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Capillary ultrastructure and mitochondrial volume density in skeletal muscle in relation to reduced exercise capacity of patients with intermittent claudication

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Abstract

Intermittent claudication (IC) is the most commonly reported symptom of peripheral arterial disease (PAD). Impaired limb blood flow is a major casual factor of lower exercise tolerance in PAD, but cannot entirely explain it. We hypothesized that IC is associated with structural changes of the capillary-mitochondria interface that could contribute to the reduction of exercise tolerance in IC-patients. Capillary and mitochondrial morphometry were performed after light and transmission electron microscopy using vastus lateralis muscle biopsies of 14 IC-patients and 10 age-matched controls and peak power output (PPO) was determined for all participants using an incremental single-leg knee-extension protocol. Capillary density was lower (411±90 mm$^{-2}$ versus 506±95 mm$^{-2}$; $P$≤0.05) in the biopsies of the IC patients than in those of the controls. The basement membrane (BM) around capillaries was thicker (543±82 nm versus 423±97 nm; $P$≤0.01) and the volume density of mitochondria was lower (3.51±0.56% versus 4.60±0.74; $P$≤0.01) in the IC-patients than the controls. In the IC-patients, a higher proportion of capillaries appeared with collapsed slit-like lumen and/or swollen endothelium. PPO was lower (18.5±9.9 W versus 33.5±9.4 W; $P$≤0.01) in the IC-patients than the controls. We suggest that several structural alterations in skeletal muscle, either collectively or separately, contribute to the reduction of exercise tolerance in IC-patients.
Introduction

Peripheral arterial disease (PAD) is an atherosclerotic disease characterized by stenosis or occlusion of the conduit arteries of the lower limbs (20, 36). Risk factors associated with PAD include advancing age, cigarette smoking, diabetes mellitus, arterial hypertension and disturbances in lipid metabolism (12). While many PAD patients are asymptomatic, the arterial narrowing leads to ischemia and hypoxia of the supplied tissues and in severe cases, this manifests as critical limb ischemia (CLI), usually requiring surgical interventions. Intermittent claudication (IC), which is typically described as a cramp-like muscle pain during exercise, is the most commonly reported symptom of PAD and is usually the first noted symptom of the disease.

Patients with IC have limited muscular strength and endurance capacities (35), reduced cardiorespiratory fitness (VO2 peak), and demonstrate reduced oxygen uptake kinetics at the onset of exercise (6). They also exhibit a significantly reduced tolerance for physical activities such as walking and cycling (2, 13). This reduction in exercise tolerance leads to functional impairments that are similar to those observed in patients with heart failure (31) and contributes to their impaired mobility, reduced quality of life and elevated risk for the development of cardiovascular complications (17).

Impaired limb blood flow is commonly assumed to be the main casual factor of the reduction in exercise tolerance in PAD, but cannot entirely explain it. For example, restoration of blood flow capacity with percutaneous endovascular revascularization did not re-establish exercise tolerance completely, with only modest (~50%) gains in maximum walking distance (39); while the very large increases in exercise capacity and walking distance (~300%) with exercise training were not accompanied by any significant hemodynamic improvements (39). A meta-analysis of 33 trials (38)
reported the mean effect of exercise therapy on the ankle to brachial blood pressure
index (ABI) to be trivial (0.03±0.06). Thus, factors other than blood flow, e.g. the
skeletal muscle structure, likely contribute the reduction in exercise tolerance in PAD
patients and the improvement seen with exercise therapy.
Peri-capillary basement membrane thickening (7, 8, 24) and alterations of the Vv_mito
(1, 15) are examples of ultrastructural changes in skeletal muscle in patients with
CLI, the late, most severe stages of PAD. However, skeletal muscle samples from
CLI patients are heterogeneous and contain large deposits of fat and connective
tissue, and there is a higher proportion of degenerated fibers compared with that
observed in patients with IC (11), which limits their potential to be used in quantitative
investigations. Skeletal muscles of patients with IC are less severely affected. Thus,
IC patients are more appropriate for the assessment of muscle ultrastructural
changes, and the relationships between morphological characteristics and functional
limitations in PAD.
We hypothesized that IC is associated with structural changes of the capillary-
mitochondria interface that potentially limit oxygen delivery and oxygen consumption
within skeletal muscle. We undertook transmission electron microscopy (TEM)
analysis of biopsy samples from the vastus lateralis muscle (VL) of patients with IC
and from control participants (without PAD) with the primary aim of quantifying the
ultrastructure by means of morphometry as well as the muscle performance capacity
of the participants.
Materials and Methods

Participants

The study was conducted at the University of the Sunshine Coast, Australia, with the approval of the local Human Research Ethics Committee (Royal Brisbane and Women's Hospital, Queensland, Australia: HREC/09/QRBW/321). Each of the participants gave written informed consent to participate in the study. The study cohort included 14 individuals with intermittent claudication (IC), which had been stable for more than a year, and 10 control participants without diagnosis of PAD. All IC patients had lesions at or above the level of the profunda femoral artery in the study leg (and therefore proximal to the sampling site of the muscle biopsy). Several of the IC patients had more than one stenosis or occlusion as identified with vascular imaging. The sites of these lesions were common iliac (n=4), internal iliac (n=2), external iliac (n=4), common femoral (n=2), superficial femoral (n=8), profunda femoral (n=4), popliteal (n=5), anterior tibial (n=2), and posterior tibial artery (n=4).

The ankle-to-brachial index (ABI), which is the ratio of arterial systolic blood pressures measured at the ankle and the arm, was measured to confirm the presence of a hemodynamic limitation and PAD. All patients with IC had an ABI < 0.9 in the biopsied limbs, whereas ABI was > 1.0 in all control limbs. Exclusion criteria included critical limb ischemia and any comorbidity for which maximal exercise is contraindicated, including unstable angina and uncontrolled hypertension. All participants were inactive, defined as participating in less than 150 minutes of light activity per week. Anthropometric and clinical data are summarized in Tab. 1. The mean age and body mass index of the individuals in the two groups did not differ significantly ($P \geq 0.05$).
Knee extension exercise protocol

Peak power output (PPO) was measured as previously described using an incremental single-leg knee extension exercise test (25). Power output commenced at 5W and increased by a further 5W every three minutes, with one-minute rest between each stage, until the cadence could not be maintained for three contractions in a row. The knee extension duty cycle was maintained at a constant rate of 60 contractions per minute throughout the test.

Biopsies

On a separate day, vastus lateralis muscle (VL) biopsies were taken from the 14 IC patients and the 10 control participants by a medical practitioner using a Bergstroem needle with suction (26, 51). All biopsies were taken from the participants at rest within a 6-week period. Specimens were chemically fixed in a 6.25% (v/v) glutaraldehyde solution buffered with 0.1 M sodium cacodylate-HCl (pH 7.4) and stored at 4°C until analysis (9).

Light Microscopy

The chemically fixed VL biopsies were divided into 2-3 pieces, each with a volume of approximately 0.5 mm$^3$, after which they were post-fixed in 1% (w/v) OsO4, stained en bloc and embedded in Epon 812 (Fluka, Buchs, Switzerland), as previously described (27). One-micrometer semithin sections were cut using a diamond knife and stained with Toluidine Blue. Examples of micrographs from such semithin sections have previously been presented (7, 8). For the morphometric analysis, transverse or slightly oblique sections through the muscle (size of approximately 1 mm$^2$) were cut from two randomly selected Epon blocks. A systematic sampling strategy was implemented to acquire 10 micrographs of each section at a
magnification of x63 in a Leica DMR light microscope (Leica Microsystems, Heerbrugg, Switzerland). Using these light micrographs, the number of capillary profiles and that of muscle fibers were quantified using the forbidden line rule (52). The mean cross-sectional fiber area (MCSFA) was estimated by point counting, and capillary profile density and capillary-to-fiber (C/F) ratio were calculated, as described previously (9).

**Transmission electron microscopy**

Ultrathin sections (50-60 nm in thickness) of the Epon blocks were prepared with an Ultracut ultramicrotome (Reichert-Jung, Bensheim, Germany), floated on 200-mesh copper grids (Plano, Wetzlar, Germany) and contrasted with uranyl acetate and lead citrate, as previously described (9, 27). Imaging was carried out using a transmission electron microscope (Philips EM 400).

**Morphometry analysis**

Ultrathin sections from two randomly selected Epon-embedded blocks of each VL biopsy were used for two morphometric analyses, namely: 1) quantification of capillary ultrastructure and 2) estimation of volume density (Vv) of mitochondria. As these two morphometric protocols require different reference structures (mitochondria: fiber volume; capillary: capillary volume), we used two different sets of micrographs taken at different magnifications. Values for Vv are provided as volume of a compartment relative to the reference volume in parentheses, according to standard stereological reporting. For example, Vv (BM, cap) refers to the volume density of the basement membrane related to the total capillary volume. As abbreviation we also used Vvmito. Principles and validity of these approaches are described elsewhere (52).
For the quantification of the capillary ultrastructure, 17-20 randomly selected capillaries were photographed on each of the ultrathin sections in the TEM at a final magnification of x14,000 using a MORADA digital camera (OSIS, Münster, Germany). The electron micrographs were overlaid with a quadratic frame (size: 6.84 µm * 6.84 µm), which was subdivided by a grid of 12 x 12 test lines. The line crossings were defined as the test points, which numbered 144 (12 * 12) in all. Thus, each point represented an area of 0.3249 µm² (0.57 µm * 0.57 µm). The number of points falling on capillary lumina, endothelial cells (ECs), pericytes (PCs) and the basement membrane (BM) were counted and expressed relative to the number of all reference points on the capillaries, thereby yielding unbiased estimates of the mean Vv of each component (52). The counting of line intersections that coincided with the abluminal side of the capillary endothelial cells (= EC/BM transition) permitted an estimate of the boundary length density per cross-sectional area of this transition, which was calculated as $B_a = 0.5 \times \pi \times I / L$ with I being the number of intersections and L the total length of the test lines within the reference space (52).

Assuming that the largely longitudinally orientated capillaries were mostly orthogonally sectioned, the point and intersection counts were used to estimate the (a) mean cross-sectional area (A) of the lumen, the ECs, the BM, the PCs and the whole capillary (where the later corresponds to the area of all compartments), (b) the mean boundary length (i.e. capillary circumference; B) the BM/endomysium interface, and (c) the mean thickness (T) of the lumen, the ECs and the BM, which were calculated as the respective mean cross-sectional areas divided by the EC/BM interface boundary length. It should be noted that the values a) to c) represent 2D-approximations of the parameters and not unbiased 3D-estimates. The PC coverage of the capillaries was expressed as the number of test lines simultaneously crossing
PCs and the EC/BM interface relative to the total number of test lines crossing the EC/BM interface.

As descriptors of the capillary structure, three additional parameters were estimated: (i) the number of capillary profiles with slit-like lumina, identified where the T of the capillary lumen was < 50 nm, expressed relative to the total number of analyzed capillary profiles; (ii) the number of capillaries with expanded/swollen ECs, identified where the T of the ECs was > 700 nm, likewise expressed relative to the total number of analyzed capillary profiles; and (iii) the number of intraluminal foldings or abluminal protrusions per capillary. These later values should be considered as semi-quantitative approximations as we did not use a highly objective stereological protocol such as isotropic uniform random sampling.

Estimation of the mean volume density (Vv) of mitochondria on 20 randomly depicted micrographs taken of each of the ultrathin sections, at a magnification of x24,000, were also carried out using established methods described previously (27).

Statistics
Numerical data are expressed as mean values together with the standard deviations. Parameters pertaining to the morphometric and performance outcomes as well as age, BMI and ABI were checked for normality (Shapiro-Wilk test) and compared between groups using a Student’s t-test in the case of cardinal variables. For categorical variables (gender, hypertension, diabetes mellitus, hypercholesterolemia and use of medication, number of skeletal muscle fibers analyzed), differences between control and IC groups were determined using chi-square or Fischer-exact tests, as appropriate. Relationships were assessed by linear regression analysis using Pearson correlation. The level of statistical significance was set at $P \leq 0.05$. 
### Results

Anthropometric and clinical characteristics of the 24 study participants are presented in Tab. 1. The control and IC groups did not differ significantly ($P > 0.05$) in age, gender, BMI, smoking activity, diabetes mellitus and hypercholesterolemia, or in the use of aspirin, Ca$^{2+}$-channel blockers, vasodilators and glucose regulators. In contrast, ABI, the incidence of hypertension and the use of beta-blockers, ACE inhibitor/AT1-receptor blockers, diuretics, statins, lipid regulators and anti-platelet therapy differed ($P \leq 0.05$) between the two study groups.

Light microscopy was performed to quantify the capillary supply in the VL biopsies of the 14 IC patients and the 10 control participants (Tab. 2). The capillary density was significantly lower (-23%; $P \leq 0.05$) in the biopsies of the IC patients than in those of the controls. In contrast, capillary/fiber (C/F)-ratio (-13%; $P > 0.05$) and MCSFA (10%; $P > 0.05$) were not significantly different between the two groups.

At the EM level, 537 capillaries of the IC patients and 382 capillaries of the control participants were evaluated. In each case, the capillary profiles included the ultrastructural features characteristic of the microcirculation: the lumen was mantled with ECs, whereas a dense BM containing the profiles of one or more PCs skirted the abluminal surface of the ECs (Fig. 1A,B). Although the capillary lumina were usually open, they occasionally had a slit-like appearance (Fig. 1C). In some instances, the ECs of capillaries were enlarged and accompanied by luminal narrowing (Fig. 1D). In many of the capillaries of all participants, one or more foldings projected from the ECs into the lumen (often pairwise in juxtaposition with tight junctions; Fig. 1E). On their abluminal side, ECs regularly sent forth short, budding protrusions into the BM, often in the vicinity to PCs (Fig. 1F).

Tab. 3 summarizes the estimates for each of the capillary-associated structural indicators determined by morphometry on the EM micrographs. The quantitative
analysis showed the volume density of BM to be to be significantly greater (+24%; \( P \leq 0.001 \); Fig. 2A) and the BM around capillaries in the VL-biopsies to be 23% thicker \( (P \leq 0.01) \) in IC patients than in the control participants \( (543 \pm 82 \, \text{nm} \text{ versus} \ 423 \pm 97 \, \text{nm}; \text{Fig. 2B}) \). The difference between the two groups was not caused by outliers but was associated with a general increase of the BM thickness (Fig. 2C). In accordance with this BM thickening in the VL capillaries, the mean cross-sectional area of the BM (+46%) was significantly greater \( (P \leq 0.001) \) in IC patients than in the control participants (Tab. 3). Furthermore, thickening of the BM in the IC patients resulted in a larger mean cross-sectional area of the capillaries relative to that of the controls (+20%; \( P \leq 0.01 \)), which was not found if only the mean cross-sectional capillary area without surrounding BM and pericytes was assessed (+5%, \( P > 0.05 \)).

No significant differences \( (P > 0.05) \) existed between the IC patients and the control participants in the numbers of intraluminal foldings and abluminal protrusions of capillaries. Furthermore, the PC coverage of the capillaries did not differ \( (P > 0.05) \) between the two groups. In contrast, more capillary profiles in the IC patients than in the control participants \( (1\% \text{ versus} \ 6\%; \ P \leq 0.001) \) manifested as a slit-like lumen. The proportion of capillaries with an enlarged swollen endothelium was higher in the IC patients than in the control group \( (14\% \text{ versus} \ 9\%; \ P \leq 0.05) \).

As shown in Fig. 3a, the mean Vv of mitochondria \( (\text{Vv (mito, fiber)} = \text{Vv}_{\text{mito}}) \) in the VL biopsies was significantly lower (-24%) in the IC patients than the control participants \( (P \leq 0.01) \). The single-leg knee-extension performance capacity of the participants was measured using an incremental test. As demonstrated in Fig. 3b, peak power output (PPO) in the IC patients was 45% lower \( (P \leq 0.01) \) than in the control participants.

Linear regression analyses revealed the combination of some indicators to be significantly \( (P \leq 0.05) \) related if the quantitative data sets obtained in this study (BM
thickness, C/F-ratio, capillary density, \( V_v^{\text{mito}} \) and PPO) of all participants were assessed (data not shown): \( V_v^{\text{mito}} \) and BM thickness \( (r = -0.60) \), capillary density \( (r = +0.57) \) and PPO \( (r = +0.41) \) as well as between C/F-ratio and PPO \( (r = +0.57) \) and C/F ratio and capillary density \( (r = +0.46) \). A tendency \( (P = 0.08) \) for a negative relationship was found between BM thickness and PPO \( (r = -0.38) \).

The significant correlation seen by linear regression analysis with the indicators were lost when both groups were analyzed separately, most likely due to the small number of subjects evaluated. However, C/F-ratio with PPO \( (r = +0.62; \ P = 0.06) \) and capillary density \( (r = +0.58; \ P = 0.08) \) and \( V_v^{\text{mito}} \) with capillary density \( (r = +0.59; \ P = 0.07) \) tended to be positively correlated in the VL biopsies of controls. ABI was significantly correlated with capillary density \( (r = +0.42) \), PPO \( (r = +0.64) \), \( V_v \) (mito) \( (r = +0.64) \), and capillary BM thickness \( (r = -0.49) \), when all data were pooled, however these relationships were not present within either the IC or control groups alone.

Morphological variables and PPO were also compared on the basis of comorbidities, medication use, smoking history and the anatomical site of disease and there were no significant differences (data not shown). Of note, three of the IC patients with diabetes mellitus were among the five IC patients with lowest C/F-ratio, lowest CD, lowest \( V_v^{\text{mito}} \), lowest PPO, and the lowest BM thickness (data not shown).
Discussion
To our knowledge, this is the first study in which capillarity and volume density of mitochondria (V_vmito) were simultaneously assessed in the skeletal muscle biopsies of intermittent claudication (IC) patients and age-matched control participants without peripheral arterial disease (PAD). Our morphometric analysis revealed four main findings: 1) The capillary density was lower (-23%) in the biopsies of the IC patients than in those of the controls, 2) The basement membrane (BM) around capillaries in biopsies of the VL was significantly thicker by 23% in IC patients than the controls, 3) V_vmito in the VL biopsies was significantly lower (-24%) in the IC patients than the controls, and 4) IC patients were found to have 45% lower peak power output (PPO) during leg extension exercise than the control participants.

This observation that PPO was lower in the IC patients than the controls is consistent with the limited muscular strength and endurance capacities of IC patients previously described (13, 35), and the well-established reduction in their tolerance for physical activities such as walking and cycling (2, 28). Several studies have investigated the mechanisms of exercise intolerance in IC patients. Besides having a limited aerobic capacity (VO_2peak), whole-body VO_2 kinetics are slowed at the onset of exercise in IC patients (4). Furthermore, muscle oxygen desaturation kinetics, assessed using near infrared spectroscopy, were slowed during plantar flexion exercise in PAD compared with controls, even during low-intensity exercise where blood flow is not generally compromised (5). These findings suggest that there is an impairment in the local supply and/or consumption of oxygen during exercise in IC, which has been attributed to a metabolic myopathy that is characterized by an altered metabolic responses to exercise such as an accumulation of acylcarnitines, compromised carbohydrate oxidation, reduced electron transport chain activity and mitochondrial
DNA injury (10, 41). Our primary aim was to extend these findings by examining the structural characteristics of the capillary-mitochondrial interface.

In accordance with our previous findings (3), the capillary density was significantly lower in the IC group than the control group, concordant with previous studies in which either capillary density or capillary-to-fiber ratio was reduced and significantly related to walking capacity and cardiorespiratory fitness (3, 11, 45). Previous findings of reduced muscle VEGF protein content (26) and elevated thrombospondin-1 in the muscle (16) and muscle extracellular fluid (26) also provides some indication that the angiogenic potential of PAD-affected muscle may be impaired. However, the capillary-to-fiber ratio and PPO during the leg extension exercise differed only non-significantly between the two groups, which is in contrast to other studies (16, 45). This discrepancy may be a result of the presence of comorbidities or the age and physical activity levels of study participants and low group sample sizes but also of the muscle sampling site where the ischemic insult of PAD is likely to be less severe, and perhaps more variable, at the vastus lateralis than at the distal muscle groups which have been sampled (3, 16, 45). It should be noted that some studies have likewise failed to demonstrate such capillary rarefaction in IC patients compared with controls (21, 30). As in IC patients, there are reports of both an increased (24) and decreased (16) muscle microvascular density in patients with severe PAD, which manifests as critical limb ischemia (CLI). This suggests that PAD severity may not necessarily have a direct impact on muscle capillary supply.

It has previously been reported by Makitie (1977) that the BM is thicker around the skeletal muscle capillaries of patients with IC (34) but this observation was made in comparison with a significantly younger control group (IC patients: 59 years; controls: 36 years), thus, the influence of age versus the presence of PAD was not deduced in this study (34). Analyses of skeletal muscle specimens obtained from amputated
limbs of patients with CLI, the most severe form of PAD, revealed an up to 50-fold greater thickness of the BM around the skeletal muscle capillaries compared to those of age-matched controls (7, 8, 24). The present finding indicates that pathological thickening of the capillary BM is already present during the earlier, less-severe stages of PAD. Although to date the physiological trigger and the functional consequences of the peri-capillary BM thickening in PAD remains unknown, contributory factors may include: 1) hypoxia, e.g. activation of the hypoxia-inducible-factor (HIF) transcription factor system may trigger the expression of collagens in other cells and tissues (18, 40), or 2) local changes in the hemodynamic environment that are a consequence of the reduction in blood flow, e.g. perturbation of stress-mediated signaling in capillary ECs, thereby limiting their functionality during, for example, angiogenesis and microvascular remodeling (37). Since thickening of the BM also has been shown to occur in individuals suffering from diabetes mellitus (46, 49, 55) and possibly hypertension (19, 22), it cannot be ruled out that one or more of the comorbidities often associated with PAD might exacerbate the process in patients with IC. Because the three IC patients with diabetes mellitus were among the five IC patients with the lowest BM thickness it is very likely that IC per se also is an important contributor to BM thickening. Furthermore, age (54, 56) and habitual activity levels (19, 54) might also contribute to the BM thickness around skeletal muscle capillaries in humans. Bearing in mind these factors that are known to influence the thickness of the microvascular BM, it is noteworthy that, in the present study, age and body mass index of the participants did not differ between the two groups, and none of the participants were trained or physically active. An interesting observation in the present study was that the IC patients had a relatively high (about 6%) proportion of capillaries with slit-like, collapsed lumina
compared to the control participants (about 1%). In rats, the appearance of such slit-like capillaries has been observed in skeletal muscle under conditions of low flow (32), and in the lung with diabetes mellitus (44). The functional consequence of slit-like lumina in the capillaries is unclear, however, it may be assumed that these capillaries are collapsed, potentially due to ongoing necrosis, and that flow through such capillaries is likely to be compromised, possibly disrupting erythrocyte passage and, thus, inhibiting tissue oxygen supply.

An additional morphological finding of note was that more capillary walls composed of swollen ECs were observed in the IC patients than in those of the controls (about 14% versus about 9%). Similar observations have previously been reported in skeletal muscles of CLI patients, where the EC swelling was exacerbated by clamp-induced ischemia before femorodistal bypass surgery (47). Treatment with iloprost, a stable prostacyclin analogue, prevented the cross clamp-induced increase in EC swelling (47). Interestingly, biopsies from muscles proximal to the site of the bypass showed similar structural changes, indicating that the EC swelling of capillaries is a systemic effect not directly related to ischemia (47). Similarly, in a rodent model of experimentally induced ischemia, ligation of the femoral artery was accompanied by substantial swelling of the capillary EC in skeletal muscles of the hind limb (14, 23, 27). Amiloride (a Na⁺/H⁺-exchange blocker) and to a lesser extent allopurinol (a xanthine oxidase inhibitor), but not vitamin E, prevented this reaction of the capillary system suggesting that EC swelling is related to acidosis (29). Interestingly, muscle fatigue after ligation was not related to the onset of EC swelling (29), which raises questions about the functional significance of EC swelling in IC patients.

We hypothesize that the changes in capillary structure (BM thickening, collapsed lumen, swollen EC) contribute to impaired microvascular muscle perfusion in PAD (33). This is likely to impede the diffusion of oxygen and nutrients into skeletal
muscle fibers of the IC patients, thereby compromising their energy supply and contractile capacity.

It has previously been reported that muscle mitochondrial enzyme activities are reduced in PAD (42) and this has been cited as potentially contributing to the impaired exercise capacity of IC patients. This proposition is supported by our finding of reduced $V_{\text{vmito}}$ in the IC patients. However, the reduced $V_{\text{vmito}}$ in the VL of IC patients does not concur with previous evidence of an increased $V_{\text{vmito}}$ in gastrocnemius muscle of IC patients compared to healthy controls (1, 15), a finding that the authors attributed to a compensation for a reduction in oxygen delivery or mitochondrial function in PAD (10). These discrepant findings reflect the broader debate about the presence (43) or absence (28) of mitochondrial impairments in PAD, and may also be influenced by factors such as sampling site, physical activity levels or the level and severity of arterial stenosis or occlusion (1).

To our knowledge, this is the first study in which the structural phenotype of the skeletal muscles determined at the EM level (capillarity, $V_{\text{vmito}}$) and the exercise capacity of IC patients, assessed as PPO of the knee-extensors, were simultaneously determined. The concurrent adjustment of these crucial components of the oxygen delivery system is an example of the symmorphosis concept (53), which proposes a close match in design between the structural organization and functional parameters of the compartments of a biological system.

**Limitations**

We are aware that some limitations may restrict the significance of our findings. To investigate the relationship of muscle structure with exercise performance during the leg extension exercise, we have chosen to obtain muscle biopsies from the vastus
lateralis (VL) muscle. As mentioned previously, this may have contributed to smaller differences between the groups than if samples were taken from the lower leg, where the ischemic insult of PAD is greater (20, 36). Thus, the present finding of ultrastructural differences in the VL would likely be even more pronounced in skeletal muscles of the lower leg, e.g. the gastrocnemius (46, 48-50), although this may not be the case in the IC patients of our study that had the vascular stenosis or occlusion at the level or proximal of the thigh.

Many of the relationships attained by linear regression analysis were not significant when both groups were analyzed separately. We assume that this lack of statistical significance was caused by the low group sample sizes. Furthermore, the determination of regression analysis is limited and does not allow identification of cause-and effect-relationships. Future studies should aim to investigate the direct effect of muscle morphological changes on exercise capacity in IC.

An additional limitation of this study is the high proportion of past-smokers in the control and the IC patients groups, which may have influenced the structural phenotype of the capillary-mitochondria interface. Whether incidence of hypertension and/or the use of beta-blockers, ACE inhibitor/AT1-receptor blockers, diuretics, statins, lipid regulators and anti-platelet therapy, which were different between the IC patients and controls, have influenced the outcome of this investigation remains elusive.

**Perspectives and Significance**

This investigation demonstrates that peri-capillary BM thickness is enlarged and the capillary density and mitochondrial volume is reduced in the thigh muscle (VL) of patients with IC compared to controls. These findings support the hypothesis that
several morphological alterations, either collectively or separately, contribute to a disturbance in the capillary-mitochondria interface. Such changes in the pathway of oxygen delivery are likely to influence the onset and progression of ischemia in IC patients, and ultimately contribute to their poor muscle function and exercise tolerance. In combination, these findings contribute to the current understanding of the etiology of IC.

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References


Figure Legends

Figure 1: Transmission electron microscopy to demonstrate the ultrastructure of capillaries in VL biopsies. Shown are representative micrographs of capillaries from VL biopsies in a control participant (A) and in a patient suffering from intermittent claudication (B). Note how much thicker the basement membrane around the endothelium is in the IC patient than the control participant. In both groups, cross-sectioned capillary profiles occasionally manifested a slit-like lumen (C) and a swollen endothelium (D). In many instances, the endothelial cells forth intraluminal foldings (E; white arrows) and abluminal protrusions towards pericytes (F; asterisks).

Figure 2: The basement membrane (BM) around capillaries is thicker in VL biopsies of IC patients than in those of control participants. A,B: Volume density (Vv (BM, cap)) and mean arithmetic thickness (T) of the peri-capillary basement membrane were significantly higher in the VL of the IC patients than in those of the control participants. Mean values given for the participants are represented together with the standard deviations. C: Each data point reflects the BM thickness of a capillary in combination with its origin (the numbers stand for assignment of the biopsies and A/B for the assignment of the blocks).

Figure 3: The mean volume density of mitochondria (Vv (mito, fiber)) in VL biopsies (A) and the peak power output (B) of IC patients and control participants. A: The Vv (mito, fiber) was morphometrically quantified using a point-counting approach. Mean values for 14 VL biopsies of IC patients and 10 biopsies of control participants are represented together with the standard deviations. B: The peak power output (PPO) of 13 IC patients and 10 control participants were determined using a unilateral incremental test. Because one IC patient suffered from pain during the testing, his PPO=0 was not included in the analysis. Mean values are represented together with the standard deviations.
Table 1: Summary of the demographic and clinical data of the study subjects. Shown are the means ± standard deviations or the number of subjects. Age, BMI and ABI were compared using a paired Student’s t-test, while differences in categorical variables (gender, hypertension, diabetes mellitus, hypercholesterolemia and use of medication) between control and IC groups were determined by chi-square analysis. ACE= angiotensin-converting enzyme; ABI= ankle-brachial-index; AT= angiotensin receptor; BMI= Body mass index; NS (not significant) = $P > 0.05$.

<table>
<thead>
<tr>
<th></th>
<th>Controls n= 10</th>
<th>IC patients n = 14</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong> (years)</td>
<td>64.0 ± 8.7</td>
<td>68.1 ± 4.5</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Gender</strong> (male / female)</td>
<td>5 / 5</td>
<td>12 / 2</td>
<td>NS</td>
</tr>
<tr>
<td><strong>BMI</strong> (kg/m²)</td>
<td>26.3 ± 2.6</td>
<td>26.2 ± 5.2</td>
<td>NS</td>
</tr>
<tr>
<td><strong>ABI of biopsied leg</strong></td>
<td>1.20 ± 0.13</td>
<td>0.73 ± 0.14</td>
<td>$P \leq 0.001$</td>
</tr>
<tr>
<td><strong>Smoking activity</strong> (Current / Past / Non)</td>
<td>0 / 8 / 2</td>
<td>4 / 9 / 1</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Hypertension</strong> (Y / N)</td>
<td>2 / 8</td>
<td>11 / 3</td>
<td>$P \leq 0.05$</td>
</tr>
<tr>
<td><strong>Diabetes mellitus</strong> (Y / N)</td>
<td>1 / 9</td>
<td>3 / 11</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Hypercholesterolemia</strong> (Y / N)</td>
<td>2 / 8</td>
<td>8 / 6</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Beta-blocker use</strong> (Y / N)</td>
<td>0 / 10</td>
<td>6 / 8</td>
<td>$P \leq 0.05$</td>
</tr>
<tr>
<td><strong>Aspirin use</strong> (Y / N)</td>
<td>3 / 7</td>
<td>9 / 5</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Ca²⁺-channel blocker use</strong> (Y / N)</td>
<td>2 / 8</td>
<td>6 / 8</td>
<td>NS</td>
</tr>
<tr>
<td><strong>ACE inhibitor / AT1-receptor blocker use</strong> (Y / N)</td>
<td>2 / 8</td>
<td>9 / 5</td>
<td>$P \leq 0.05$</td>
</tr>
<tr>
<td><strong>Vasodilator use</strong> (Y / N)</td>
<td>1 / 9</td>
<td>3 / 11</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Diuretics use</strong> (Y / N)</td>
<td>0 / 10</td>
<td>5 / 9</td>
<td>$P \leq 0.05$</td>
</tr>
<tr>
<td><strong>Glucose regulator use</strong> (Y / N)</td>
<td>1 / 9</td>
<td>2 / 12</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Statin use</strong> (Y / N)</td>
<td>1 / 9</td>
<td>11 / 3</td>
<td>$P \leq 0.05$</td>
</tr>
<tr>
<td><strong>Lipid regulator use</strong> (Y / N)</td>
<td>3 / 7</td>
<td>12 / 2</td>
<td>$P \leq 0.05$</td>
</tr>
<tr>
<td><strong>Anti-platelet agent use</strong> (Y / N)</td>
<td>3 / 7</td>
<td>11 / 3</td>
<td>$P \leq 0.05$</td>
</tr>
</tbody>
</table>
Table 2: The capillary supply in the VL of intermittent claudication (IC) patients and control subjects. The C/F-ratio, capillary density and mean cross-sectional fiber area (MCSFA) were determined on the semithin sections of the VL-biopsies obtained from the two cohorts. Shown are the means ± standard deviations as determined applying morphometrical light microscopy on semithin sections. NS (not significant) = \( P > 0.05 \).

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>IC patients</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C/F-ratio</strong></td>
<td>1.32 ± 0.31</td>
<td>1.15 ± 0.30</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Capillary density, mm²</strong></td>
<td>506 ± 95</td>
<td>411 ± 90</td>
<td>( P \leq 0.05 )</td>
</tr>
<tr>
<td><strong>MCSFA, µm²</strong></td>
<td>2675 ± 584</td>
<td>2934 ± 806</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Number of skeletal muscle fibers analyzed</strong></td>
<td>169 ± 65</td>
<td>154 ± 83</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 3: Summary of the quantitative stereological analysis. Transmission electron micrographs of the capillaries in VL biopsies of IC patients and controls were subjected to a morphometric analysis of the structural indicators by point and intersection counting. Means ± standard deviations are represented. Abbreviations: A = area; Vv = volume density; T = mean arithmetic thickness; B = mean arithmetic boundary/perimeter; EC = endothelial cells; PC = pericytes; BM = basement membrane; NS (not significant) = $P > 0.05$. Note that Vv (BM; cap) and T (BM) are also presented in Fig. 2.

<table>
<thead>
<tr>
<th></th>
<th>Controls n = 10</th>
<th>IC patients n = 14</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A (cap), µm²</strong></td>
<td>21.9 ± 1.8</td>
<td>26.2 ± 2.8</td>
<td>$P \leq 0.001$</td>
</tr>
<tr>
<td><strong>A (cap without PC and BM), µm²</strong></td>
<td>13.7 ± 0.7</td>
<td>14.4 ± 1.5</td>
<td>NS</td>
</tr>
<tr>
<td><strong>A (lumen), µm²</strong></td>
<td>7.7 ± 0.9</td>
<td>7.6 ± 1.3</td>
<td>NS</td>
</tr>
<tr>
<td><strong>A (EC), µm²</strong></td>
<td>6.0 ± 0.8</td>
<td>6.8 ± 0.7</td>
<td>$P \leq 0.05$</td>
</tr>
<tr>
<td><strong>A (PC), µm²</strong></td>
<td>1.0 ± 0.2</td>
<td>1.2 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td><strong>A (BM), µm²</strong></td>
<td>7.2 ± 1.8</td>
<td>10.5 ± 1.6</td>
<td>$P \leq 0.001$</td>
</tr>
<tr>
<td><strong>Vv (lumen, cap), %</strong></td>
<td>35.0 ± 6.6</td>
<td>28.1 ± 2.7</td>
<td>$P \leq 0.01$</td>
</tr>
<tr>
<td><strong>Vv (EC, cap), %</strong></td>
<td>27.3 ± 2.7</td>
<td>26.1 ± 2.7</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Vv (PC, cap), %</strong></td>
<td>5.0 ± 0.6</td>
<td>5.2 ± 1.5</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Vv (BM, cap), %</strong></td>
<td>32.8 ± 6.1</td>
<td>40.6 ± 3.4</td>
<td>$P \leq 0.001$</td>
</tr>
<tr>
<td><strong>T (BM), nm</strong></td>
<td>423 ± 97</td>
<td>543 ± 82</td>
<td>$P \leq 0.01$</td>
</tr>
<tr>
<td><strong>T (EC), nm</strong></td>
<td>488 ± 79</td>
<td>410 ± 48</td>
<td>$P \leq 0.01$</td>
</tr>
<tr>
<td><strong>B (BM/endomysium transition), µm</strong></td>
<td>22.9 ± 1.6</td>
<td>26.0 ± 2.4</td>
<td>$P \leq 0.01$</td>
</tr>
<tr>
<td><strong>B (EC/BM transition), µm</strong></td>
<td>13.8 ± 0.9</td>
<td>14.2 ± 1.4</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Pericyte coverage, %</strong></td>
<td>18.2 ± 4.2</td>
<td>20.0 ± 3.9</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Capillaries with slit-like lumen, %</strong></td>
<td>1.08 ± 0.22</td>
<td>6.10 ± 4.59</td>
<td>$P \leq 0.001$</td>
</tr>
<tr>
<td><strong>Capillaries with swollen EC, %</strong></td>
<td>9.0 ± 6.8</td>
<td>14.0 ± 5.1</td>
<td>$P \leq 0.05$</td>
</tr>
<tr>
<td><strong>Intraluminal foldings per capillary</strong></td>
<td>1.23 ± 0.19</td>
<td>1.42 ± 0.22</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Abluminal protrusions per capillary</strong></td>
<td>0.66 ± 0.12</td>
<td>0.63 ± 0.06</td>
<td>NS</td>
</tr>
</tbody>
</table>
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Fig. 1
A. Mitochondria Morphometry

Vv (mito, fiber), %

Controls: 4.60 ± 0.74
IC patients: 3.51 ± 0.56

P ≤ 0.01

B. PPO

Power (W)

Controls: 33.5 ± 9.4
IC patients: 18.5 ± 9.9

P ≤ 0.01

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Fig. 3