Leg Blood Flow and Skeletal Muscle Microvascular Perfusion Responses to Submaximal Exercise in Peripheral Arterial Disease

Running Title: Skeletal Muscle Microvascular Perfusion in PAD

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ABSTRACT

Peripheral arterial disease (PAD) is characterized by stenosis and occlusion of the lower limb arteries. While leg blood flow is limited in PAD, it remains unclear whether skeletal muscle microvascular perfusion is affected. We compared whole-leg blood flow and calf muscle microvascular perfusion following cuff occlusion and submaximal leg exercise between PAD patients (n=12, 69±9 years) and healthy age-matched control participants (n=12, 68±7 years). Microvascular blood flow (microvascular volume x flow velocity) of the medial gastrocnemius muscle was measured before and immediately after: 1) 5 min of thigh-cuff occlusion; and 2) a 5-min bout of intermittent isometric plantar-flexion exercise (400N) using real-time contrast-enhanced ultrasound (CEU). Whole-leg blood flow was measured after thigh-cuff occlusion and during submaximal plantar-flexion exercise using strain-gauge plethysmography. Post-occlusion whole-leg blood flow and calf muscle microvascular perfusion were lower in PAD patients than controls, and these parameters were strongly correlated ($r=0.84$; $p<0.01$). During submaximal exercise, total whole-leg blood flow and vascular conductance were not different between groups. There were also no group differences in post-exercise calf muscle microvascular perfusion, although microvascular blood volume was higher in PAD patients than control (12.41±6.98 vs 6.34±4.98 aU; $p=0.03$). This study demonstrates that the impaired muscle perfusion of PAD patients during post-occlusion hyperemia is strongly correlated with disease severity, and is likely mainly determined by the limited conduit artery flow. In response to submaximal leg exercise, microvascular flow volume was elevated in PAD patients, which may reflect a compensatory mechanism to maintain muscle perfusion and oxygen delivery during recovery from exercise.

KEYWORDS: Peripheral arterial disease, skeletal muscle, microcirculation, reactive hyperemia, exercise, ultrasound.

NEW AND NOTEWORTHY

This study suggests that PAD has different effects on the microvascular perfusion responses to cuff occlusion and submaximal leg exercise. PAD patients have impaired microvascular perfusion following cuff occlusion, similar to that previously reported following maximal exercise. In response to submaximal exercise, however, the microvascular flow volume response was elevated in PAD patients compared with control. This finding may reflect a compensatory mechanism to maintain perfusion and oxygen delivery during recovery from exercise.

INTRODUCTION

Peripheral arterial disease (PAD) is an atherosclerotic disease characterized by stenosis or occlusion of the conduit arteries of the lower limbs. PAD is associated with severe exercise intolerance (2), which cannot be fully explained by hemodynamic impairments. Patients with PAD have impaired endothelial function (4), and alterations in their skeletal muscle phenotype that likely contribute to their exercise intolerance (2, 25). There have been some reports that skeletal muscle capillarization is reduced in PAD patients (2, 25, 26), and positively correlated to exercise capacity in these patients (2, 26). There are, however, some contrasting reports that muscle capillarization is similar (10), or even greater in PAD patients (20) than controls. It is therefore unclear what effect PAD has on microvascular structure and function, and in particular what role microvascular dysfunction plays in the exercise intolerance of PAD patients.

Contrast-enhanced ultrasound (CEU) is widely used for the assessment of microvascular blood flow (perfusion) in various human tissue beds (29), and a limited number of studies have used this technique to assess leg muscle perfusion in PAD patients (1,

6, 7, 16, 18, 30). These studies have shown that PAD is associated with a reduction in peak muscle perfusion capacity in response to ischemic provocation tests, such as thigh-cuff occlusion (1), limiting (maximal pain) treadmill walking exercise (16), and relatively high-intensity (20 W) plantar-flexion exercise (6). These studies of peak perfusion responses demonstrate the strong diagnostic potential of CEU and the ability to discriminate between people with and without PAD. However, as CEU provides a measure of end-organ (i.e. muscle) perfusion, it is not clear to what extent the limited perfusion responses in PAD patients can be attributed to a microvascular impairment or the impaired upstream conduit artery flow due to stenosis or occlusion.

In order to distinguish the microvascular from the conduit artery contribution, it is important to consider whole-leg blood flow when interpreting microvascular perfusion responses, which has not been considered in previous studies of PAD patients (1, 6, 7, 16, 18, 30). Whole-limb (33) and muscle microvascular blood flow (6) are generally not limited in PAD patients with intermittent claudication under resting conditions, and it is not until exercise is performed that a limit in blood flow is revealed. For a given patient, the point at which this limit is reached depends on the intensity of exercise, and patients usually self-select low, submaximal intensities to carry out activities of daily living (9). It is likely that the limit in peak muscle perfusion reported in previous studies was due to the relatively high exercise intensities used, at which point conduit artery blood flow limits are achieved. To better understand the microvascular perfusion limit as it applies to typical activities of daily living, and to better distinguish the microvascular blood flow limit associated with PAD, there is a need for further studies. Specifically, an investigation of the microvascular perfusion in conjunction with whole-limb blood flow, in response to submaximal exercise conditions that can be sustained within the symptomatic and hemodynamic limits of patients.

Therefore, the primary aim of this study was to compare calf muscle microvascular perfusion following cuff occlusion and submaximal leg exercise between PAD patients and control participants using continuous real-time CEU imaging, and also to assess whole-limb blood flow and its relationship with the muscle perfusion responses.

METHODS

Participant recruitment

Twelve patients with PAD and symptoms of intermittent claudication and 12 healthy control participants were recruited to take part in this study. Participants that were current smokers, had uncontrolled hypertension, unstable angina, congestive heart failure or critical limb ischemia were excluded. PAD patients unable to complete the 5-min leg exercise test due to intolerable calf muscle pain were also excluded. All participants gave written informed consent to participate in the study, which was approved by the Prince Charles Hospital (HREC/14/QPCH/122) and University of the Sunshine Coast (A/15/706) Human Research Ethics Committees. All procedures were conducted in accordance with the Declaration of Helsinki.

Study overview

Participants initially underwent screening and baseline measures, and completed a seven-day physical activity recall (3). Resting ankle-brachial index (ABI) of each leg was measured, and estimated calf muscle mass was determined using established anthropometric measures (5). Participants were familiarized with the plantar-flexion exercise protocol and performed a maximum force test. Participants attended an experimental session (session 1) for the assessment of whole-leg blood flow at rest and following thigh-cuff occlusion using strain-gauge plethysmography. Then, contraction-by-contraction whole-leg blood flow was

recorded during a 5-min bout of intermittent isometric plantar-flexion exercise. Exercise was submaximal and conducted at the same absolute (400 N) and relative (60±18% maximum force) intensity in both groups. Participants attended two further experimental sessions, on separate days, where muscle microvascular perfusion of the medial gastrocnemius was assessed using CEU before and after 5-min of thigh-cuff occlusion (session 2), and before and after the same 5-min plantar-flexion exercise protocol (session 3). Within each session, test procedures were conducted twice, separated by 15 min, and average responses were used for analysis. The non-dominant leg of the control participants, and the leg with the lowest ABI of PAD patients was assessed. Sessions were conducted at the same time of day, ~72 h apart, and participants were instructed to avoid exercise and caffeine or alcohol consumption for 24 h before each session.

Plantar-flexion exercise

Participants performed isometric plantar-flexion contractions in the seated position with their foot on an immovable footplate. A calibrated load cell (Xtran S1W, Applied Measurement, Melbourne, Australia) was positioned over the distal thigh, and contraction force was displayed on a monitor. After familiarization, participants performed five maximum voluntary contractions (MVC), each separated by 60 s rest, and the average was used as the maximum force. The 5-min bouts of plantar-flexion exercise were performed at a fixed contraction intensity of 400 N (contraction:relaxation duty cycle: 2:3 s). After each exercise bout participants were instructed to rate their exercise-associated calf muscle pain as “no pain”, “mild pain”, “moderate pain” or “maximal pain”.

Whole-leg blood flow

Whole-leg blood flow was assessed using strain-gauge plethysmography (EC6 Plethysmograph, Hokanson, Bellevue, USA). A rapid inflation cuff (Hokanson, Bellevue, USA) was placed around the upper thigh, and a mercury-in-silastic strain gauge was placed around the calf at the largest circumference. Measurements were performed during supine rest and following five min of thigh-cuff occlusion at a pressure of 200 mmHg, as previously described (14).

Contraction-by-contraction leg blood flow was measured throughout exercise as previously described (23). Blood flow was assessed following each contraction as the change in limb volume over the first cardiac cycle, free of movement artefact, from the onset of the 3-s relaxation phase. Single lead electrocardiogram, heart rate (ADInstruments, Sydney, Australia) and beat-by-beat finger blood pressure (Finapres Medical Systems, the Netherlands) were continuously monitored during exercise. Leg vascular conductance was calculated at rest and during exercise as leg blood flow divided by mean arterial pressure. Contraction-by-contraction whole-leg blood flow and vascular conductance were averaged across the repeated within-session trials and fitted to a biphasic exponential response curve (Table Curve 2D v4, SPSS, Illinois, USA) to determine the total rise in whole-leg blood flow during exercise (23). The goodness of fits for the whole-leg blood flow (n=24) and vascular conductance data (n=24), based on the adjusted R^2, were 0.77±0.12 and 0.73±0.15, respectively.

Calf muscle microvascular perfusion

Calf muscle microvascular perfusion was measured using CEU. Images were obtained from the medial gastrocnemius muscle at the point of the greatest leg circumference, with the ultrasound transducer secured to the leg using a foam holder. Continuous harmonic power-Doppler imaging (Philips Diagnostic Ultrasound Systems model iE33, Philips Medical

Systems, Andover, USA) was performed with a linear-array transducer (Philips L9-3), using a low mechanical index (0.10), 87% gain and 5 cm depth. Contrast solution consisting of 1.5 mL of lipid shelled octafluoropropane microbubbles (Definity, Bristol-Myers Squibb Medical Imaging, New York, USA) mixed to 50mL with saline was infused intravenously (antecubital fossa) at a constant rate of 200 mL/hour, using a syringe pump (Alaris PK, Auckland, New Zealand) that was continuously mechanically rocked to prevent agent sedimentation. Each CEU measurement required a total of three min of contrast microbubble infusion, with the first two min of infusion required to achieve a steady-state concentration of microbubbles. This was followed by a pulse of high-energy ultrasound (mechanical index = 1.07) for microbubble destruction, and at least 50 s (range 50-60 s) of image acquisition to assess the kinetics of microbubble replenishment. Examples of original CEU images are presented in Figure 1.

Microvascular image analysis

QLAB software (Philips Healthcare, Bothell, USA) was used to generate time-intensity curves for analysis of replenishment kinetics. An examiner (A.L.M.) manually selected a representative quadrilateral region of interest (839±163 mm^2) encompassing the medial gastrocnemius muscle that was automatically transposed for repeated measurements. Time-intensity data were exported for background subtraction (Excel 15.0, Microsoft Corporation), where the background intensity was set at 0.98 s (resting data) or 0.49 s (cuff occlusion and exercise data) from the moment of bubble-destruction to exclude the contributions of the faster-filling, larger non-capillary vessels from the background signal intensity (27). Using a 3-s moving average, we identified the time-to-peak acoustic intensity as the duration from the onset of change in microbubble acoustic intensity after microbubble

destruction until its peak. The area under the curve was calculated as the total accumulated change in acoustic intensity during the first 50 s following cuff occlusion or exercise.

Averaged time-intensity data from repeat trials were fitted to an exponential function $y=A[1-exp^{-\beta t}]$ for the analysis of the time-intensity curves, where: y is the video intensity at time t (in seconds); A is the peak video intensity, which estimates microvascular blood volume; and β is the rate of appearance of the microbubbles (i.e. the rate constant), which estimates microvascular flow velocity (35). Skeletal muscle microvascular blood flow (perfusion) was calculated as the product of blood volume multiplied by velocity ($A*\beta$). Curve fitting was performed using Sigmaplot software version 13.0 (Systat Software, San Jose, USA). The adjusted R^2 for the CEU microvascular blood flow (n=24) data was 0.92±0.10. The within-session intra-class correlation coefficient indicated an excellent reliability of measurements of muscle microvascular perfusion (n=24) obtained with CEU under resting conditions (0.91 [95% CI 0.83-0.96]) and in response to cuff occlusion (0.96 [95% CI 0.91-0.99]) and exercise (0.95 [95% CI 0.88-0.98]) (8).

Statistical analysis

Skeletal muscle microvascular perfusion was the primary outcome of interest and therefore a priori sample size calculations were performed based on the mean of previously reported differences between PAD patients and controls for reactive hyperemia and exercise (10±4 and 10±9 $aU.s^{-1}$, respectively) (18, 31). Assuming a power of 80% and an alpha level of 0.05, a minimum sample size of 6-11 participants in each group was required to detect these differences.

The Gaussian distribution and homogeneity of variance of the data were confirmed using Shapiro Wilk and Levene tests. Baseline comparisons were performed using independent sample t-tests and chi-square tests for continuous and categorical variables,

respectively. Whole-leg blood flow and microvascular perfusion parameters were analyzed using a 2-way (group x time) mixed ANOVA, followed by Tukey's post-hoc test when there were significant main effects or interactions. Correlations were assessed using Pearson´s correlation coefficient. $P<0.05$ was considered statistically significant. Statistical analyses were performed using SPSS version 22.0 for Windows and power calculations were performed using GPower version 3.1. Data are presented as mean and standard deviation (SD) unless stated otherwise.

RESULTS

Participant characteristics

Participant characteristics are shown in Table 1. Five PAD patients (42%) had arterial stenoses or occlusions in multiple artery segments, while the remaining patients had significant unilateral or bilateral stenoses or occlusions in the aorto-iliac (n=2; 17%), femoral (n=4; 33%) or popliteal (n=1; 8%) segments. Self-reported habitual physical activity levels tended to be lower in PAD patients but were not significantly different between groups (PAD: 1.6±1.6; Control: 2.8±2.2 h/week; p=0.16).

INSERT TABLE 1

Plantar-flexion force

Plantar-flexion MVC force was not significantly different between groups, meaning that the relative intensity of the target force during the bouts of plantar-flexion exercise was not different between groups (Table 1). The results remained unchanged after correcting MVC force for estimated calf muscle mass. The 5-min submaximal exercise bouts were well tolerated by all control participants, and 11 of the 12 PAD patients (92%) reported moderate to maximal calf muscle pain towards the end of the tests.

Post-occlusion whole-leg blood flow and vascular conductance

Baseline (supine rest) whole-leg blood flow and vascular conductance were not significantly different between groups (Table 2). Post-occlusion whole-leg blood flow and vascular conductance were significantly lower in PAD patients compared with controls (Table 2).

INSERT TABLE 2

Post-occlusion calf muscle microvascular perfusion

Mean CEU time-intensity curves following cuff occlusion in PAD patients and controls are shown in Figure 2. Time-to-peak acoustic intensity was longer in PAD patients (41.15±12.37 s, $p<0.01$) compared with controls (17.79±3.98 s). Area under the response curve tended to be lower in the PAD group compared to controls, but the difference was not statistically significant (PAD: 413±343; Control: 659±452 aU.s; $p=0.08$).

Calf muscle microvascular perfusion parameters, at baseline and following cuff occlusion, are shown in Figure 3. Baseline (pre-occlusion) muscle microvascular blood volume, flow velocity and perfusion were not significantly different between groups ($p>0.05$). In comparison with baseline, post-occlusion microvascular blood volume increased ($p<0.01$; Figure 3A) in both groups, and despite the slower rise (Figure 2), peak volume in the PAD group was not significantly different to controls ($p=0.14$, Figure 3A). Post-occlusion microvascular flow velocity increased in controls ($p<0.01$; Figure 3B), but did not change significantly in PAD patients ($p=0.29$), resulting in a lower microvascular perfusion response in PAD patients than in controls ($p<0.01$; Figure 3C).

INSERT FIGURE 2

INSERT FIGURE 3

Exercise whole-leg blood flow and vascular conductance

Baseline (seated rest) whole-leg blood flow was not different between groups (p=0.43; Table 2). Total whole-leg blood flow during submaximal exercise was not significantly different between PAD patients and controls (p=0.10; Table 2). Results remained unchanged when differences in blood pressure between groups were accounted for by expressing the blood flow responses as leg vascular conductance (p=0.75; Table 2).

Post-exercise calf muscle microvascular perfusion

The mean CEU time-intensity curves for the period immediately following submaximal plantar-flexion exercise in PAD patients and controls are shown in Figure 4. Time-to-peak acoustic intensity was not different between groups (PAD: 39.67±16.18; Control: 30.35±14.46 s; p=0.13). The area under the curve was greater in PAD patients (576±343 aU.s; p=0.01) than controls (264±224 aU.s).

Calf muscle microvascular perfusion parameters, before and after submaximal exercise, are shown in Figure 5. Baseline (pre-exercise) microvascular parameters were not different between groups (p>0.05). Microvascular blood volume increased (p<0.01) with exercise in both groups, and was significantly higher in the post-exercise period in PAD patients than controls (p=0.03; Figure 5A). Microvascular flow velocity increased (p<0.01) with exercise in both groups, with no significant difference between PAD patients and controls (p=0.91; Figure 5B). Microvascular perfusion also increased (p<0.01) with exercise in both groups, with no significant difference between PAD patients and controls (p=0.35; Figure 5C).

INSERT FIGURE 4

INSERT FIGURE 5

Relationships between variables

ABI was significantly correlated with muscle microvascular blood volume ($n=24$; $r=0.41$; $p=0.04$), flow velocity ($n=24$; $r=0.80$; $p<0.01$) and perfusion ($n=24$; $r=0.67$; $p<0.01$). Post-occlusion whole-leg blood flow was significantly correlated with muscle microvascular blood volume ($n=24$; $r=0.58$; $p<0.01$), flow velocity ($n=24$; $r=0.76$; $p<0.01$) and perfusion ($n=24$; $r=0.84$; $p<0.01$). All correlations remained significant when whole-leg blood flow was expressed as leg vascular conductance ($p<0.05$). However, these correlations were not significant when considering PAD and control groups separately. There were no significant correlations between post-exercise CEU microvascular parameters and ABI or whole-leg blood flow.

DISCUSSION

Previous studies that have reported reductions in muscle microvascular perfusion capacity in PAD patients in response to cuff occlusion and exercise have not reported whole-leg or conduit artery blood flow. Consequently it was not known whether the microvascular perfusion limitation merely reflected the limited (stenotic) arterial flow capacity. Moreover, it was not known whether muscle microvascular perfusion was limited in PAD patients during submaximal exercise, at workloads that reflect activities of daily living and that are able to be sustained within the symptomatic and hemodynamic limits of the patient. This study demonstrated that: (i) post-occlusion whole-leg blood flow and calf muscle microvascular perfusion were lower in PAD patients compared with controls, and these parameters were strongly correlated; and (ii) in response to submaximal plantar-flexion exercise, PAD patients exhibited similar whole-leg and post-exercise muscle microvascular perfusion responses

compared with controls, but muscle microvascular perfusion was achieved through a greater increase in microvascular volume in PAD patients.

We found that calf muscle microvascular perfusion following 5-min of cuff occlusion was impaired in PAD patients. Post-occlusion time-to-peak acoustic intensity was longer in PAD patients than controls, which verifies the findings of a previous CEU study by Amarteifio et al. (1), and is consistent with the perfusion limit reported in studies of PAD patients using magnetic resonance imaging (19, 31). In healthy individuals, post-occlusion reactive hyperemia is predominantly determined by vascular resistance at the level of the microvessels (28), whereas in PAD patients the conduit artery resistance at the site of the arterial stenosis also likely contributes to the hyperemic response. In the present study, the impairment in post-occlusion hyperemia in PAD patients was evident throughout the vascular tree, with lower whole-leg blood flow and calf muscle microvascular perfusion compared with controls. The lower post-occlusion microvascular perfusion in PAD patients was mainly attributable to a lower microvascular flow velocity (parameter β) compared with controls. Therefore, microvascular flow velocity might be determined by limb blood flow or perfusion pressure, and this is supported by the strong correlation between microvascular flow velocity and whole-leg blood flow. While previous findings of an impaired post-occlusion CEU response in PAD patients have been attributed to a microvascular impairment (1), our findings suggest that the low post-occlusion microvascular flow velocity may largely reflect disease severity and the impaired whole-limb blood flow response. This is further supported by the strong relationship between ABI and the post-occlusion microvascular velocity and whole-leg blood flow parameters observed in the current study.

During tightly matched submaximal plantar-flexion exercise at the same absolute (400N = ~8W) and relative (60±18% maximum force) intensity, steady-state whole-leg blood flow did not differ between PAD patients and controls. Patients were able to sustain this

exercise for 5 min, indicating that the intensity was within their hemodynamic and symptomatic limits, and at a level that is typical of their daily activities (9). In response to this level of exercise we found that post-exercise calf muscle microvascular perfusion is preserved in PAD patients and did not differ compared with healthy control participants. From this we assume that the observed microvascular responses are largely independent of whole-limb blood flow. In healthy individuals it has previously been shown that exercise-induced microvascular hyperemia is accompanied by changes in total limb blood flow at higher (80% max) but not lower (25% max) exercise intensities (32). It was therefore suggested that perfusion at lower exercise intensities is regulated through a localized redistribution of blood flow, possibly at the level of the terminal arterioles, and with more intense exercise there are also changes in the upstream resistance of the arteriolar network with consequent changes in total limb blood flow (32). Whether maximal exercise-induced muscle ischemia in PAD patients might disrupt this relationship cannot be determined in this study as the exercise test was submaximal and within the limits of patients; however, it is notable that there was no significant relationship between exercise whole-leg blood flow and post-exercise muscle microvascular perfusion in PAD patients and controls.

While muscle perfusion responses to submaximal exercise did not differ between groups, an important finding was that the increase in perfusion relied on a larger increase in microvascular volume (parameter A) in PAD patients compared with controls. The increased microvascular volume, characterized by an increase in CEU microbubble density, may reflect a reduction in microvascular resistance and a redistribution of blood flow within the working skeletal muscle. It has also been suggested that an increase in microvascular volume may reflect *de-novo* recruitment of previously non-flowing capillaries (21), although this is debatable (24). In PAD patients, the increased microvascular volume may serve to enhance capillary flow and the endothelial surface area available to support the delivery of oxygen and

nutrients to myocytes (24), and therefore compensate for microvascular flow velocity, which tended to be lower in PAD patients compared to controls. Similar compensatory responses have been reported in ischemic heart studies using CEU, where myocardial microvascular perfusion is maintained with increasing coronary artery stenosis severity via increases in microvascular blood volume (17).

Recent studies by Kundi et al. 2017 (16) and Davidson et al. 2017 (6) have demonstrated the clinical utility of CEU to differentiate between individuals with and without PAD on the basis of peak exercise muscle perfusion. Their findings of a *lower* peak muscle perfusion response, compared with controls, to (maximal) limiting treadmill walking (16) and a short bout (2 min) of high-intensity (~20 W) plantar-flexion exercise (6), provide support for the use of peak exercise CEU responses in the diagnosis of PAD. The exercise intensity used in those studies was not well matched between groups (16) and was notably higher than the submaximal intensity used in the present study (400 N, ~8 W). At these higher intensities, patients were likely working at a level above their conduit artery blood flow limit, which is reflected in the lower (end-organ) muscle perfusion relative to controls. This cannot be directly verified as those studies did not include measures of whole-limb or conduit artery blood flow, as was done in the present study. However, in a subgroup of PAD patients in the study by Davidson et al. 2017 (6) who underwent surgical revascularization to restore conduit artery blood flow, there was a marked increase in the muscle perfusion response to exercise. This suggests that at this relatively high exercise intensity (20 W), the muscle perfusion response is largely determined by the conduit artery flow limit in PAD patients, and this response would be expected to be exacerbated in patients with more severe disease (e.g. ABI < 0.6), such as those in the study by Davidson et al. (6).

We used real-time CEU imaging in conjunction with a continuous infusion of the microbubble contrast agent, which has not been done in previous studies of PAD patients.

This method enables the assessment of rapid changes in muscle microvascular flow, such as during the period immediately following exercise. The incremental imaging approach used by Davidson et al. 2017 (6) and the bolus infusion approach used by Kundi et al. 2017 (16) are limited to use under steady-state conditions, and are therefore not ideally suited to studies of short periods of exercise (2 min) (6) and exercise recovery (16). Our study groups were well matched for age, whereas the mean age of the control groups in previous studies were 15-23 years younger than the PAD groups (6, 16). In addition, there was a high proportion (89%) of diabetic patients in the PAD group of the study by Davidson et al. 2017 (6). Given the known influence of age (13, 15) and diabetes (27) on muscle microvascular structure and function, these factors are important to control for in studies of PAD. Patients with uncontrolled hypertension were not considered for the present study because of the known association between high blood pressure and capillary rarefaction (11). Blood pressure tended to be lower (not significant) in PAD patients at rest and during exercise, which we attribute to the greater use of anti-hypertensive medications in that group.

Using real-time CEU, we have shown that PAD has a significant effect on the muscle microvascular perfusion responses to cuff-induced ischemia and submaximal exercise. Further research is needed to understand the mechanisms responsible for these responses, and specifically to determine to what extent the microvascular perfusion alterations in PAD are associated with changes in capillary density and structure. Future studies should test the effect of exercise intensity (e.g. maximal vs submaximal intensities) to better understand the influence on conduit artery flow and microvascular responses, and determine whether CEU derived measures of microvascular volume reflect muscle capillary supply, as has been proposed (34). There is also an opportunity for future research to focus on microvascular function as a treatment target in PAD patients. Adding exercise as an adjunct therapy following lower-limb surgical or endovascular revascularization for PAD promotes additional

improvements in symptoms and exercise capacity (22). Computational modelling of the PAD vasculature suggest that these gains are partially dependent on improvements in microvascular function (12), however this has yet to be confirmed.

Limitations of this study include the relatively small study sample, which might have contributed to the failure to detect significant differences in some secondary outcomes, such as AUC of the image intensity response. Nevertheless, this study was adequately powered to detect differences in our primary outcome of muscle microvascular perfusion. The study sample did not allow for subgroup analysis of variables that could possibly affect calf muscle microvascular perfusion, such as cardiovascular risk factors, medication use and site of stenosis. Additionally, it was not possible to make simultaneous measurements of whole-leg blood flow and calf muscle microvascular perfusion, which would provide a better indication of the role of whole-leg blood flow in determining microvascular perfusion responses to exercise.

This study suggests that the impaired muscle perfusion of PAD patients during post-occlusion hyperemia is strongly associated with disease severity, and is likely determined by the limited conduit artery flow. In response to submaximal leg exercise, muscle microvascular flow volume was elevated in PAD patients, which may reflect a compensatory mechanism to maintain perfusion and oxygen delivery during recovery from exercise. This study has highlighted novel alterations in microvascular flow that are likely to be of functional relevance in PAD patients and contribute to their capacity to undertake activities of daily living. Further research is required to understand the mechanism responsible for these findings and the impact of treatments, such as lower limb revascularization and exercise therapy, on muscle microvascular perfusion.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Conception and design of study (A.L.M, K.G, C.D.A); collection, analysis and interpretation of data (A.L.M., M.C.Y.N., T.B., R.M., J.G., Y.H., M.K., K.G., C.D.A.); initial draft of the manuscript (A.L.M, C.D.A); critical revision of the manuscript for important intellectual content (A.L.M., M.C.Y.N., T.B., R.M., J.G., Y.H., M.K., K.G., C.D.A.). All authors approved the final version of the manuscript.

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FIGURE LEGENDS

Figure 1. Representative presentation of original CEU images from PAD and control participants at baseline and during peak microvascular perfusion following cuff occlusion and submaximal plantar-flexion exercise. Post-occlusion peak acoustic intensity occurs earlier in the control compared to PAD participant. Post-exercise peak acoustic intensity occurs at a similar time in PAD and control participants, but seems to be enhanced in the patient with PAD despite similar submaximal workload performed by both participants.

Figure 2. Post-occlusion CEU time-intensity curves in PAD patients (n=12; open circles) and controls (n=12; shaded circles). Data points represent group mean and error bars are SEM.

Figure 3. Calf muscle microvascular blood volume (panel A), flow velocity (panel B) and perfusion (panel C) at baseline and following cuff occlusion in PAD patients (n=12) and controls (n=12). Values are mean and SD. Dotted lines indicate individual participant responses. *Significantly different from baseline ($p<0.05$); †Significantly different to control participants ($p<0.05$).

Figure 4. Post-exercise CEU time-intensity curves in PAD patients (n=12; open circles) and controls (n=12; shaded circles). Data points represent group mean and error bars are SEM.

Figure 5. Calf muscle microvascular blood volume (panel A), flow velocity (panel B) and perfusion (panel C) before and following plantar-flexion exercise in PAD patients (n=12) and controls (n=12). Values are mean and SD. Dotted lines indicate individual participant

599 responses. *Significantly different from baseline ($p<0.05$); †Significantly different to control

600 participants ($p<0.05$).

601

602 **TABLES**

603 Table 1. Participant characteristics.

Parameter	Control (n=12)	PAD (n=12)	*P* Value
Age, y	68±7	69±9	0.72
Male gender, n (%)	6 (50)	8 (67)	0.34
Weight, kg	79.7±12.0	85.4±22.0	0.44
Height, cm	172.3±7.0	170.6±7.5	0.57
Body mass index, $kg.m^{-2}$	26.9±4.0	29.1±6.1	0.32
Resting ABI[a]	1.16±0.12	0.69±0.21	<0.01
ECMM, kg	1.98±0.22	1.86±0.37	0.36
Plantar-flexion MVC force, N	908±366	908±203	0.99
Plantar-flexion MVC force, % MVC	63±22	58±13	0.40
Plantar-flexion MVC force/ ECMM, N/kg	455±154	512±170	0.39
Cardiovascular risk factors			
Previous-smoker, n (%)	2 (17)	10 (83)	<0.01
Dyslipidemia, n (%)	2 (17)	11 (92)	<0.01
Hypertension, n (%)	2 (17)	8 (67)	0.02
Diabetes, n (%)	0 (0)	2 (17)	0.24
History of coronary artery disease, n (%)	0 (0)	4 (33)	0.05
Resting haemodynamics			
Heart rate, beats/min	61±9	63±10	0.55
Systolic BP, mmHg	136±18	134±12	0.75
Diastolic BP, mmHg	77±7	69±9	0.02
Mean arterial pressure, mmHg	97±11	88±9	0.05

Blood Biochemistry			
Fasting glucose, mmol.L^{-1}	5.16±0.37	6.48±1.72	<0.01
Total cholesterol, mmol.L^{-1}	5.73±0.74	4.28±0.93	<0.01
LDL cholesterol, mmol.L^{-1}	3.62±0.65	2.21±0.79	<0.01
HDL cholesterol, mmol.L^{-1}	1.53±0.45	1.35±0.38	0.31
Triglycerides, mmol.L^{-1}	1.33±0.94	1.53±1.01	0.67
Medications			
Aspirin or clopidogrel, n (%)	2 (17)	9 (75)	<0.01
Beta blocker, n (%)	0 (0)	4 (33)	0.05
ACE inhibitor or ARB, n (%)	2 (17)	9 (75)	<0.01
Statin, n (%)	2 (17)	11 (92)	<0.01
Calcium channel blocker, n (%)	2 (17)	4 (33)	0.32
Metformin, n (%)	0 (0)	2 (17)	0.24

Values are expressed as mean ± SD or n (%).

ABI = ankle brachial index; ACE = angiotensin-converting enzyme inhibitor; ARB = angiotensin II receptor blocker; BP = blood pressure; ECMM = estimated calf muscle mass; HDL = high-density lipoprotein; LDL = low-density lipoprotein; MVC = maximum voluntary contraction; PAD = peripheral arterial disease.

[a] ABI measured in the non-dominant limb of control participants, and in the more affected limb of PAD patients.

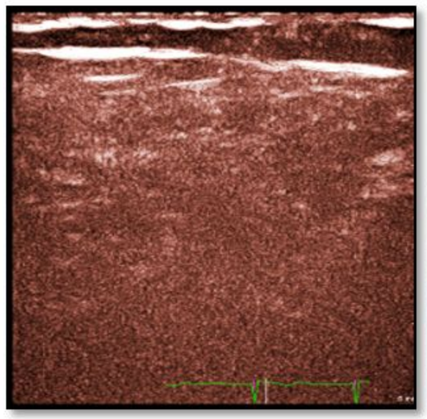
612 Table 2. Whole-leg blood flow and vascular conductance.

Parameter	Control (n=12)	PAD (n=12)	*P* value
Post-occlusion measures (supine)			
Baseline blood flow, $ml.100ml^{-1}.min^{-1}$	1.94±0.65	2.10±0.78	0.61
Baseline vascular conductance, $ml.100ml^{-1}.min^{-1}.mmHg$	0.02±0.01	0.02±0.01	0.33
Post-occlusion blood flow, $ml.100ml^{-1}.min^{-1}$	16.55±6.02	4.52±3.59	<0.01
Post-occlusion vascular conductance, $ml.100ml^{-1}.min^{-1}.mmHg$	0.18±0.07	0.05±0.04	<0.01
Exercise measures (seated)			
Baseline blood flow, $ml.100ml^{-1}.min^{-1}$	1.73±0.95	2.04±0.89	0.43
Baseline vascular conductance, $ml.100ml^{-1}.min^{-1}.mmHg$	0.02±0.01	0.02±0.02	0.90
Exercise blood flow (plateau), $ml.100ml^{-1}.min^{-1}$	33.12±8.55	29.02±8.32	0.10
Exercise vascular conductance (plateau), $ml.100ml^{-1}.min^{-1}.mmHg$	0.27±0.06	0.29±0.09	0.75
Exercise mean arterial pressure, mmHg	117±22	108±16	0.15
Exercise heart rate, beats/min	61±13	71±14	0.50

613 Values are mean ± SD

614

CONTROL



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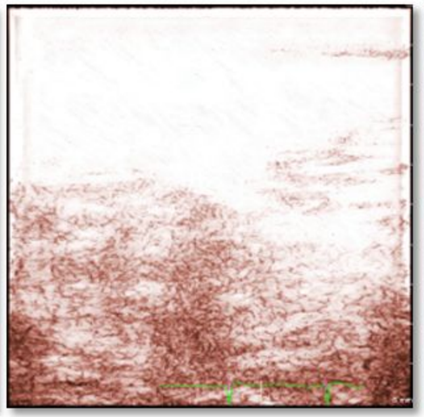
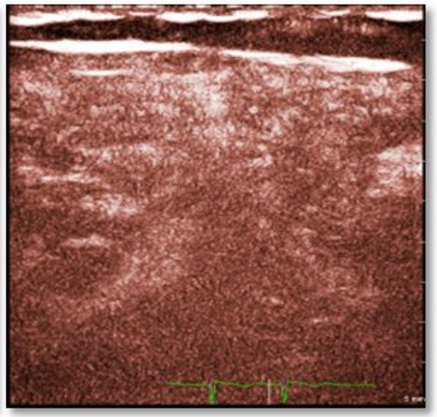
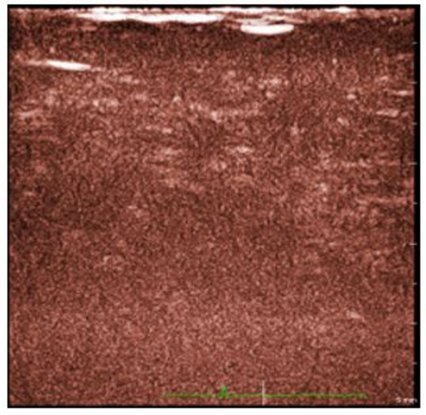


Microbubble destruction



Post-occlusion peak acoustic intensity (15s)

CONTROL



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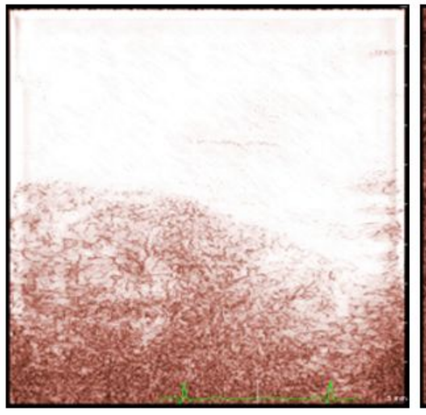

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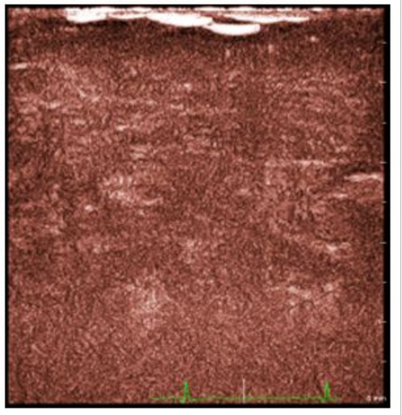
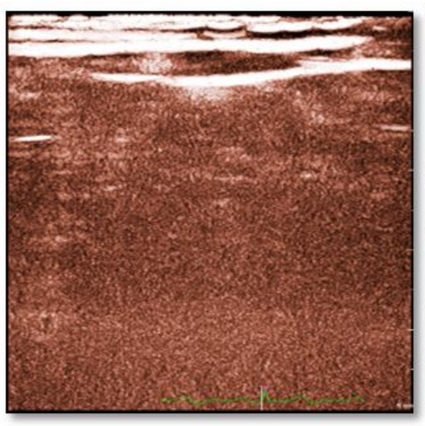
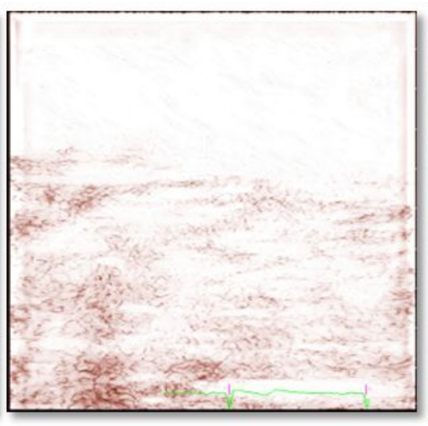


Post-exercise peak acoustic intensity (40s)

PAD



Baseline



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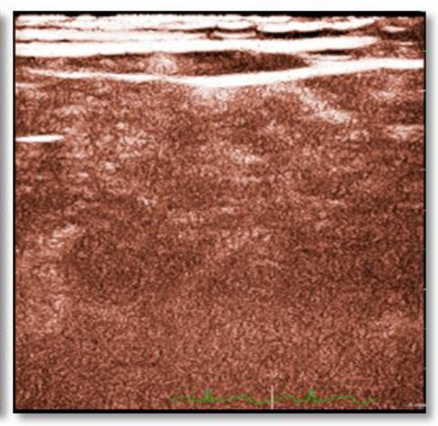
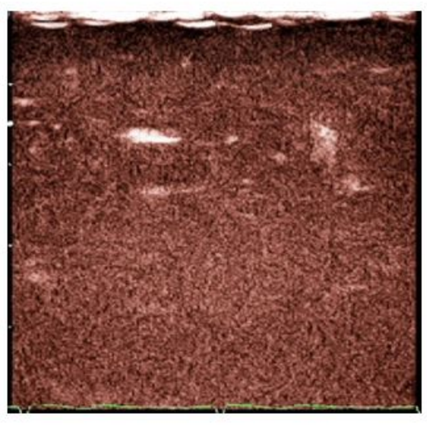
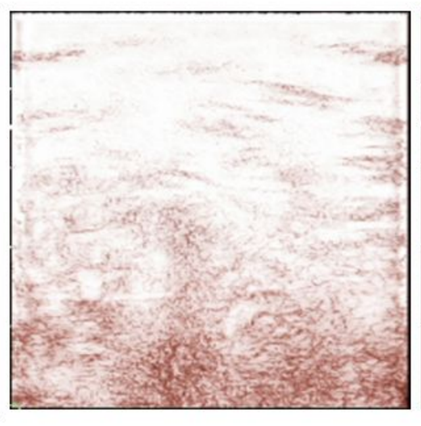


Post-occlusion peak acoustic intensity (34s)

PAD



Baseline



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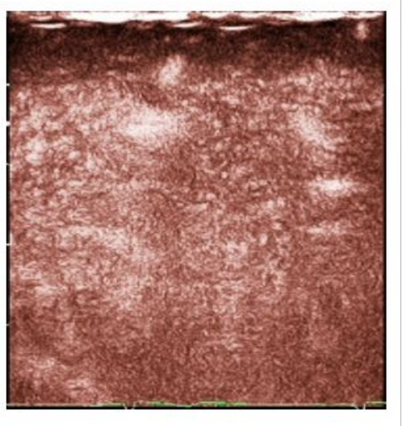
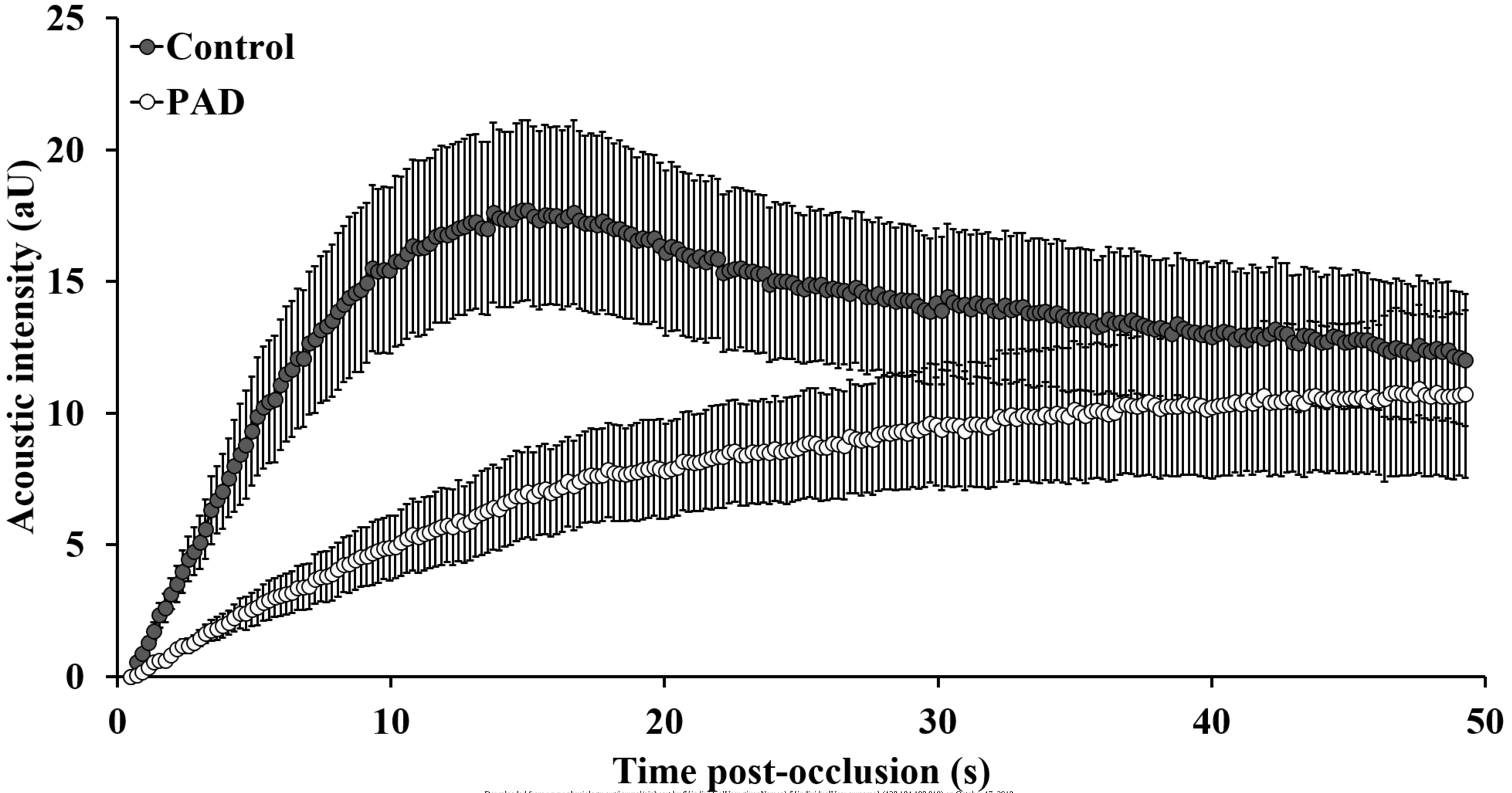


Post-exercise peak acoustic intensity (40s)



A

Microvascular blood volume (A value, aU)

60
50
40
30
20
10
0

Baseline
Post-occlusion

*
*

Control
PAD

B

Microvascular flow velocity (β value, s^{-1})

0.30
0.25
0.20
0.15
0.10
0.05
0.00

Baseline
Post-occlusion

*
†

Control
PAD

C

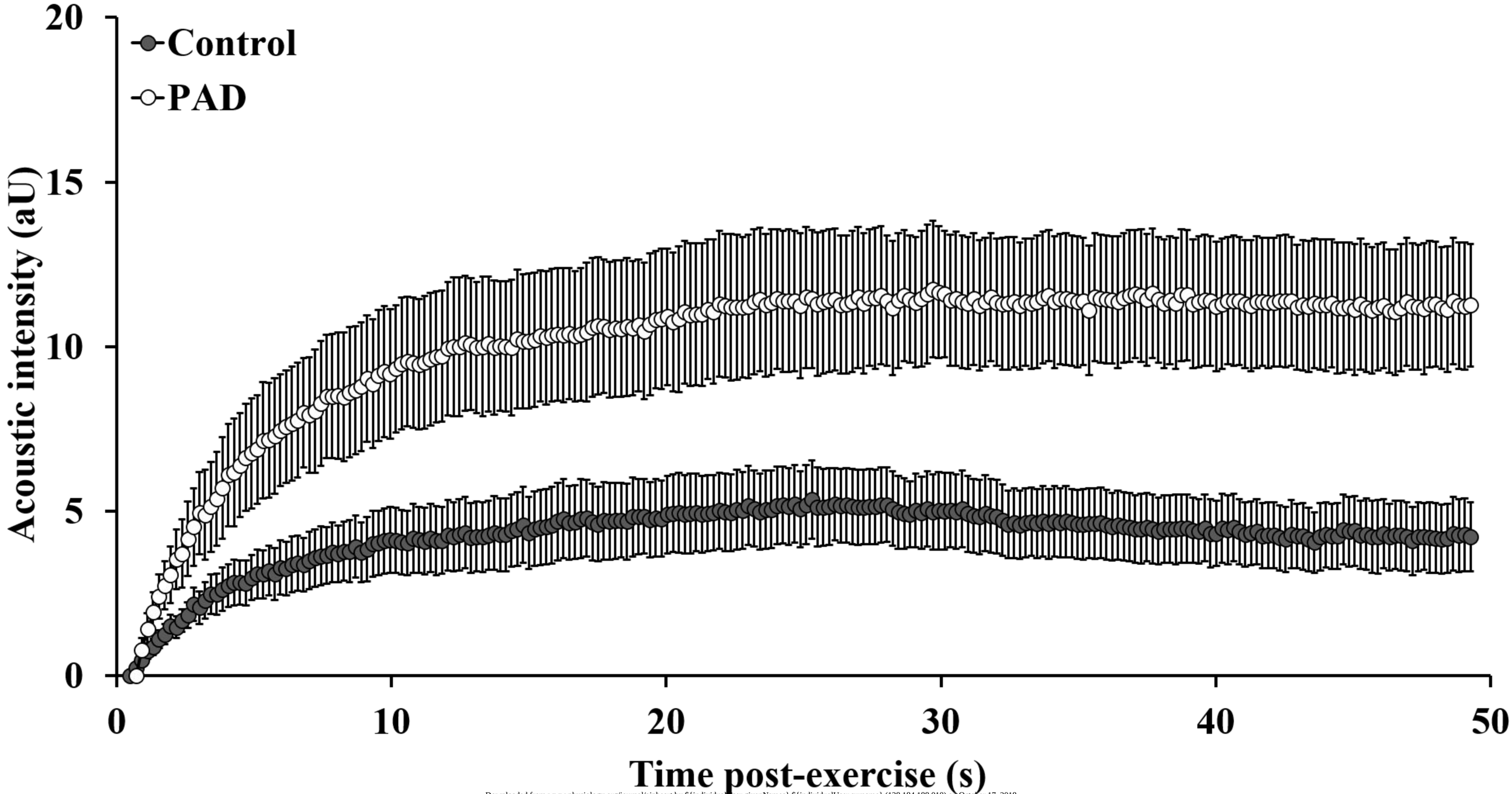
Microvascular perfusion (A.β, $aU.s^{-1}$)

10
8
6
4
2
0

Baseline
Post-occlusion

*
*†

Control
PAD



A

Microvascular blood volume (A value, aU)

30
20
10
0

Baseline
Post-exercise

*
*†

Control
PAD

B

Microvascular flow velocity (β value, s^{-1})

0.60
0.50
0.40
0.30
0.20
0.10
0.00

Baseline
Post-exercise

*
*

Control
PAD

C

Microvascular perfusion (A.β, $aU.s^{-1}$)

10
8
6
4
2
0

Baseline
Post-exercise

*
*

Control
PAD