Relationship between leg muscle capillary density and peak hyperemic blood flow with endurance capacity in peripheral artery disease


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PERIPHERAL ARTERY DISEASE (PAD) is a major health problem that affects over eight million people in the United States (42). PAD is caused by atherosclerosis, which leads to arterial obstruction and decreased blood flow to the lower extremities. The most frequent clinical symptomatic manifestation of PAD is intermittent claudication (IC), which is defined as ischemic leg pain that occurs with walking and improves with rest (21). Patients with IC are known to have impaired exercise capacity and decreased ability to perform activities of daily living (31).

Despite the role diminished arterial flow has in claudication, altered hemodynamics of the affected limbs do not entirely explain the degree of functional limitation in patients with IC (6, 7, 11, 29, 41). Surgical revascularization does not completely normalize exercise performance (31), and exercise training, which does not modify hemodynamics, has been shown to improve walking capacity in patients with PAD (40). These observations have prompted investigations of a mechanism distal to the arterial occlusion to explain these phenomena.

A potential mechanistic target for study is the microcirculation as approximated by skeletal muscle capillary density. Capillary density has been shown to partially explain exercise tolerance in other disease states such as heart failure and chronic obstructive pulmonary disease (9, 10, 23, 38). Although the relationship between capillary density and walking capacity in this population has not been extensively studied, it is of particular interest because it represents the interface between blood flow and the exercising muscle. This interface may explain the alterations in skeletal muscle metabolism that have been hypothesized to contribute to the functional impairment observed in PAD (6, 20). Unfortunately, no study has investigated the contribution of both peak hyperemic blood flow and skeletal muscle capillary density to exercise intolerance in one group of PAD and normal control subjects. The purpose of this study was to further explore differences in capillary density in patients with PAD compared with controls and investigate if functional capacity in PAD is more related to skeletal muscle capillary density than peak hyperemic blood flow. We hypothesized that capillary density is lower in the calf muscle of PAD patients compared with controls and that the lower capillary density is associated with functional impairment in this population.

METHODS

Subjects. Subjects with PAD and controls free of complicating illnesses were recruited from the clinics and community at Duke University Medical Center in Durham, North Carolina, and the University of Colorado, School of Medicine in Aurora, Colorado. PAD subjects were selected based on symptom-limiting IC and an ankle brachial index (ABI) < 0.90 at rest or a 20% decrease in ABI postexercise or angiographic evidence of PAD. PAD subjects were required to be on a stable medical regimen including a statin medication (if tolerated) and antplatelet and antihypertensive medications (as indicated). Exclusion criteria included critical limb ischemia, severe peripheral neuropathy, revascularization for PAD within 3 mo, unstable angina or severe coronary artery disease, or other conditions that would prohibit cardiopulmonary exercise (CPX) testing. Subjects with diabetes mellitus were also excluded as this risk factor may influence the measure of capillary density in muscle. Controls had an initial screening history and physical exam by a physician to document the absence of the same exclusion criteria and an ABI > 0.90.
and < 1.30. Subjects were excluded if they participated in structured exercise ≥1 day/wk at the time of enrollment. Subjects were informed of testing protocols and the potential risks and benefits of participation. Each subject provided written informed consent before enrollment in the study. The Institutional Review Boards at Duke University and University of Colorado approved the research protocols.

Exercise testing. All subjects underwent a maximal CPX with a 12-lead electrocardiogram and expired gas analysis on a treadmill.Expired gases were analyzed continuously using a ParvoMedics (Sandy, UT) or a MedGraphics (St. Paul, MN) unit and averaged in 15-s intervals. For PAD subjects, the Gardner protocol, which maintains 2 mph with a 2% grade increase every 2 min, was used. This protocol is specifically designed for a claudication-limited PAD population (12). To achieve maximal functional capacity in the control group, a Storey protocol, which consists of larger workload increases per stage, was used. In addition to peak oxygen consumption (V\textsubscript{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{o}}}}}}}}}}}}}O_2\text{}}}, claudication onset time (COT) and peak walking time (PWT) were measured for subjects with PAD.

Peak hyperemic limb blood flow. In a subset of (48 PAD and 47 normal) subjects, calf muscle peak hyperemic blood flow was measured in the supine position by venous occlusion strain-gauge plethysmography (DE Hokanson, Issaquah, WA) at rest and during reactive hyperemia (RH) immediately after release of 5 min cuff occlusion, as described below (15). The most severely diseased leg was supported just above the level of the heart, and a mercury-in-Silastic strain gauge was placed around the widest part of the calf. Before all assessments an ankle cuff was inflated to 50 mmHg above systolic pressure for 60 s to eliminate foot circulation from the measurement. A pneumatic cuff was placed on the thigh and inflated to 30 mmHg to achieve venous occlusion. The cuff occlusion was maintained for several cardiac cycles (4–6 cycles) to obtain resting blood flow measurements. Resting and peak hyperemic blood flow was expressed as milliliters per 100 ml tissue per minute. Resting blood flow was calculated as the average of five separate measurements in each limb. Peak hyperemic blood flow was determined after limb ischemia induced by the proximal thigh cuff that was inflated 50 mmHg above systolic blood pressure for 5 min. Postocclusion hyperemic blood flow measurements were made every few seconds, and the highest value achieved was taken as the peak value.

Skeletal muscle biopsies. Skeletal muscle biopsies were taken from the medial aspect of the gastrocnemius muscle. The biopsied leg was the same as the leg measured via plethysmography for each subject. Biopsies were obtained at least 24 h after CPX testing to ensure that the skeletal muscle was in a resting metabolic state. A modified Bergstrom needle technique was utilized to obtain 40–50 mg of skeletal muscle following local anesthesia with 2% lidocaine and a 1-cm skin incision (5). Samples were embedded in cross-section using optical cutting temperature (OCT) tissue freezing medium (Tissue-Tek, Sakura Finetek, Torrance, CA), snap-frozen in liquid nitrogen, and stored at −80°C.

Histological analysis. The capillary density of each section was determined by the number of endothelial cells/mm\textsuperscript{2}, calculated by dividing the total number of CD31-positive (fluoroscein-stained) cells by the area (mm\textsuperscript{2} of tissue), which was measured with a stage micrometer and Image-Pro Plus. A minimum of 100 endothelial cells was counted per sample. Endothelial cells were identified in histological sections using immunofluorescence techniques with an established endothelial cell-specific monoclonal antibody in methods described.

Immunofluorescence. Cryostat sections (7 μm) were placed on microscope slides (Snowcoat X-tra; Surgipath, Richmond, IL) and stored in a −80°C freezer until immunofluorescent analysis. Sections were removed from the freezer and allowed to reach room temperature (RT). Sections were fixed by immersion into 100% ice-cold acetone for 10 min, air dried for 10 min (RT), and then rehydrated in PBS for 5 min. Sections were blocked for 30 min with 10% normal goat serum in PBS containing 0.5% cold-water fish skin gelatin (Sigma). Endothelial cells were detected using a mouse anti-human CD31 (clone: 9G11, 20 μg/ml; R&D) followed by goat anti-mouse Alexa-Fluor-488 (40 μg/ml; Invitrogen). Hybridoma lines BA-D5 and SC-71 (39) were obtained from the ATCC (Manassas, VA). Hybridomas were cultured and purified by the Lymphocyte Culture Center at the University of Virginia. BA-D5 (8.9 μg/ml) and SC-71 (12.3 μg/ml) were coincubated overnight at 4°C in blocking solution plus 5% normal mouse serum. Slides were washed twice in PBS and coverslips were applied using Prolong-Gold (Invitrogen). Images were captured using a Zeiss LSM 510 UV confocal microscope at 100× magnification (final). A blinded observer analyzed the images using Image-Pro Plus 4.5.1.

Statistical analysis. Differences in demographic and clinical characteristics, skeletal muscle capillary density, and peak hyperemic blood flow between normal and PAD subject groups were determined using a Student’s t-test. To adjust for differences between the PAD and control groups, a multiple logistic regression model that included age, sex, smoking, ABI, peak hyperemic blood flow, hypertension, and current exercise was created. For categorical variables, differences were determined by chi square analysis. Pearson correlations were performed to determine the relationship between the measures of capillary density and peak hyperemic blood flow and functional capacity for each group. All tabular data are presented as means ± SD. P values of <0.05 were considered significant for all tests.

RESULTS

Patient demographics. Demographic and clinical characteristics of the 120 subjects (64 PAD and 56 control) included in the analysis are presented in Table 1. As expected, PAD subjects had a significantly lower peak VO\textsubscript{2} compared with the control group. The PAD cohort had a mean PWT of 529 ± 305 s and a COT of 208 ± 172 s. Controls did not have claudication and underwent a different exercise testing protocol; thus PWT and COT were not recorded.

Differences in capillary density and peak hyperemic blood flow measures. Capillary density (endothelial cells/mm\textsuperscript{2}) was ~18% lower in PAD patients compared with controls (PAD vs. controls; 247.9 ± 80.2 vs. 302.1 ± 91.7; P < 0.001). There was no difference in resting blood flow between control and PAD subjects (2.50 ± 1.54 and 2.47 ± 1.25 ml100 ml tissue\textsuperscript{−1} min\textsuperscript{−1}, respectively). Peak hyperemic blood flow was reduced 22% in PAD relative to controls (PAD vs. controls: 10.1 ± 5.9 vs. 13.0 ± 7.7; P < 0.05), but this distinction was lost after adjusting for differences in baseline demographics (P = 0.19) in the full model.

Table 1. Demographic and clinical characteristics

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>PAD</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n = 56</td>
<td>n = 64</td>
</tr>
<tr>
<td>Age, yr</td>
<td>58 ± 9</td>
<td>68 ± 10*</td>
</tr>
<tr>
<td>Sex, % male</td>
<td>34%</td>
<td>61%*</td>
</tr>
<tr>
<td>Race, % Caucasian</td>
<td>77%</td>
<td>80%</td>
</tr>
<tr>
<td>Body mass index, kg/m\textsuperscript{2}</td>
<td>27 ± 4</td>
<td>27 ± 6</td>
</tr>
<tr>
<td>Current smoking, %</td>
<td>5%</td>
<td>33%*</td>
</tr>
<tr>
<td>Statin use, %</td>
<td>20%</td>
<td>67%*</td>
</tr>
<tr>
<td>Ankle brachial index</td>
<td>1.10 ± 0.11</td>
<td>0.67 ± 0.19*</td>
</tr>
<tr>
<td>Peak oxygen consumption, ml·kg\textsuperscript{−1}·min\textsuperscript{−1}</td>
<td>24.8 ± 5.5</td>
<td>15.7 ± 3.7*</td>
</tr>
<tr>
<td>Peak walking time, s</td>
<td>N/A</td>
<td>529 ± 305</td>
</tr>
<tr>
<td>Claudication onset time, s</td>
<td>N/A</td>
<td>208 ± 172</td>
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</table>

Values are means ± SD. *P < 0.05 between peripheral artery disease (PAD) and control groups.
The relationship between capillary density and peak hyperemic blood flow to functional capacity. The primary functional endpoint was peak $\dot{V}O_2$. Therefore, we performed both univariate and full model analysis predicting peak $\dot{V}O_2$. Table 2 demonstrates the univariate correlations of interest. In the PAD group, univariate correlations for peak $\dot{V}O_2$ demonstrated relationships with peak hyperemic blood flow, capillary density, and ABI. In the control group, peak $\dot{V}O_2$ was only correlated with peak hyperemic blood flow ($P < 0.05$).

Given the imbalances in baseline demographics between PAD and controls, statistical modeling was performed to further evaluate the relationship between capillary density and exercise performance in an attempt to adjust for age, sex, smoking, and hypertension, as well as their interactions. This analysis demonstrated that, although sex contributed to the population difference, the overall fully adjusted model remained highly significant ($P < 0.001$). The other variables did not contribute to the model and were therefore dropped.

After adjustment for covariates, capillary density, ABI and sex were independently related to peak $\dot{V}O_2$. In PAD, PWT also demonstrated univariate relationships with capillary density ($P < 0.0001$) and peak hyperemic blood flow ($P < 0.05$). Capillary density but not peak hyperemic blood flow was related to COT. In contrast to the above, control subjects demonstrated a univariate correlation between peak hyperemic blood flow and peak $\dot{V}O_2$, but no relation between capillary density and peak $\dot{V}O_2$. There was no relation between capillary density and peak hyperemic blood flow in either group.

**DISCUSSION**

Although exercise tolerance is clearly reduced in PAD patients with IC, the mechanism behind this impairment is not fully understood. The primary findings from this study were 1) patients with PAD had a decreased skeletal muscle capillary density compared with controls, and 2) capillary density had a relationship with three key measures of functional capacity in PAD patients with IC. These findings suggest that skeletal muscle capillary density may help understand the mechanisms of functional disability associated with PAD.

ABI and segmental Doppler testing are routinely used for the diagnosis of PAD. Although these are valuable diagnostic tools, there remains considerable debate about the strength of relationship between these measures and functional capacity in patients with PAD (7, 18, 19, 25, 29, 44). Similar to these previous findings, peak hyperemic blood flow did not relate to COT in PAD patients in the present study. Peak hyperemic blood flow was, however, related to peak $\dot{V}O_2$ in both normal and PAD populations (Fig. 1). This is not surprising due to the strong influence blood flow has on peak oxygen consumption (1, 13, 35). However, this relationship is not widely known since peak $\dot{V}O_2$ is rarely measured in patients with PAD. While it appears to be true that peak hyperemic blood flow relates to peak $\dot{V}O_2$ in PAD, our findings suggest that this relationship also exists in a control population and therefore is not distinct to the disease.

Skeletal muscle capillary density was different between PAD patients and controls in both number of capillaries per area and its relationship to measures of functional capacity (peak $\dot{V}O_2$, PWT, COT). The most clinically significant finding was that capillary density was related to all aspects of functional performance including COT, PWT, and peak $\dot{V}O_2$ in PAD patients (Fig. 2). In contrast, capillary density did not relate to peak $\dot{V}O_2$ in controls. In the past, there have been conflicting results reported in the correlation between capillary density and peak $\dot{V}O_2$.

Table 2. **Univariate correlations by treatment group**

<table>
<thead>
<tr>
<th>Variable 1</th>
<th>Variable 2</th>
<th>Group</th>
<th>r</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ankle brachial index</td>
<td>Peak oxygen consumption</td>
<td>Control</td>
<td>0.183</td>
<td>0.177</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PAD</td>
<td>0.292</td>
<td>0.02</td>
</tr>
<tr>
<td>Capillary density</td>
<td>Peak oxygen consumption</td>
<td>Control</td>
<td>0.08</td>
<td>0.556</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PAD</td>
<td>0.375</td>
<td>0.002</td>
</tr>
<tr>
<td>Peak hyperemic blood flow</td>
<td>Peak oxygen consumption</td>
<td>Control</td>
<td>0.359</td>
<td>0.013</td>
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<tr>
<td></td>
<td></td>
<td>PAD</td>
<td>0.372</td>
<td>0.009</td>
</tr>
<tr>
<td>Peak hyperemic blood flow</td>
<td>Peak walking time</td>
<td>Control</td>
<td>−0.028</td>
<td>0.849</td>
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<tr>
<td></td>
<td></td>
<td>PAD</td>
<td>0.115</td>
<td>0.438</td>
</tr>
</tbody>
</table>

Fig. 1. The relationship of peak hyperemic blood flow (ml-100 ml tissue $^{-1}$ min$^{-1}$) and peak oxygen consumption ($\dot{V}O_2$). Both normal control ($r = 0.36$, $P < 0.05$) and peripheral artery disease (PAD) subjects ($r = 0.37$, $P < 0.05$) had a positive relationship between peak hyperemic blood flow and peak $\dot{V}O_2$. 
Our findings coupled with the lack of finding of a relation between capillary density and exercise capacity in controls further supports the physiological importance of capillary density particularly in PAD.

While capillary density does not directly influence peak performance in normal subjects, it is associated with both PWT and COT in patients with PAD. This suggests that in the presence of restricted conduit blood flow, there may be an increased reliance on the microvasculature to perfuse and meet the metabolic demands of the active skeletal muscle. Microvascular adaptations improving oxygen extraction and diffusion capacity from limited available flow may become necessary in conjunction with the disease (43). Due to the increased importance placed on perfusion at this level, it is not surprising that the capillary supply to the affected muscle is related to multiple measures of exercise capacity in patients with PAD. In support of this, capillary density was related to all three measures of exercise capacity after controlling for age. Given that surrogate measures of blood flow are poor predictors of exercise tolerance, capillary density of the affected muscle, although not currently thought of as a clinical measure, may be an important biological marker in PAD.

Askew et al. (2) is the only previous study to demonstrate the importance of capillary supply to functional capacity in PAD. However, this study measured capillary to fiber ratio in relationship to maximal indexes: peak VO\textsubscript{2} and PWT. Our study adds that the onset time for claudication (COT) is directly related to capillaries per area in the gastrocnemius muscle. The measurement of COT is of clinical value because it occurs at a point when activities of daily living are likely affected in contrast to findings at maximal exercise (32). Capillaries directly perfuse the ischemic muscle during exercise, which is likely the reason its density relates to the time pain occurs at that level of intensity. Although PWT is a validated and important measure for PAD patients, the sub-maximal measurement of COT provides insight into the daily disability of the patient.

It was somewhat unexpected that PAD and control subjects were not different in terms of peak hyperemic blood flow via plethysmography after controlling for age. Although many previous reports (3, 4, 37) have detected a difference between these groups, careful inspection of these studies demonstrate that age is rarely statistically taken into account. It has been reported that peak hyperemic flow is reduced with age (27, 30, 33), which could explain why the difference between groups is no longer significant after age is considered. In addition, Ogren et al. (28) reported that 76% of IC patients in his study had normal peak hyperemic flow. This causes overlap in values for PAD and normal subjects thereby lessening the difference between groups. These reports coupled with our data suggest that peak hyperemic flow may be more related to age and peak VO\textsubscript{2} than the prevalence of PAD.

Within the published literature on capillary density in lower extremity peripheral skeletal muscle, there are reports that both contradict and agree with our finding that capillary density is lower in PAD subjects compared with controls (2, 8, 14, 16, 24, 26). Only one of these studies examined capillary density as measured by capillaries per area (26). Contrary to our results, this study found capillary density per area to be higher in PAD patients relative to normal controls. There are several potential explanations that may have contributed to our opposing finding. Methodological issues such as sample size or staining technique could account for some of this divergent finding. We used a highly specific immunofluorescence staining technique compared with amylase periodic acid-Schiff typically used in older studies. In addition, it is possible that the PAD subjects were of higher functional capacity than the control subjects included in the present study. Although PWT was not measured by McGuigan et al. (26), the average reported time to claudication was almost twice that achieved in our cohort of PAD subjects. We have found capillary density to have a positive relationship to functional capacity in this population. Therefore, the superior functional ability in a cohort of PAD subjects could explain their elevated levels of capillary density. While we recognize that the present study occurred at different altitudes and with different equipment, we do not think that these factors contribute to our findings since the peak VO\textsubscript{2} measurements are not significantly different between sites.

Summary. There has been a longstanding debate concerning whether peripheral hemodynamics or skeletal muscle play a major role in limiting functional capacity in PAD. Our findings provide a significant contribution to this literature because this study investigated both a measure of limb blood flow and skeletal muscle capillary density in relation to all relevant
measures of functional capacity in both PAD as well as a control population. These data suggest that large-vessel peak hyperemic blood flow relates to peak $\dot{V}O_2$ in both normal and PAD populations. In contrast, the relationship between capillary density in the calf muscle and functional measures in PAD patients suggests that it is an important contributor to overall functional performance. These findings support that while large vessel peak hyperemic blood flow has a clear impact on peak exercise performance across populations, microvascularature at the skeletal muscle level is particularly germane to this disease. Therefore, additional clinical insight into the functional anomalies observed in these patients may be gained with the knowledge of skeletal muscle capillary density. Understanding the importance of capillary density associated with this disease could have important influences on established or investigational studies that target microvascular endothelium in muscle, including exercise or the direct administration of angiogenic agents.

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The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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