standardized at 3 kg; the contractions lasted 1 s and were performed every 3 s over 3 min (Naylor *et al.* 2005). As done in the FMD tests, arterial D and V_{mean} were recorded during IEx at baseline, 30 s prior to cuff deflation, and continued for 5 min post-deflation. The same parameters as in the FMD tests were calculated.

Acute passive stretching. The participants rested in a supine position during stretching for the knee extensor (Venturelli *et al.* 2019) and in a prone position for plantar flexor muscles (Kruse *et al.* 2016) for 20 min before starting the data collection and remained in this position throughout the entire duration of the data collection. The two stretching bouts were separate by 60 min rest, during which the participant remained at rest on a medical bed. PS protocol consisted of 5 min of resting baseline followed by passive static elongations for 45 s and passive relaxations for 15 s, repeated five times. During the entire PS protocol, the muscles were stretched by the same operator up to a point of discomfort similar to those required during the PS training. The knee and ankle joint angles were continuously recorded using a biaxial goniometer (model no. TSD 130A; Biopac Systems). Force output between the passively stretched leg and the operator arms was recorded during the protocol by a load cell (model SM-2000 N; Interface, Crowthorne, UK). Specifically, the load cell was positioned 5 cm above the ankle or on the metatarsum of the stretched limb, and a member of the research team pushed perpendicularly the load cell to stretch the leg extensor for 45 s. The passive force output during the 45 s of the consecutive flexion of the PS protocol was displayed on a PC screen so as to maintain constant the passive force throughout the elongations. During the two stretching bouts, the artery's D and the antegrade and retrograde V_{mean} were measured from the femoral and the popliteal artery. Antegrade, retrograde and mean \dot{Y} during the 5 elongations and relaxation phases were then calculated as in the FMD tests.

Statistical analysis

Statistical analysis was performed using a statistical software package (IBM-SPSS Statistics v. 26, Armonk, NY, USA). The Shapiro-Wilk test was used to check normal distribution of the data. To determine inter-session reliability in vascular function parameters, intra-class correlation coefficient (ICC) and percentage change of the standard error of the measurement (SEM%) were calculated. The ICC was interpreted as follows: >0.90 , *very high*; $0.89-0.70$, *high*; $0.69-0.50$, *moderate* (Munro, 2004). The minimal detectable change at 95% confidence interval ($MDC_{95\%}$) was used to detect the sensitivity of the effects on vascular function before and after the stretching intervention (Donoghue *et al.* 2009). To assess significant effects of stretching, two-way ANOVA (within-group factor: time, 4 levels (Pre, week 6, week 12, and follow-up); between-groups factor: intervention, 4 levels (Ctrl, PS_{Bil}, PS_{MonoSL}, PS_{MonoCL})). To calculate the difference in changes between the groups, analysis of covariance (ANCOVA) was performed, entering the baseline (Pre) values as covariate. A one-way ANOVA for repeated measures (within-group factor: time, 5 levels) was applied to check for differences in \dot{Y} in elongation and relaxation phases occurring during acute passive stretching bout. Multiple comparisons were performed by applying Bonferroni's correction. Statistical significance was set at $P < 0.05$. Data are presented as mean \pm standard deviation. Cohen's d effect size was calculated and interpreted as: $0.00-0.19$, *trivial*; $0.20-0.59$, *small*; $0.60-1.19$, *moderate*; $1.20-1.99$, *large*; 2.00 , *very large* (Hopkins *et al.* 2009). The 95% CI of d is reported (<https://www.cem.org/effect-size-calculator>). A Pearson correlation test was applied to check for possible correlations between the percentage changes in ankle and knee ROM, PWA and PWV variables, and in sPLM, FMD and ischaemic exercise tests. The magnitude of correlations was interpreted as follows: (R) < 0.1 , *trivial*; $0.10-0.30$, *low*; $0.31-0.50$, *moderate*; $0.51-0.70$, *high*; $0.71-0.90$, *very high*; $0.91-0.99$, *nearly perfect*; 1 , *perfect* (Hopkins *et al.* 2009).

Results

Participant compliance

Attendance was about 93% (56/60 training sessions). Three participants dropped out because of injury (unrelated to the training protocol) and lack of time. They were immediately replaced to maintain the sample size.

Reliability

Table 1 presents the intersession reliability for PWA, PWV, and vascular function. ICC and SEM% in PWA and

PWV ranged from 0.893 and 0.9% to 0.987 and 5.0%, respectively. ICC and SEM% in sPLM ranged from 0.947 and 1.7% to 0.958 and 2.2%, respectively. ICC and SEM% in popliteal and brachial FMD ranged between 0.945 and 0.6% and between 0.981 and 1.6%, respectively. ICC and SEM% in popliteal and brachial HEx ranged from 0.881 and 0.5% to 0.991 and 3.0%, respectively. $MDC_{95\%}$ ranged between 1.3 and 13.9%.

ROM and MVC

ANOVA revealed a significant time \times intervention interaction for the ankle ($F = 5.46$, $P < 0.001$) and knee ROM ($F = 5.67$, $P < 0.001$). Main effects for time were found in ankle ($F = 9.13$, $P < 0.001$) and knee joint ROM ($F = 4.12$, $P < 0.001$). Ankle joint ROM was increased at week 12 (PS_{Bil}: $+3.3 \pm 0.71$ deg, $d = 1.06$ (0.17/1.95); PS_{MonoSL}: $+3.2 \pm 0.96$ deg, $d = 1.02$ (0.09/1.95), $p = 0.01$ in PS_{Bil} and PS_{MonoSL}), and knee joint ROM in the PS_{Bil} and the PS_{Mono} group was increased at week 6 (PS_{Bil}: $+8.5 \pm 3.4$ deg, $d = 0.92$ (0.13/1.70); PS_{MonoSL}: $+8.9 \pm 2.6$ deg, $d = 0.95$ (0.02/1.87), $p = 0.02$ in PS_{Bil} and PS_{MonoSL}), whereas no changes were noted for the Ctrl and PS_{MonoCL} groups. ROM of the ankle and the knee joint returned to pre-training levels at follow-up assessment. The changes in knee and ankle joint ROM at any time were greater for the PS_{Bil} and the PS_{MonoSL} group than for the Ctrl group ($P < 0.001$). Neither time \times intervention interaction nor main effect for time was found for MVC. Plantar flexor and knee extensor muscle MVC remained unchanged during the study in all groups.

Acute passive stretching

Table 2 provides the changes in antegrade, retrograde, and mean \dot{Y} of the femoral and popliteal artery occurring during the 5 elongations and relaxations of the acute passive stretching bout involving the knee extensor and plantar flexor muscles, respectively. Independently from the artery, during the elongation phase the antegrade and mean \dot{Y} increased during the first stretch ($P < 0.001$) and then decrease from baseline ($P < 0.001$), while the retrograde \dot{Y} increased ($P < 0.001$). On the contrary, during the relaxation phase the antegrade and mean \dot{Y} increased ($P < 0.001$) and retrograde \dot{Y} decreased ($P < 0.001$).

PWA and PWV

Figures 3 and 4 present PWA and PWV measurements. ANOVA disclosed a significant time \times intervention interaction for SBP ($F = 2.25$, $p = 0.02$), DBP ($F = 8.18$, $P < 0.001$), MAP ($F = 9.12$, $p = 0.002$), HR ($F = 3.15$, $p = 0.002$), PP ($F = 5.67$, $P < 0.001$), AIx75 ($F = 3.04$

Table 1. Intersession reliability (ICC), and sensitivity (MDC95%) in the main parameters calculated during pulse wave analysis (PWA) and velocity (PWV), single passive limb movement (sPLM), flow-mediated dilatation (FMD) and ischaemic exercise (IEx) tests

		Trial 1 (mean ± SD)	Trial 2 (mean ± SD)	ICC	SEM%	MDC ₉₅ %
PWA/PWV	SBP (mm Hg)	109.0 ± 6.0	108.0 ± 6.1	0.974	0.9	1.7
	DBP (mm Hg)	76.0 ± 7.1	76.0 ± 7.7	0.946	2.2	4.6
	HR (bpm)	73.0 ± 9.2	74.0 ± 8.3	0.968	2.1	4.1
	AA (mm Hg)	21.9 ± 2.3	19.7 ± 2.5	0.923	2.9	8.0
	Aix (%)	20.1 ± 2.6	19.7 ± 2.5	0.987	1.5	4.1
	TTI (ms)	357.0 ± 16.0	357.7 ± 15.0	0.912	1.3	2.5
	PWV _{CF} (m s ⁻¹)	6.8 ± 0.9	6.5 ± 0.8	0.960	2.6	7.2
	PWV _{CR} (m s ⁻¹)	7.5 ± 1.2	7.7 ± 1.2	0.893	5.0	13.9
sPLM	<i>D</i> _{fem, bas} (mm)	7.8 ± 0.6	7.5 ± 0.6	0.947	1.7	4.7
	<i>V</i> _{peak} (cm s ⁻¹)	34.6 ± 3.8	34.7 ± 3.5	0.958	2.2	6.0
FMD popliteal artery	<i>D</i> _{pop, bas} (mm)	5.0 ± 0.2	4.9 ± 0.2	0.972	0.6	1.7
	<i>D</i> _{pop, peak} (mm)	6.1 ± 0.2	6.0 ± 0.2	0.958	0.7	2.0
	<i>V</i> _{peak} (cm s ⁻¹)	34.8 ± 2.3	34.6 ± 2.0	0.947	1.4	4.0
FMD brachial artery	<i>D</i> _{brach, bas} (mm)	3.1 ± 0.2	3.1 ± 0.2	0.981	0.8	2.3
	<i>D</i> _{brach, peak} (mm)	3.7 ± 0.2	3.6 ± 0.2	0.945	1.5	4.1
	<i>V</i> _{peak} (cm s ⁻¹)	70.3 ± 6.7	70.0 ± 5.5	0.971	1.6	4.3
IEx popliteal artery	<i>D</i> _{pop, bas} (mm)	5.0 ± 0.3	5.0 ± 0.2	0.991	0.5	1.3
	<i>D</i> _{pop, peak} (mm)	6.1 ± 0.3	6.0 ± 0.3	0.962	0.8	2.3
	<i>V</i> _{peak} (cm s ⁻¹)	64.1 ± 5.4	63.1 ± 3.6	0.899	2.2	6.2
IEx brachial artery	<i>D</i> _{brach, bas} (mm)	3.1 ± 0.2	3.1 ± 0.2	0.989	0.6	1.7
	<i>D</i> _{brach, peak} (mm)	3.8 ± 0.2	3.8 ± 0.3	0.956	1.4	4.0
	<i>V</i> _{peak} (cm s ⁻¹)	119.3 ± 12.3	121.9 ± 8.8	0.881	3.0	8.4

n = 39. MDC95%, minimum detectable change at 95% confidence interval; ICC, intraclass correlation coefficient; SEM%, standard error of measurement as a percentage; CF, carotid-femoral; CR, carotid-radial; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; PP, pulse pressure; Aix, augmentation index; TTI, tension time index; *D*, diameter; *bas*, baseline; *V*_{peak} peak velocity. **P* < 0.05 vs. Pre, #*P* < 0.05 vs. Ctrl.

p = 0.011), TTI (*F* = 4.48, *p* = 0.001), PWV_{CF} (*F* = 4.09, *P* < 0.001), and PWV_{CR} (*F* = 4.47, *P* < 0.001).

A main effect for time was found for SBP (*F* = 6.81, *p* = 0.001), DBP (*F* = 100.15, *P* < 0.001), MAP (*F* = 5.01, *P* < 0.001), HR (*F* = 5.24, *P* < 0.001), PP (*F* = 5.87, *P* < 0.001), Aix75 (*F* = 8.21, *P* < 0.001), AA (*F* = 7.19, *P* < 0.001), TTI (*F* = 6.27, *p* = 0.002), PWV_{CF} (*F* = 6.32, *P* < 0.001), and PWV_{CR} (*F* = 6.94, *P* < 0.001).

SBP, DBP and MAP were decreased significantly in the PS_{Bil} group at week 6 (MAP: -4.6 ± 0.9 mmHg, *d* = -1.06 ($-1.95/-0.17$), *p* = 0.004) and in the PS_{Mono} group at week 12 (MAP: -4.8 ± 1.0 mmHg, *d* = -1.20 ($-2.15/-0.25$), *P* < 0.001) and returned to baseline at follow-up assessment. No changes in HR were observed in any group. PP, Aix75 and TTI were decreased in the PS_{Bil} and the PS_{Mono} group at week 12 (TTI. PS_{Bil}: -23 ± 5.6 ms, *d* = -1.70 ($-2.64/-0.77$), *P* < 0.001; PS_{Mono}: -12 ± 6.6 ms, *d* = -0.74 ($-1.57/-0.08$), *p* = 0.004). PP remained reduced at follow-up, whereas Aix75 and TTI returned to baseline values. AA was decreased in the PS_{Bil} and the PS_{Mono} group at week 6 (PS_{Bil}: -2.4 ± 0.6 mmHg, *d* = -1.59 ($-2.51/-0.67$), *p* = 0.003; PS_{Mono}: -2.5 ± 0.9 mmHg, *d* = -1.14 ($-2.00/-0.28$), *p* = 0.002) and at week 12 (PS_{Bil}:

-2.9 ± 0.7 mmHg, *d* = -1.88 ($-2.84/-0.92$), *P* < 0.001; PS_{Mono}: -3.7 ± 0.9 mmHg, *d* = -1.72 ($-2.65/-0.78$), *P* < 0.001) and returned to baseline at follow-up. PWV_{CF} and PWV_{CR} were significantly decreased in all PS training groups at week 6 and week 12, respectively (*P* < 0.001). They remained reduced in the PS_{Bil} and PS_{Mono} group at follow-up (*p* = 0.002 and 0.005). Changes in SBP, DBP and MAP at any time were greater in the PS_{Bil} and the PS_{Mono} group than in the Ctrl group (*P* < 0.001). The changes in PWA and in PWV_{CF} at week 12 were greater in the PS_{Bil} and the PS_{Mono} group than in the Ctrl group (*P* from 0.005 to < 0.001).

sPLM

Figure 5 presents the results of femoral artery vascular function testing. ANOVA disclosed significant time × intervention interactions for $\dot{Q}_{fem, bas}$ (*F* = 6.11, *P* < 0.001), $\dot{Q}_{fem, peak}$ (*F* = 5.37, *P* < 0.001), $\Delta \dot{Q}_{fem}$ (*F* = 5.54, *P* < 0.001), and AUC (*F* = 6.66, *P* < 0.001). Main effects for time were found for $\dot{Q}_{fem, bas}$ (*F* = 5.34, *P* < 0.001), $\dot{Q}_{fem, peak}$ (*F* = 7.82, *P* < 0.001), $\Delta \dot{Q}_{fem}$ (*F* = 7.56, *P* < 0.001), and AUC (*F* = 8.15, *P* < 0.001). In all PS training groups, the other parameters except for

Table 2. Changes in the antegrade, retrograde and mean shear rate values in the femoral and popliteal artery during the elongation and relaxation phases of the acute stretching bout administered to the knee extensor and plantar flexor muscle

	Shear rate (s ⁻¹)	Baseline	Elongation phase					One-way ANOVA RM (F; P)
			1st	2nd	3rd	4th	5th	
Femoral artery	Antegrade	79 ± 17	117 ± 14*	70 ± 17 [†]	47 ± 13 [†]	43 ± 12 [†]	32 ± 13 [†]	11.04; <0.001
	Retrograde	-3 ± 1	-6 ± 3*	-5 ± 2*	-4 ± 2	-5 ± 3*	-4 ± 3	7.15; <0.001
	Mean	76 ± 15	111 ± 12*	65 ± 15 [†]	43 ± 10 [†]	38 ± 11 [†]	28 ± 11 [†]	8.94; <0.001
Popliteal artery	Antegrade	47 ± 5	70 ± 14*	42 ± 9 [†]	28 ± 5 [†]	26 ± 6 [†]	19 ± 5 [†]	10.7; <0.001
	Retrograde	-5 ± 1	-6 ± 2	-7 ± 2*	-7 ± 3*	-6 ± 2	-8 ± 3*	8.30; <0.001
	Mean	42 ± 4	64 ± 13*	35 ± 8 [†]	21 ± 5 [†]	12 ± 5 [†]	11 ± 3 [†]	9.1; <0.001
	Shear rate (s ⁻¹)	Baseline	Relaxation phase					One-way ANOVA RM (F; P)
			1st	2nd	3rd	4th	5th	
Femoral artery	Antegrade	79 ± 17	189 ± 27*	167 ± 16*	171 ± 25*	169 ± 17*	130 ± 12*	12.5; <0.001
	Retrograde	-3 ± 1	-1 ± 1 [†]	-0.7 ± 1 [†]	-1 ± 1 [†]	-0.6 ± 1 [†]	-0.9 ± 1 [†]	7.36; <0.001
	Mean	76 ± 15	188 ± 25*	166 ± 16*	170 ± 23*	168 ± 16*	129 ± 10*	9.60; <0.001
Popliteal artery	Antegrade	47 ± 5	113 ± 14*	100 ± 13*	103 ± 13*	101 ± 15*	78 ± 12*	12.29; <0.001
	Retrograde	-5 ± 1	-2 ± 2 [†]	-2 ± 1 [†]	-1.5 ± 2 [†]	-1 ± 2 [†]	-2 ± 1 [†]	5.50; <0.001
	Mean	42 ± 4	111 ± 12*	98 ± 11*	101 ± 10*	100 ± 14*	76 ± 11*	11.10; <0.001

* $P < 0.05$ higher than baseline; [†] $P < 0.05$ lower than baseline ($n = 20$). Data are presented as mean ± standard deviation.

$D_{fem, bas}$ were significantly increased at week 6 ($\Delta \dot{Q}_{fem}$ PS_{Bil}: +52 ± 11.2 ml·min⁻¹, $d = 1.34$ (0.67/1.77), $P < 0.001$; $\Delta \dot{Q}_{fem}$ PS_{Mono}: +31 ± 5.6 ml·min⁻¹, $d = 1.14$ (0.28/2.00), $P < 0.001$) and remained elevated up to follow-up ($P < 0.001$ for all parameters). Changes in $\dot{Q}_{fem, bas}$ at week 6 and week 12 for the PS_{Bil} and the PS_{Mono}SL group were greater than for the Ctrl group (P from 0.003 to <0.001). Changes in $\dot{Q}_{fem, peak}$ were greater for the PS_{Bil} and the PS_{Mono}SL group than for the Ctrl and the PS_{Mono}CL group (P from 0.004 to <0.001). Changes in $\Delta \dot{Q}_{fem}$ were greater for the PS_{Bil} and the PS_{Mono}SL group than for the Ctrl group at any time and at week 12 and follow-up than for the PS_{Mono}CL group (P from 0.01 to <0.001). Changes in AUC_{fem} were greater at week 6 for the PS_{Bil} and the PS_{Mono}SL group than for the Ctrl group, and at week 12 for the PS_{Bil}, the PS_{Mono}SL, and the PS_{Mono}CL than for the Ctrl group (P from 0.009 to <0.001).

FMD

Figures 6 and 7 present FMD test results for the popliteal and the brachial artery, respectively. For the popliteal and the brachial artery, significant time × interventions interaction were observed in D_{bas} ($F = 7.03$ and 6.57, $P < 0.001$), D_{peak} ($F = 4.58$ and 4.46, $P < 0.001$), FMD% ($F = 2.34$ and 2.80, $p = 0.017$ and 0.005), AUC ($F = 7.40$ and 7.47, $P < 0.001$), $\dot{Y}AUC$ ($F = 3.59$ and 3.91, $P < 0.001$), FMD/ \dot{Y} ($F = 2.56$ and 2.02, $p = 0.010$ and 0.042), and \dot{Q}_{peak} ($F = 7.11$ and 7.35, $P < 0.001$). Main effects for time were found in D_{bas} ($F = 6.27$ and 6.62, $P < 0.001$),

D_{peak} ($F = 7.02$ and 7.44, $P < 0.001$), FMD% ($F = 3.56$ and 8.99, $p = 0.022$ and <0.001), AUC ($F = 7.37$ and 7.41, $P < 0.001$), $\dot{Y}AUC$ ($F = 7.07$ and 7.35, $P < 0.001$), and \dot{Q}_{peak} ($F = 7.34$ and 6.73, $P < 0.001$).

$D_{pop, bas}$ and $\dot{Q}_{pop, bas}$ were significantly increased in the PS_{Bil} and the PS_{Mono}SL group at week 12 (P from 0.01 to 0.04). $D_{pop, peak}$ and FMD_{pop}% ($p = 0.002$) were significantly increased in the PS_{Bil} and the PS_{Mono}SL group at week 6 ($D_{pop, peak}$ PS_{Bil}: +0.16 ± 0.024 mm, $d = 0.89$ (0.22/1.52), $P < 0.001$; $D_{pop, peak}$ PS_{Mono}SL: +0.17 ± 0.015 mm, $d = 0.99$ (0.31/1.71), $P < 0.001$) and in the PS_{Mono}CL group at week 12 ($D_{pop, peak}$ PS_{Mono}CL: +0.18 ± 0.06 mm, $d = 0.71$ (-0.11/1.54, $p = 0.02$), before returning to baseline at follow-up. $\dot{Q}_{pop, peak}$ and AUC_{pop} were increased in the PS_{Bil} and the PS_{Mono}SL group at week 6 ($\dot{Q}_{pop, peak}$ PS_{Bil}: +33 ± 1.5 ml·min⁻¹, $d = 1.21$ (0.57/1.72), $P < 0.001$; $\dot{Q}_{pop, peak}$ PS_{Mono}SL: +34 ± 2.0 ml·min⁻¹, $d = 1.24$ (0.62/1.81), $P < 0.001$) and week 12 in the PS_{Mono}CL group up to follow-up ($\dot{Q}_{pop, peak}$ PS_{Mono}CL: +29 ± 2.6 ml·min⁻¹, $d = 1.01$ (0.45/1.44, $p = 0.003$). \dot{Y}_{pop} AUC was decreased in the PS_{Bil} and the PS_{Mono}SL group at week 6 until follow-up (\dot{Y}_{pop} AUC PS_{Bil}: -2.91 ± 0.61 s⁻¹, $d = -0.74$ (-1.56/-0.09), $p = 0.03$; \dot{Y}_{pop} AUC PS_{Mono}SL: -2.57 ± 0.78 s⁻¹, $d = -0.76$ (-1.59/-0.06), $p = 0.005$). Except for $D_{pop, bas}$, changes in all the other parameters were greater at week 6 and week 12 in the PS_{Bil} and the PS_{Mono}SL group than in the Ctrl group (P from 0.004 to <0.001).

$D_{brach, bas}$ and $\dot{Q}_{brach, bas}$ remained unchanged in all groups, whereas $D_{brach, peak}$ was significantly increased in the PS_{Bil} and the PS_{Mono} at week 12 until follow-up

($D_{\text{brach,peak}} \text{PS}_{\text{Bil}}$: $+0.22 \pm 0.041 \text{ mm}$, $d = 0.88 (0.04/1.71)$, $p = 0.004$; $D_{\text{pop,peak}} \text{PS}_{\text{MonoSL}}$: $+0.18 \pm 0.056 \text{ mm}$, $d = 0.82 (0.01/1.65)$, $p = 0.007$). $\text{FMD}_{\text{brach}}\%$ was increased at week 12 in the PS_{Bil} and the PS_{Mono} ($p = 0.006$ and 0.01), before returning to baseline at follow-up. $\dot{Q}_{\text{brach,peak}}$ and $\text{AUC}_{\text{brach}}$ were increased in the PS_{Bil} and the PS_{Mono} group at week 12 up to follow-up (P from 0.02 to 0.002). \dot{Y}_{brach} AUC was decreased in the PS_{Bil} and the PS_{Mono} group at week

12, returning to baseline at follow-up (\dot{Y}_{pop} AUC PS_{Bil} : $-30 \pm 5.1 \text{ s}^{-1}$, $d = -1.21 (-2.08/-0.34)$, $p = 0.003$; \dot{Y}_{pop} AUC $\text{PS}_{\text{MonoSL}}$: $-23 \pm 5.1 \text{ s}^{-1}$, $d = -1.30 (-2.18/-0.34)$, $p = 0.001$). $\text{FMD}/\dot{Y}_{\text{brach}}$ was increased in the PS_{Bil} and the PS_{Mono} group in week 12 returning to baseline at follow-up ($p = 0.002$ to 0.004). Changes in \dot{Y}_{brach} AUC were greater for the PS_{Bil} and the PS_{Mono} than for the Ctrl. group at week 12 and follow-up ($P < 0.001$ for both comparisons).

Pulse wave analysis

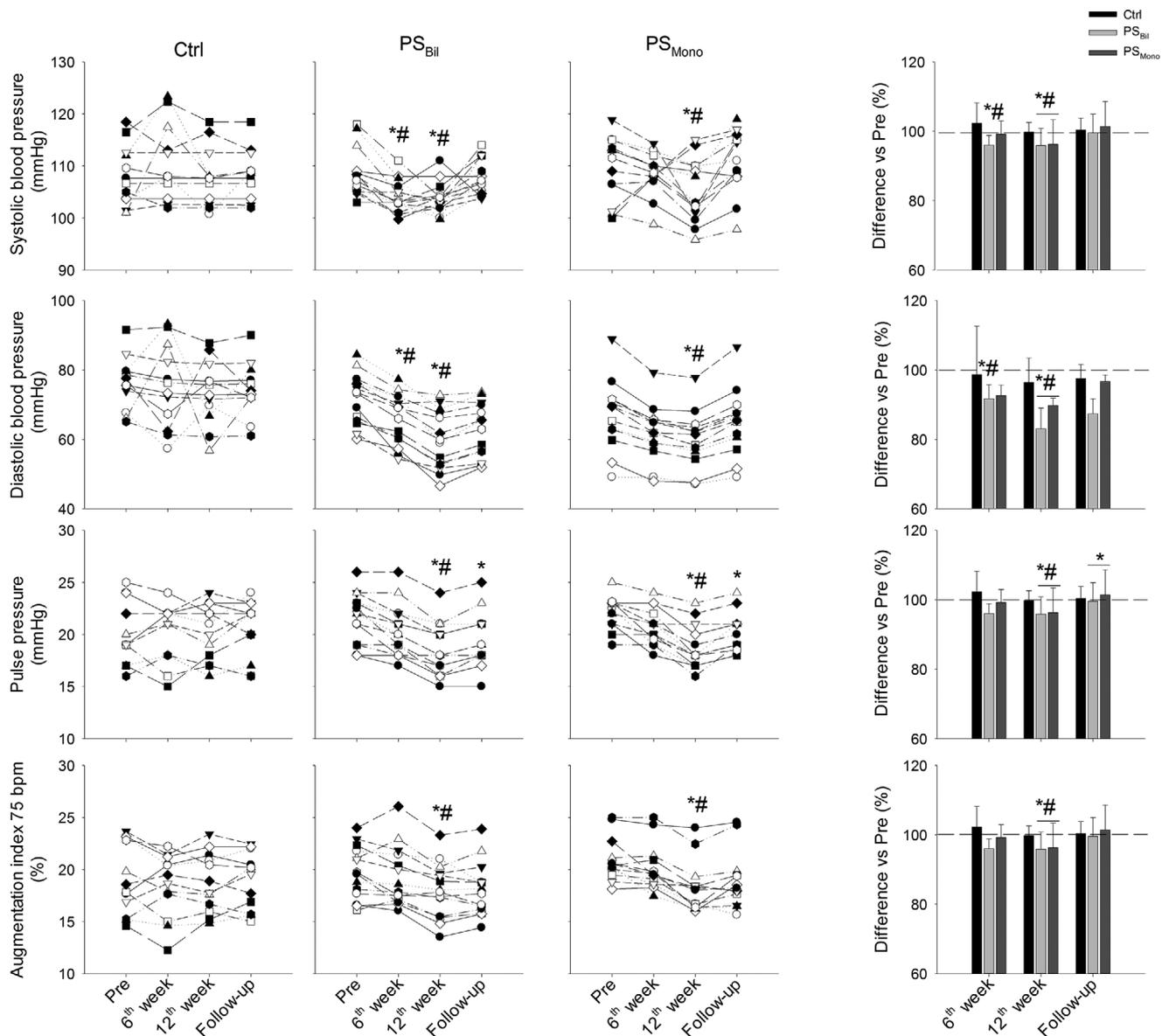


Figure 3. Pulse wave analysis

Individual data for systolic blood pressure, diastolic blood pressure, pulse pressure, and augmentation index normalised at 75 beats min^{-1} in the control (Ctrl), the bilateral passive stretching (PS_{Bil}), and the unilateral passive stretching group (PS_{Mono}) at baseline (Pre), at weeks 6 and 12, and at week 6 of follow-up. Histograms report the percentage changes in respect to Pre for the systolic pressure, systolic blood pressure, diastolic blood pressure, pulse pressure and augmentation index normalised at 75 beats min^{-1} in the control (Ctrl), the bilateral passive stretching (PS_{Bil}), and the unilateral passive stretching group (PS_{Mono}) at weeks 6 and 12, and at week 6 of follow-up (* $P < 0.05$ vs. Pre, # $P < 0.05$ vs. Ctrl).

IEx

Figure 8 reports the IEx test results for the popliteal and the brachial artery, respectively. Time \times interventions interaction was observed in the brachial artery the IEx% ($F = 2.89$, $p = 0.004$). In the popliteal and the brachial artery, main effects for time were found in IEx % ($F = 3.47$ and 8.90 , $p = 0.024$ and < 0.001). IEx_{pop}% was significantly increased at week 12 in the PS_{Bil}, the PS_{MonoSL}, and the PS_{MonoCL} group (P from 0.005 to < 0.001), before returning to baseline at follow-up. IEx/ \dot{Y}_{pop} was increased in all groups at week 12 (P from 0.004 to 0.003), before returning to baseline at follow-up. Changes in IEx_{pop}% and IEx/ \dot{Y}_{pop} were greater for the PS_{Bil} and the PS_{MonoSL} than for the Ctrl group at week 12 (P from 0.003 to < 0.001).

IEx_{brach}% was increased in the PS_{Bil} and the PS_{Mono} group ($P < 0.001$ for both groups) at week 12 before returning to baseline at follow-up. IEx/ \dot{Y}_{brach} was increased in all groups at week 12 (P from 0.005 to 0.003), before returning to baseline at follow-up. Changes in IEx_{brach}% and IEx/ \dot{Y}_{brach} were greater for the PS_{Bil} and the PS_{Mono} than for the Ctrl group at week 12 ($p = 0.003$ and to 0.004, respectively).

Correlations

Table 3 provides the correlations between the changes in ankle and knee joint ROM and in PWA, PWA, and in the variables calculated in sPLM, FMD and IEx tests. Inverse correlations ranging from moderate to high were found between ankle and knee joint ROM and the DBP, PP, AIx75, PWV_{CF} and PWV_{CR}. Direct correlations spanning from moderate to high were retrieved between ankle and knee joint ROM and several variables in sPLM, FMD and IEx variables.

Discussion

Improvement in blood pressure, arterial stiffness and vascular function was noted in the arteries of the body parts directly and not directly involved in PS training of the lower limbs. Blood pressure was decreased, central and peripheral arterial stiffness was reduced, and vascular function was increased after 12 weeks of PS training. Such changes suggest PS training-induced local and systemic cardiovascular adjustments. Interestingly, systemic changes, in particular in the vessels not directly

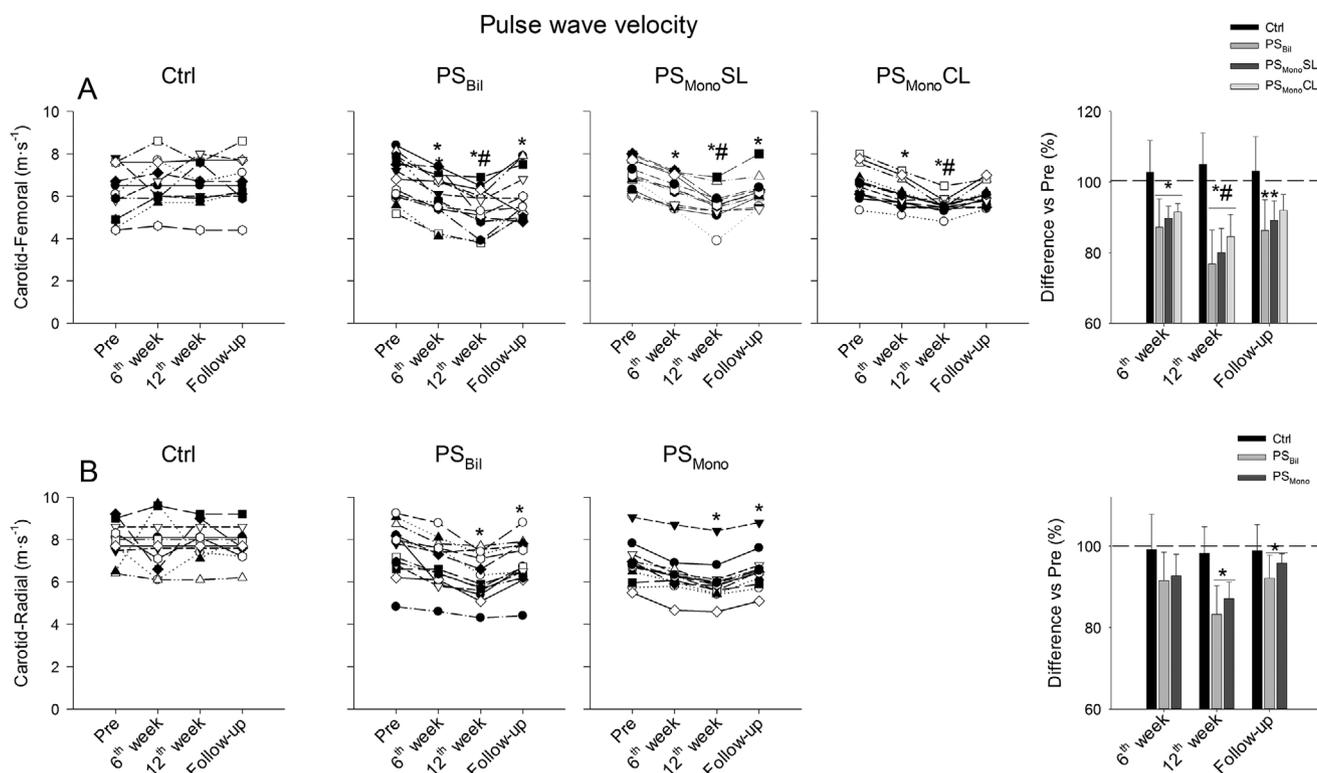


Figure 4. Pulse wave velocity

Individual data for carotid-femoral and carotid-radial pulse wave velocity in the control (Ctrl), the bilateral passive stretching (PS_{Bil}), and the unilateral passive stretching group (stretched limb, PS_{MonoSL} and contralateral limb PS_{MonoCL}) at baseline (Pre), at weeks 6 and 12, and at week 6 of follow-up. Histograms report the percentage changes in respect to Pre for the carotid-femoral and carotid-radial pulse wave velocity in the control (Ctrl), the bilateral passive stretching (PS_{Bil}), and the unilateral passive stretching group (PS_{Mono}) at weeks 6 and 12, and at week 6 of follow-up (* $P < 0.05$ vs. Pre, # $P < 0.05$ vs. Ctrl).

involved in PS training, seemed to have a shorter duration in comparison to local adaptations, which are maintained in the arteries directly involved in PS training even after 6 weeks from its cessation.

Preliminary considerations

After PS training, ankle and knee joint ROM was increased in the stretched but not in the contralateral, unstretched limb. Increased ROM with stretching is a well-described phenomenon that is more likely the result of a rise in stretch tolerance (reduced mechanoreceptor and nociceptor activity during stretching) of the muscles than a decrease in muscle/tendon stiffness or a change in muscle architecture (Freitas *et al.* 2018). At follow-up assessment 6 weeks after stretching cessation, ROM was noted to have returned to pre-training levels. As this is the first study to investigate the effects of chronic PS cessation on joint ROM, no comparison with previous studies can be made. Furthermore, our observation of no effect of

PS training on maximum isometric force of plantar flexor and knee extensor muscles is shared by previous reports (Medeiros & Lima, 2017).

PS training effects on blood pressure and central and peripheral arterial stiffness

We observed positive readjustment in blood pressure and central and peripheral arterial stiffness after PS training. Arterial stiffness was decreased in both limbs of the PS training groups, albeit with some time differences between central and peripheral readjustment: PWV_{CF} was decreased starting at week 6 of PS training, while PWV_{CR} and other blood pressure parameters were reduced starting at week 12. Several mechanical and neural adaptations may elucidate these changes. The reduction in central artery stiffness may be explained by the reduction in central pressure in view of the strong two-way street relation between the two variables. Indeed, PS training, by inducing repetitive stimulation of transient sympathetic

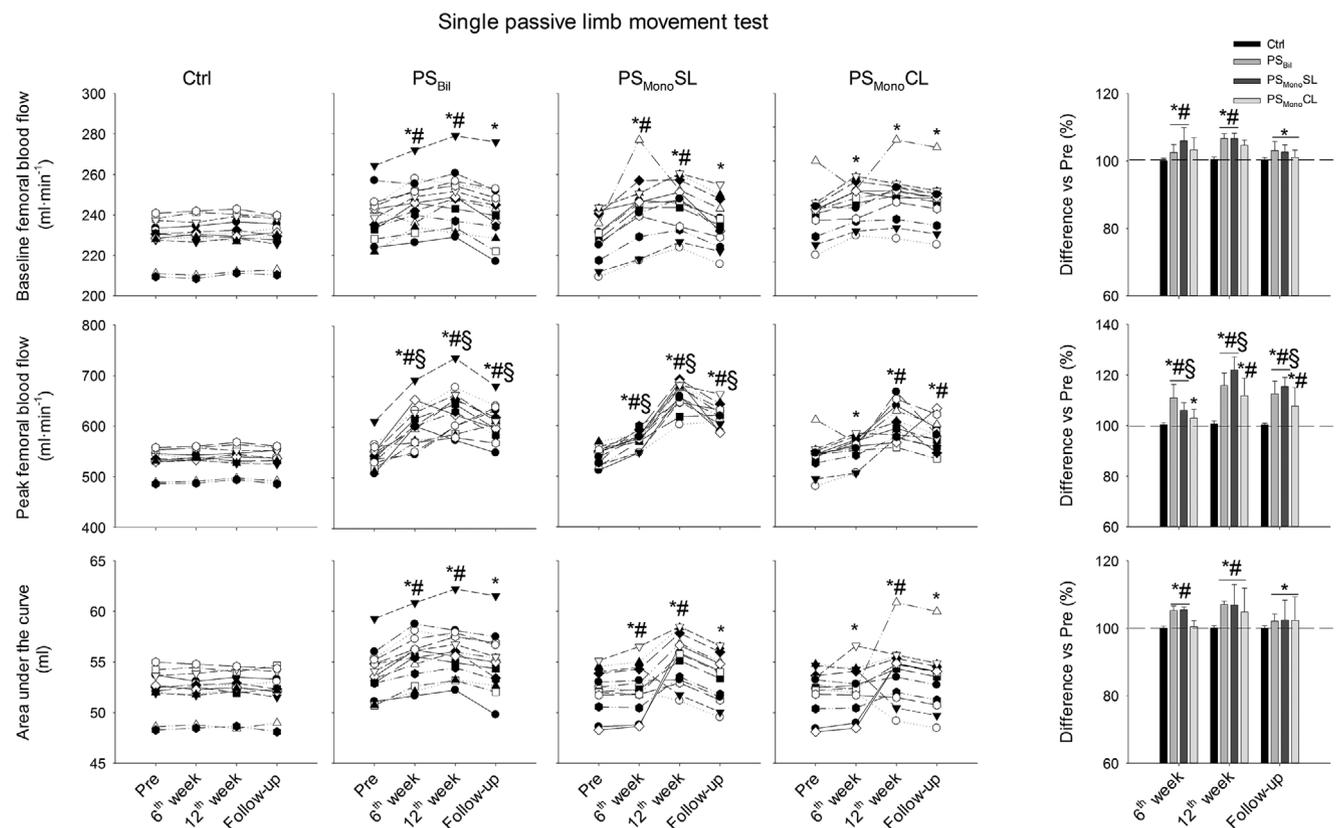
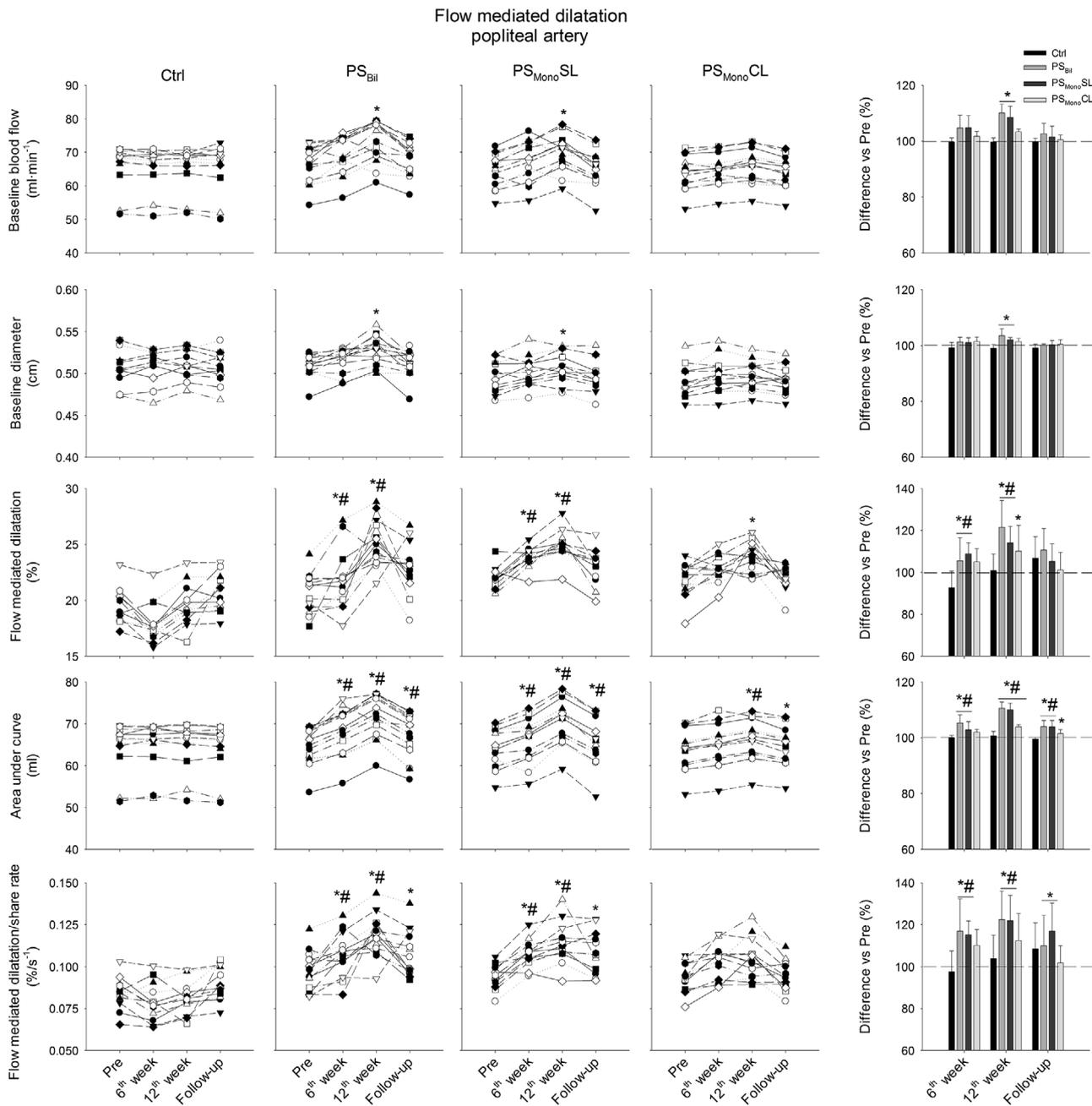
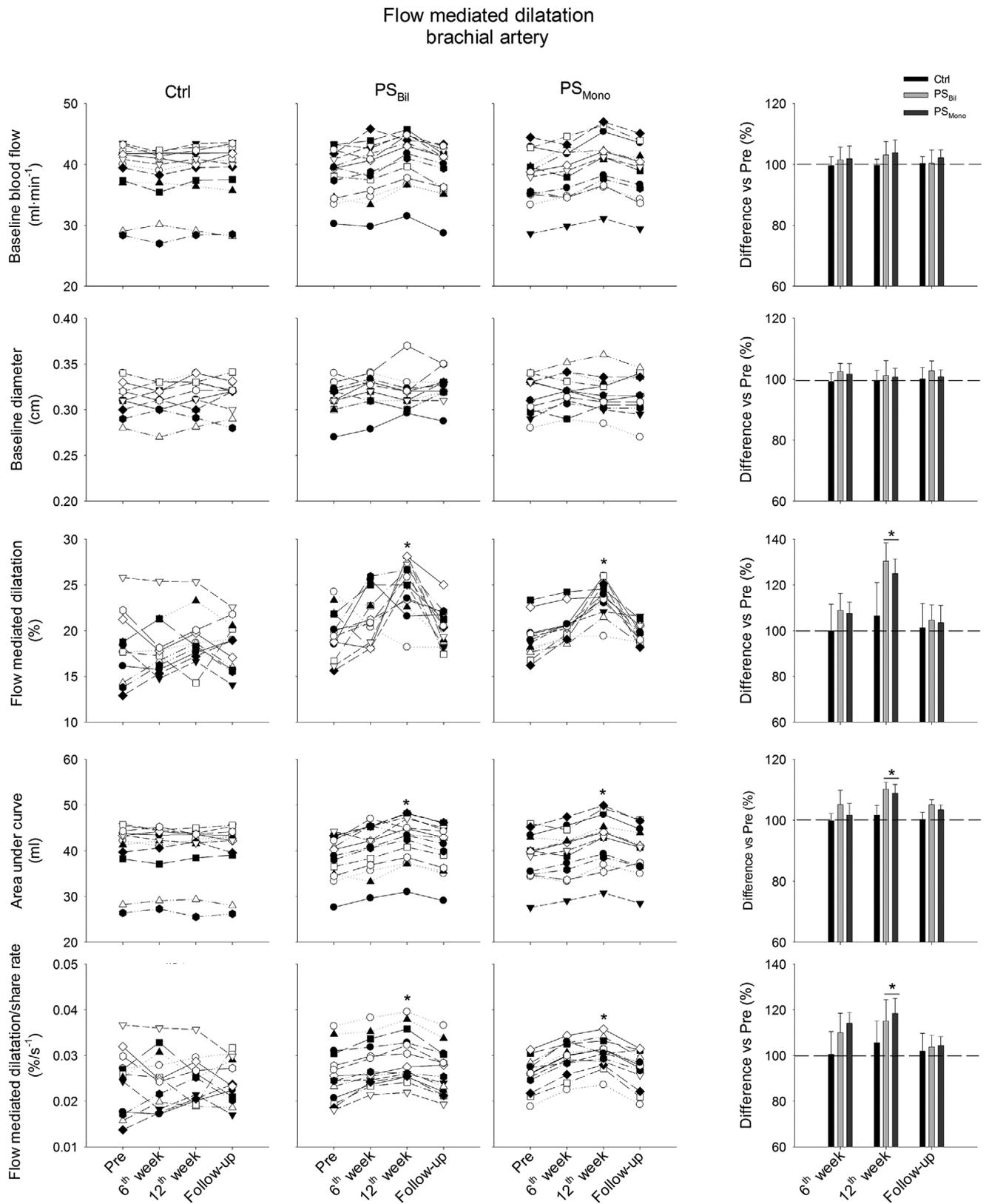


Figure 5. Single passive limb movement test
 Individual data for baseline blood flow, peak blood flow, and area under the curve in femoral artery in the control (Ctrl), the bilateral passive stretching (PS_{Bil}), and the unilateral stretching group (stretched limb, PS_{MonoSL}), and contralateral limb (PS_{MonoCL}) at baseline (Pre), at weeks 6 and 12, and at week 6 of follow-up. Histograms report the percentage changes in respect to Pre for the baseline blood flow, peak blood flow, and area under the curve in femoral artery in the control (Ctrl), the bilateral passive stretching (PS_{Bil}), and the unilateral stretching group (stretched limb, PS_{MonoSL}), and contralateral limb (PS_{MonoCL}) at weeks 6 and 12, and at week 6 of follow-up (**P* < 0.05 vs. Pre, #*P* < 0.05 vs. Ctrl, §*P* < 0.05 vs. PS_{MonoCL}).

excitation (Cui *et al.* 2006), may have chronically reduced the central autonomic mechanism of \dot{Q} control, thus also decreasing arterial stiffness in the vessels not directly involved in PS training and aortic blood pressure with related parameters (PP, MAP and TTI). Moreover, the

decrease in TTI, an indirect index of myocardial oxygen consumption (Chemla *et al.* 2008) may be indicative of a reduction in afterload and, probably, in overall cardiac work. Although the present study does not provide direct measurement of a PS-induced reduction in the





central autonomic mechanism of \dot{Q} control, its possible contribution to reducing arterial stiffness and blood pressure cannot be ruled out. The changes in arterial stiffness and blood pressure are partially in agreement with previous work (Williams *et al.* 2013; Wong & Figueroa, 2014). Similar changes were noted in an elderly but not in a young population (Yamamoto *et al.* 2009; Williams *et al.* 2013; Wong & Figueroa, 2014; Shinno *et al.* 2017). Such a discrepancy may be attributed to PS training duration and sample size. Previous studies had shorter training periods and/or training sessions than our study, with a lower total amount of stretching (Wong & Figueroa, 2014; Shinno *et al.* 2017). Additionally, previous studies did not mention participant adherence to the protocol (Williams *et al.* 2013; Wong & Figueroa, 2014; Shinno *et al.* 2017), which in our case was set at 80% to ensure a minimum of involvement by all participants. PS training may have reduced arterial stiffness by inducing an increase in elastin and collagen content in the arterial wall as a result of sustained axial elongation of the arteries involved in chronic stretching (Jackson *et al.* 2002; Nichols *et al.* 2011; Marti *et al.* 2012). This explanation could be supported by evidence for a further increase in vasodilatation capacity after IEx and by the correlations between the changes in ankle and knee joint ROM, and the changes in central and peripheral arterial stiffness, suggesting mechanical remodeling of the arterial wall in the vessels directly involved in PS training (Naylor *et al.* 2005). Independently from the mechanisms underlying the PS training-induced changes in arterial stiffness and blood pressure, blood pressure returned to baseline within 6 weeks after training cessation, while the central and peripheral arterial stiffness were still reduced at the end of the follow-up. The different time course between blood pressure and arterial stiffness after PS training cessation may suggest a different relative weight played by the central and local (mechanical) mechanisms underlying their decrement.

PS training-induced changes in vascular function

The present findings provide novel evidence for the impact of PS training on vascular function, as assessed by FMD (Harris *et al.* 2010) and by the more recent sPLM methodology (Venturelli *et al.* 2017b). FMD is a well-recognized means to assess vascular function and estimate cardiovascular risk (Harris *et al.* 2010; Broxterman *et al.* 2019). FMD response is dependent on both reduction in sympathetic outflow towards the vessel (Hijmering *et al.* 2002) and on bioavailability of local vaso-

dilatory molecules such as NO (Green, 2005; Wray *et al.* 2013). In contrast, sPLM response is more representative of a microvascular assessment and closely related to local factors (see Methods). Thus, its response seems to be only marginally influenced by the sympathetic activity (Venturelli *et al.* 2017a,b). Previous studies reported enhanced vascular function of the stretched limb after PS training only indirectly as measured with the reactive hyperaemia peripheral tonometry index (Hotta *et al.* 2013; Kato *et al.* 2017; Shinno *et al.* 2017), a technique mainly related to better NO bioavailability due to shear stress-induced vasodilatation (Matsuzawa *et al.* 2015). FMD results in the present study are in line with the previously published data: indeed, an improvement in vascular function in the arteries directly involved with PS training was noted for both training groups under investigation. The improvement may stem from repeated changes in \dot{Y} during PS and from its effect on NO bioavailability. PS training repeatedly exposed the vessels to increased levels of \dot{Y} (Kruse *et al.* 2016; Venturelli *et al.* 2017a, 2019), thus stimulating endothelial mechano-transduction signalling in smooth muscle cells that results in vasodilatation (2017, Green *et al.* 2017a,b). Such repeated alterations in \dot{Y} over time can be considered as a kind vascular preconditioning that may lead to increase NO bioavailability (Green *et al.* 2017a; Bisconti *et al.* 2019). It was demonstrated that vascular function increases during skeletal muscle contraction because of an increase in mean \dot{Y} due to elevated antegrade \dot{Y} (Tinken *et al.* 2009). A previous study characterized the oscillatory nature of \dot{Y} during elongation and relaxation cycles intrinsic in stretching manoeuvres, and reported that a marked hyperaemic response occurred immediately after a bout of stretching, which coincided with elevated antegrade and mean \dot{Y} and attenuated retrograde \dot{Y} (Kruse *et al.* 2016). The increment of antegrade and mean \dot{Y} and the attenuation of retrograde \dot{Y} shown in our study during a single PS administration further support this mechanism, suggesting a pivotal role for \dot{Y} in triggering an adjustment in vascular function after PS training. Additionally, some studies reported that these positive effects depend on \dot{Y} magnitude (Green *et al.* 2017a). In our study, the FMD (%) and its relative AUC were significantly increased in the popliteal artery of the involved limb, as well as in the popliteal and the brachial artery of the limbs not involved in PS training, indicating a widespread systemic effect. Notably, the changes in vascular function in the arteries directly involved in PS training occurred earlier than in those not involved. Nevertheless, after 12 weeks

weeks 6 and 12, and at week 6 of follow-up. Histograms report the percentage change in respect to Pre for the baseline blood flow, baseline diameter, flow-mediated dilatation, area under the curve, and flow-mediated dilatation/shear rate in the brachial artery in the control (Ctrl), the bilateral passive stretching (PS_{Bi}), the unilateral stretching group (stretched limb, PS_{Mono}SL), and contralateral limb (PS_{Mono}CL) at weeks 6 and 12, and at week 6 of follow-up (**P* < 0.05 vs. Pre).

of PS training, no significant differences in FMD response were observed between the arteries directly involved in PS training and the contralateral arteries (non-involved), suggesting a major contribution of the systemic/central mechanisms in explaining the increase in FMD response. This increase returned to baseline within 6 weeks from PS training cessation, indicating a limited duration of the aforementioned mechanisms.

Our study demonstrated a clear improvement in all sPLM parameters at week 12 of PS training of the involved and uninvolved limbs, thus reinforcing the hypothesis for a systemic effect. As stated above, the sPLM response relates to microvasculature reactivity induced by increased perfusion pressure and peripheral vasodilatation (Mortensen *et al.* 2012; Trinity *et al.* 2012). Enhancement of the microvasculature, together with lower

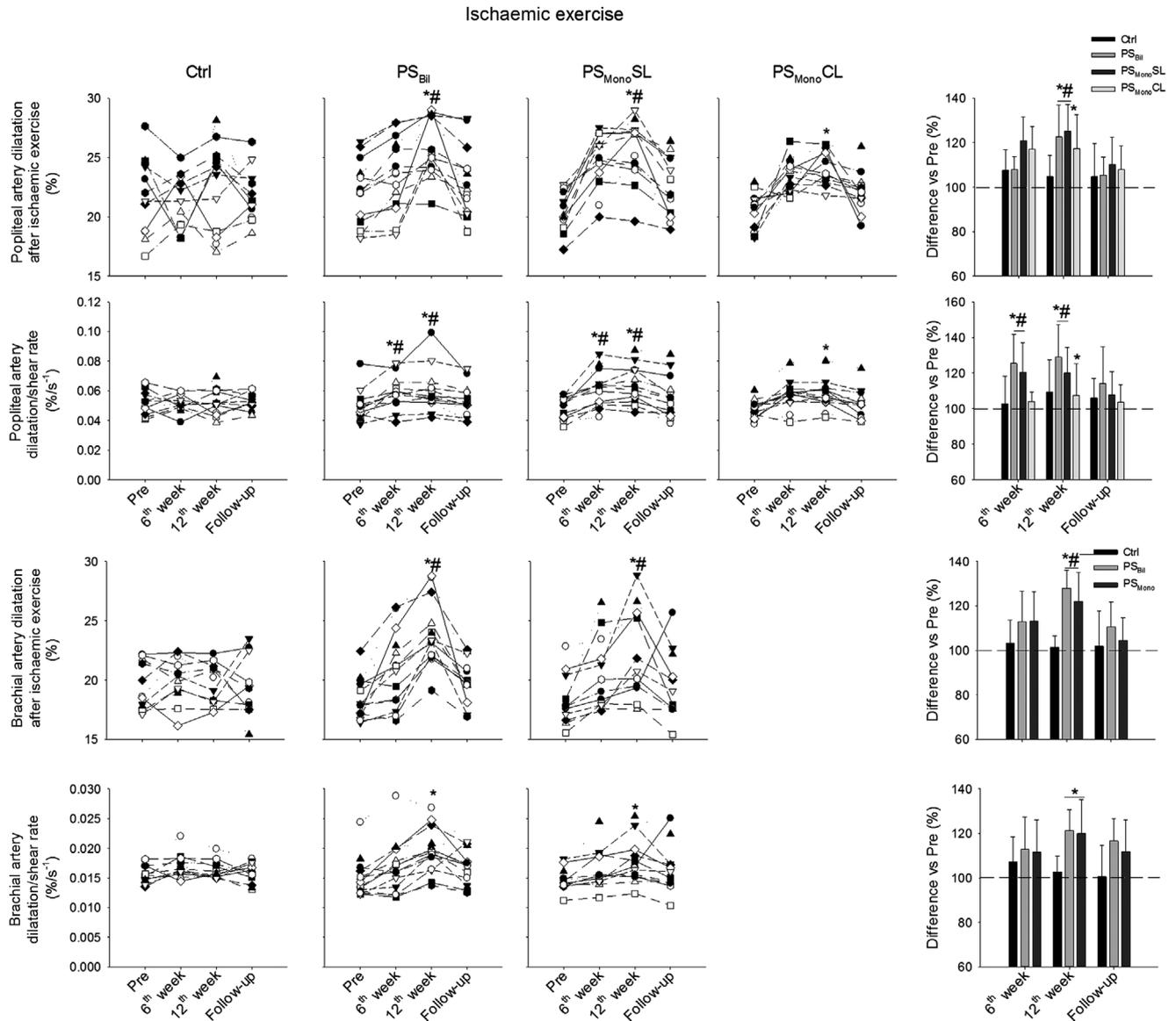


Figure 8. Ischaemic exercise

Individual data for artery dilatation, and dilatation/shear rate after ischaemic exercise in the popliteal artery (upper panels) and in the brachial artery (lower panels) in the control (Ctrl), the bilateral passive stretching (PS_{Bil}), the unilateral stretching group (stretched limb, PS_{MonoSL}), and contralateral limb (PS_{MonoCL}) at baseline (Pre), at weeks 6 and 12, and at week 6 of follow-up. Histograms report the percentage changes in respect to Pre for the artery dilatation, and dilatation/shear rate after ischaemic exercise in the popliteal artery (upper graphs) and in the brachial artery (lower graphs) in the control (Ctrl), the bilateral passive stretching (PS_{Bil}), the unilateral stretching group (stretched limb, PS_{MonoSL}) and contralateral limb (PS_{MonoCL}); in the brachial artery in the control (Ctrl), the bilateral passive stretching (PS_{Bil}), the unilateral stretching group (stretched limb, PS_{Mono}) at weeks 6 and 12, and at week 6 of follow-up.

Table 3. Correlations between the changes at week 12 vs. Pre in ankle and knee range of motion (ROM) and single passive limb movement (sPLM), flow mediated dilatation (FMD), and ischaemic exercise (IEx) variables

PWA/PWV		SBP	DBP	PP	Aix75	PWV _{CF}	PWV _{CR}
Ankle ROM	R (CI _{95%})	-0.233 (-0.477/-0.019)	-0.492 (-0.697/0.167)	-0.391 (-0.613/-0.204)	-0.301 (-0.560/-0.060)	-0.632 (-0.765/-0.484)	-0.515 (-0.715/-0.272)
	P R (CI _{95%})	0.153 -0.212 (-0.489/0.40)	0.001 -0.242 (-0.498/0.061)	0.014 -0.352 (-0.534/-0.10.8)	0.063 -0.377 (-0.574/-0.125)	<0.001 -0.533 (-0.686/-0.350)	0.001 -0.383 (-0.627/-0.088)
sPLM femoral artery	P	0.195	0.138	0.028	0.018	<0.001	0.016
	R (CI _{95%})	$\dot{Q}_{fem,bas}$ 0.603 (0.427/0.751)	$\dot{Q}_{fem,peak}$ 0.662 (0.496/0.801)	AUC 0.448 (0.251/0.657)	AUC 0.448 (0.251/0.657)	<0.001 0.004 0.354 (0.021/0.631)	<0.001 0.004 0.354 (0.021/0.631)
Knee ROM	P R (CI _{95%})	<0.001 0.557 (0.326/-754)	<0.001 0.697 (0.525/0.816)	0.004 0.354 (0.021/0.631)	0.004 0.354 (0.021/0.631)	<0.001 0.658 (0.450/0.797)	<0.001 0.453 (0.187/0.668)
	P	<0.001	<0.001	0.007	0.001	<0.001	<0.001
FMD popliteal artery	R (CI _{95%})	FMD% 0.521 (0.291/0.718)	$\dot{Q}_{pop,bas}$ 0.673 (0.501/0.803)	$D_{pop,bas}$ 0.380 (0.135/0.574)	AUC 0.720 (0.561/0.845)	FMD/ȳ 0.565 (0.347/0.755)	FMD/ȳ 0.565 (0.347/0.755)
	P R (CI _{95%})	0.002 0.389 (0.131/0.608)	<0.001 0.541 (0.340/0.717)	0.017 0.482 (0.232/0.687)	0.001 0.658 (0.450/0.797)	<0.001 0.453 (0.187/0.668)	<0.001 0.453 (0.187/0.668)
FMD brachial artery	P R (CI _{95%})	0.014 FMD% 0.275 (0.011/0.497)	<0.001 $\dot{Q}_{brach,bas}$ 0.652 (0.462/0.791)	0.002 $D_{brach,bas}$ 0.194 (-0.033/0.384)	<0.001 AUC 0.611 (0.428/0.773)	FMD/ȳ 0.117 (-0.380/533)	FMD/ȳ 0.117 (-0.380/533)
	P R (CI _{95%})	0.090 0.407 (0.186/0.614)	<0.001 0.541 (0.302/0.731)	0.236 0.084 (-0.239/376)	<0.001 0.650 (0.419/0.809)	0.477 0.322 (0.037/0.556)	0.477 0.322 (0.037/0.556)
IEx popliteal/ brachial artery	P R (CI _{95%})	0.010 IEx% _{pop} 0.369 (0.067/0.582)	<0.001 IEx% _{brach} 0.600 (0.424/0.757)	0.612 0.612 (-0.239/376)	<0.001 0.650 (0.419/0.809)	0.045 0.045 (0.037/0.556)	0.045 0.045 (0.037/0.556)
	P R (CI _{95%})	0.021 0.274 (-0.060/0.547)	<0.001 0.373 (0.058/0.653)	0.612 0.612 (-0.239/376)	<0.001 0.650 (0.419/0.809)	0.045 0.045 (0.037/0.556)	0.045 0.045 (0.037/0.556)

R coefficient with 95% confidence interval and P values are provided for each correlation ($n = 39$). PWA, pulse wave analysis; PWV, pulse wave velocity; CF, carotid-femoral; CR, carotid-radial; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; Aix, augmentation index normalised to 75 bpm; \dot{Q}_{bas} , baseline blood flow; \dot{Q}_{peak} , peak blood flow; AUC, area under the curve; D_{bas} , baseline diameter; \dot{Y} , shear rate; IEx%, maximum dilatation capacity.

MAP, could be ascribed to a reduction in total peripheral vascular resistance. Of note, vessel tortuosity is closely related to augmented peripheral vascular resistance in large and small blood vessels and to \dot{Q} distribution (Poole & Mathieu-Costello, 1989). Vessel tortuosity describes how twisted the capillaries are and how many turns and bends they have, on which basis a physiological index of the capillary extension reserve is defined (Poole & Mathieu-Costello, 1989; Poole *et al.* 1997). However, changes in muscle sarcomere length significantly reduce the capillary extension reserve (i.e. tortuosity) (Poole & Mathieu-Costello, 1989; Poole *et al.* 1997). Specifically, extensive muscle lengthening (as in PS) results in repeated cycles of vessel elongation and compression, resulting in a reduction of vessel resistance and capillary diameter, as well as of \dot{Q} and O_2 supply (Poole *et al.* 1997). Such acute vessel distortion constitutes an important stimulus for long-term vascular adaptation, induced by PS training. While our study did not supply direct evidence for changes in vessel tortuosity, a reduction following PS training cannot be ruled out. In addition to these mechanisms, a significant increase in angiogenesis was observed after PS training (30 min day⁻¹, 5 days week⁻¹, for 4 weeks) in a murine model (Hotta *et al.* 2018), a period far shorter than our study protocol. Further studies are needed to clarify the occurrence of this mechanism also in humans. Interestingly, the change in sPLM response was higher in the artery of the limb directly involved in PS training than in the contralateral artery (non-involved). This difference may underline a larger increase in vascular function in the area where a greater shear stress stimulus occurred. Moreover, an increased sPLM response was also observed at follow-up, with the maintenance of greater response in the artery of the limb directly involved compared to the contralateral (non-involved) artery, probably suggesting more persisting effects of the local mechanisms.

Lastly, femoral artery resting \dot{Q} was increased in all training groups in the limbs directly and not directly involved in PS training. Such a finding suggests that not only vascular function but also muscle perfusion improved after PS training. The correlations found in the present study between the changes in ankle and knee ROM and the resting \dot{Q} in all the three investigated arteries may further support this possible occurrence.

Study limitations

The present study has several limitations. First, direct assessment of muscle sympathetic nerve activity and of NO bioavailability would have evidenced possible stretch-induced remodulation of sympathetic vessel tone and endothelial function, respectively. The lack of a direct assessment of these measurements did not allow us to establish a clear balance between central and local

mechanisms underlying the positive changes in vascular function. Second, measurements on female participants were always made during the early follicular phase of the menstrual cycle, implying that tests may have occurred with a ± 5 day-dispersion from the exact testing week. However, in view of the strong vascular responsiveness fluctuation induced by the menstrual cycle, we preferred to test female participants during the same period of the menstrual cycle. Lastly, a larger sample size may have helped to detect possible differences in PS training response between female and male participants.

Conclusions

The present study clearly demonstrates that 12-week PS training is effective in improving vascular function and decreasing stiffness of the directly involved arteries (i.e. femoral and popliteal arteries of the stretched limbs) and the arteries not directly involved (i.e. contralateral femoral and popliteal arteries and brachial artery) in PS training. These improvements encompassed both the modification of central and local \dot{Q} control mechanisms. However, the central mechanisms influencing blood pressure and vascular function (i.e. PWA and FMD) had a shorter duration compared to those mainly driven by local control (i.e. sPLM), as the former returned to baseline within 6 weeks from PS training cessation, while the latter were still present at the end of follow-up. PS has been shown to be an effective means to improve vascular function, with practical implications for its use as a novel non-pharmacological treatment for improving vascular health, reducing the overall cardiovascular risk, especially in individuals with limited mobility.

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Additional information

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no professional relationship with companies or manufacturers that might receive help from the results of the

present study. The authors declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

Authors contributions

All experiments were conducted at the Physiology Labs of the School of Sport Science, Università degli Studi di Milano. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. Conception or design of the study: A.V.B., E.C., S.L., F.E. Acquisition, analysis or interpretation of data for the study: A.V.B., E.C., S.L., M.V., G.C., E.L., C.D., S.R., F.E. Drafting the manuscript or revising it critically for important intellectual content: A.V.B., E.C., S.L., M.V., G.C., E.L., C.D., S.R., F.E. All the authors have approved the final version of the manuscript and agree to be accountable for all aspects of the study in ensuring that questions related to the accuracy or integrity of any part of the study are appropriately investigated and resolved.

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Keywords

arterial stiffness, endothelial function, flow-mediated dilatation, muscle stretching, single passive limb movement

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Statistical Summary Statement