

PERIPHERAL VASCULAR DISEASE COMPENDIUM

# Walking Exercise Therapy Effects on Lower Extremity Skeletal Muscle in Peripheral Artery Disease

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**ABSTRACT:** Walking exercise is the most effective noninvasive therapy that improves walking ability in peripheral artery disease (PAD). Biologic mechanisms by which exercise improves walking in PAD are unclear. This review summarizes evidence regarding effects of walking exercise on lower extremity skeletal muscle in PAD. In older people without PAD, aerobic exercise improves mitochondrial activity, muscle mass, capillary density, and insulin sensitivity in skeletal muscle. However, walking exercise increases lower extremity ischemia in people with PAD, and therefore, mechanisms by which this exercise improves walking may differ between people with and without PAD. Compared with people without PAD, gastrocnemius muscle in people with PAD has greater mitochondrial impairment, increased reactive oxygen species, and increased fibrosis. In multiple small trials, walking exercise therapy did not consistently improve mitochondrial activity in people with PAD. In one 12-week randomized trial of people with PAD randomized to supervised exercise or control, supervised treadmill exercise increased treadmill walking time from 9.3 to 15.1 minutes, but simultaneously increased the proportion of angular muscle fibers, consistent with muscle denervation (from 7.6% to 15.6%), while angular myofibers did not change in the control group (from 9.1% to 9.1%). These findings suggest an adaptive response to exercise in PAD that includes denervation and reinnervation, an adaptive process observed in skeletal muscle of people without PAD during aging. Small studies have not shown significant effects of exercise on increased capillary density in lower extremity skeletal muscle of participants with PAD, and there are no data showing that exercise improves microcirculatory delivery of oxygen and nutrients in patients with PAD. However, the effects of supervised exercise on increased plasma nitrite abundance after a treadmill walking test in people with PAD may be associated with improved lower extremity skeletal muscle perfusion and may contribute to improved walking performance in response to exercise in people with PAD. Randomized trials with serial, comprehensive measures of muscle biology, and physiology are needed to clarify mechanisms by which walking exercise interventions improve mobility in PAD.

**Key Words:** exercise ■ ischemia ■ mitochondria ■ peripheral artery disease ■ perfusion ■ plasma ■ skeletal muscle

Lower extremity peripheral artery disease (PAD), characterized by atherosclerotic or thrombotic obstructions of lower extremity arteries, affects nearly 250 million people worldwide.<sup>1</sup> Risk factors for PAD include older age, diabetes, smoking, hypertension, and hyperlipidemia.<sup>2</sup> In people with PAD, ischemia of the lower extremities during walking activity typically causes pain, tightness, weakness, or other discomfort in the legs and buttocks, impairing the ability to walk even short distances without stopping to rest.<sup>3</sup> Lower extremity

ischemia is associated with adverse lower extremity skeletal muscle (ie, gastrocnemius) characteristics.<sup>4–16</sup> Compared with people without PAD, those with PAD have greater mitochondrial injury, abundance of fibrosis and oxidative stress in lower extremity skeletal muscle.<sup>4–16</sup> Lower extremity skeletal muscle in people with PAD has an increased proportion of myofibers with central nuclei, suggesting ongoing muscle repair, and reduced muscle mass compared with people without PAD.<sup>8–16</sup> Both increased capillary density and reduced

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## Nonstandard Abbreviations and Acronyms

<b>4E-BP1</b>	4E-binding protein 1
<b>ABI</b>	ankle brachial index
<b>AMPK</b>	AMP-activated protein kinase
<b>HIF-1</b>	hypoxia inducible factor 1
<b>mTORC1</b>	mTOR complex 1
<b>NF-κB</b>	nuclear factor κB
<b>NO</b>	nitric oxide
<b>PAD</b>	peripheral artery disease
<b>PGC-1α</b>	proliferator-activated receptor γ coactivator 1α
<b>PPARα</b>	proliferator-activated receptor α
<b>REST</b>	RE1-silencing transcription factor
<b>ROS</b>	reactive oxygen species
<b>S6 K1</b>	S6 kinase
<b>VEGF-A</b>	vascular endothelial growth factor

capillary density have been reported in skeletal muscle of people with PAD, compared with those without PAD.<sup>6,12</sup> Some adverse gastrocnemius muscle characteristics, such as reduced muscle mass, smaller myofiber size, fewer inflammatory-associated (ie, CD206-) macrophages, and fewer capillary contacts with type I and type IIa fibers were associated with greater walking impairment and increased rates of mobility loss in people with PAD.<sup>6–8,15,17,18</sup> Although causal evidence is not available, ischemia-related damage of lower extremity skeletal muscle may exacerbate walking impairment in people with PAD.

Walking exercise is first line therapy for people disabled by lower extremity PAD.<sup>19</sup> Compared with a control group that did not exercise, supervised treadmill exercise interventions improved 6-minute walk distance by a mean of 15 to 35 meters, while effective home-based walking exercise interventions improved 6-minute walk distance by 45 to 55 meters.<sup>20–25</sup> Walking exercise interventions have also improved treadmill walking performance, participant perceived walking ability, and physical activity.<sup>20–25</sup> Thus, even while inducing lower extremity ischemia, walking exercise significantly improved walking performance and other outcomes in patients with PAD. This review summarizes evidence regarding the effects of walking exercise on lower extremity skeletal muscle characteristics in people with PAD without chronic limb threatening ischemia. This review focuses on PAD without chronic limb threatening ischemia because lower extremity muscle characteristics differ substantially between people with PAD without chronic limb threatening ischemia and because walking exercise is typically not recommended for people with chronic limb threatening ischemia.<sup>19,26,27</sup>

## EVIDENCE OF ISCHEMIA-RELATED LOWER EXTREMITY SKELETAL MUSCLE ABNORMALITIES IN PAD

Atherosclerotic obstructions in the lower extremities restrict delivery of oxygen and nutrients during walking exercise. While direct measurement of ischemia within lower extremity skeletal muscle during walking activity in people with PAD is not typically feasible, evidence for ischemic damage of lower extremity skeletal muscle related to walking includes the following. First, people with PAD typically report lower extremity skeletal muscle discomfort, tightness, weakness, or other adverse leg symptoms during walking activity, when oxygen demand exceeds supply, and these symptoms resolve within minutes of rest, when oxygen supply is sufficient to meet demand.<sup>28</sup> Second, in 10 patients with unilateral PAD and 6 patients without PAD, skeletal muscle biopsies showed a higher abundance of short-chain acylcarnitines and lactate in the leg affected by PAD, compared with the contralateral leg without PAD and compared with the patients without PAD, suggesting ineffective oxidative metabolism in ischemic legs of patients with PAD.<sup>29</sup> Third, animal models of hindlimb ischemia showed reduced skeletal muscle mass, impaired mitochondrial function, and increased oxidative stress, findings that were also observed in skeletal muscle of patients with PAD.<sup>4,11–14,30,31</sup>

## OVERVIEW OF WALKING EXERCISE AND LOWER EXTREMITY SKELETAL MUSCLE IN PAD

The fact that walking exercise induces lower extremity ischemia while simultaneously improving walking performance in people with PAD appears paradoxical, since lower extremity ischemia has been associated with lower extremity skeletal muscle pathology in both animals and in humans with PAD.<sup>4,11–14,30,31</sup> Yet recent evidence supports a hypothesis that the lower extremity ischemia induced by walking exercise may be necessary for improving walking performance in people with PAD. In the LITE (low intensity exercise intervention in PAD) randomized clinical trial, 305 participants with PAD were randomized to ischemic pain inducing high-intensity walking exercise, low-intensity walking exercise without ischemic leg symptoms, or a control group that did not exercise for 12 months.<sup>25</sup> While both exercise groups were coached to walk for exercise 5 days per week for up to 30 minutes per session, those randomized to high-intensity exercise walked significantly fewer minutes per week than those randomized to low-intensity exercise (77 versus 145 minutes/wk,  $P < 0.01$ ). Despite fewer minutes of walking exercise, high-intensity ischemic-symptom inducing walking exercise significantly improved 6-minute walk distance, compared with the

control group: (between group difference: 49.6 meters (95% CI, 24.3–74.9,  $P < 0.001$ )) and compared with the low-intensity exercise group ( $P < 0.001$ ). In contrast, the low-intensity exercise group, which walked for exercise at a pace to avoid ischemic leg symptoms, did not improve 6-minute walk more than the control group and declined significantly in walking ability compared with the high-intensity exercise group (between group difference with high intensity exercise group: -40.9 meters, [95% CI -61.7, to -20.0]  $P < 0.001$ ).<sup>25</sup> No significant differences were observed in gastrocnemius muscle biopsy measures of citrate synthase activity, cytochrome C oxidase activity, or nitrotyrosine abundance across the 3 exercise groups in the 47 participants who underwent skeletal muscle biopsy at baseline and follow-up.<sup>25</sup> While biologic pathways responsible for improved walking performance in response to ischemia-inducing walking exercise, compared with walking exercise without ischemic symptoms, remain unclear in people with PAD, the LITE randomized trial supports the hypothesis that lower extremity ischemia induced by walking exercise may be necessary to stimulate biologic pathways that improve walking performance in people with PAD. In this review, evidence regarding the effects of walking exercise on lower extremity skeletal muscle in PAD is discussed in the context of evidence about the effects of walking exercise on lower extremity skeletal muscle in older people without PAD. While some pathophysiologic changes in lower extremity skeletal muscle of people with PAD may be an extreme version of skeletal muscle changes that occur with aging in people without PAD, the effects of exercise on lower extremity skeletal muscle may differ between older people with and without PAD.

## EFFECTS OF LOWER EXTREMITY ISCHEMIA ON GASTROCNEMIUS SKELETAL MUSCLE IN HUMANS

Lower extremity skeletal muscle in people with PAD is characterized by heterogeneity of myofiber type distribution, but multiple studies have documented overall loss of myofiber area and number, increased abundance of fibrosis or collagen, and increased oxidative stress in gastrocnemius muscle of people with PAD, compared with those without PAD.<sup>5–8,12–16</sup> In 16 patients with PAD (mean ankle brachial index [ABI], 0.58) and 13 control patients without PAD who underwent gastrocnemius muscle biopsy, the relative area of muscle occupied by type I fibers was significantly lower (49% versus 64%), while the relative area of muscle occupied by type IIA fibers was significantly higher (29% versus 16%) in patients with PAD.<sup>15</sup> There was no difference in the relative area of muscle occupied by type IIX fibers in this study.<sup>15</sup> In contrast, a study by Koutakis et al<sup>14</sup> of 53 patients with PAD (including 25 with advanced PAD) and 25 control patients

without PAD reported a higher relative area of type I fibers and reduced type 2 fibers in gastrocnemius muscle from people with PAD, compared with those without PAD. In this study by Koutakis et al,<sup>14</sup> patients with PAD had a greater abundance of carbonyl content in gastrocnemius myofibers, compared with those without PAD, and greater myofiber oxidative damage was associated with greater loss of structural integrity and smaller size of the myofibers in PAD. Ha et al<sup>16</sup> documented an increased abundance of collagen density, fibroblast accumulation, and TGF- $\beta$ 1 (transforming growth factor  $\beta$ 1) abundance in myofibers of 45 patients with PAD, compared with 20 control patients without PAD. The 45 patients with PAD included 25 patients with claudication (Fontaine Stage II) and 20 with chronic limb threatening ischemia (Fontaine Stage IV).<sup>16</sup> More severe PAD (ie, those with chronic limb threatening ischemia) was associated with a greater abundance of collagen density and fibroblast accumulation, and greater TGF $\beta$ 1 abundance was associated with a greater collagen abundance in the patients with PAD but not in those without PAD.<sup>16</sup> Collagen density was concentrated around microvessels of patients with PAD.<sup>16</sup> It is important to note, however, that differences between the patients without PAD, with claudication, and with chronic limb threatening ischemia in this study may have been due in part to confounding, since there were substantial differences in age, presence of diabetes, and other characteristics between the groups.<sup>16</sup> Reported associations of PAD with capillary density and mitochondria activity have varied and are discussed further below.

## EFFECTS OF EXERCISE ON SKELETAL MUSCLE MYOFIBERS, CAPILLARY DENSITY, AND MITOCHONDRIAL ACTIVITY IN OLDER PEOPLE WITHOUT PAD

Some characteristics of gastrocnemius muscle pathology in PAD may represent an extreme of skeletal muscle changes observed with aging. In people without PAD, aging is associated with declines in myofiber size and abundance, reduced mitochondrial mass and function, declines in capillary density, intramuscular fat accumulation, and metabolic derangements consistent with insulin resistance.<sup>32</sup> In older people without PAD, walking and other aerobic exercise partially reverse these adverse effects of aging by increasing muscle mass, muscle strength, insulin sensitivity, capillary density, mitochondrial activity, mRNA levels of mitochondrial genes, mitochondrial biogenesis, and mitochondrial proteins.<sup>32–37</sup> In a nonrandomized clinical trial, Coggan et al<sup>33</sup> studied 23 healthy men and women age 60 to 70 who ran or jogged for 45 minutes 4 days per week for 9 to 12 months and 7 control participants age 60 to 70 who did not exercise over the same time period. Among the older participants who exercised, gastrocnemius muscle biopsies collected

before and after exercise showed that the proportion of type IIX muscle fibers (fast twitch fibers used for high-intensity exercise) significantly decreased (from 19.1% [SD: 9.1] to 15.1% [SD: 8.1],  $P<0.001$ ), while type IIA muscle fibers, that use both aerobic and anaerobic energy, significantly increased (from 22.1% [SD: 7.7] to 29.6% [SD: 9.1],  $P<0.05$ ). The proportion of type I fibers did not change significantly, but the cross-sectional area of type I and type 2 fibers increased by 12% ( $P<0.001$ ) and 10% ( $P<0.05$ ), respectively, and capillary density increased (from 257 capillaries/mm<sup>2</sup> [SD: 43] to 310 capillaries/mm<sup>2</sup> [SD: 48],  $P<0.001$ ) in those who exercised. Lactate dehydrogenase activity decreased by 21% ( $P<0.001$ ), while the mitochondrial enzymes succinate dehydrogenase, citrate synthase, and  $\beta$ -hydroxyacyl-CoA dehydrogenase increased by 49% ( $P<0.001$ ), 23% ( $P<0.05$ ), and 39% ( $P<0.001$ ), respectively. In contrast, skeletal muscle characteristics did not meaningfully change in the 7 participants who did not exercise.<sup>33</sup> A separate study compared the effects of 6 weeks of aerobic exercise training on changes in skeletal muscle in 11 healthy elderly people (mean age 80 years, SD: 4) with 10 healthy young people (mean age 24 years, SD: 3).<sup>34</sup> Aerobic training for 6 weeks improved maximal workload, maximal oxygen consumption, and exercise economy in the elderly group by  $\approx$ 34%, 13%, and 12%, respectively, and by 26%, 9%, and 10% in the young group. Both the older and the younger participants had significant increases in skeletal muscle citrate synthase, a measure of mitochondrial abundance and activity, and in activity of mitochondrial complexes I to IV, without differences between the older and younger participants.<sup>34</sup> However, 8 weeks after training, older patients experienced statistically significant declines in activity of mitochondrial complexes I (by 40%), II (by 8%), III (by 25%), and IV (by 26%) while activity of only mitochondrial complexes I (by 26%) and II (by 9%) declined significantly in the younger individuals.<sup>34</sup> Together, these studies demonstrate significant increases in skeletal muscle mitochondrial activity, myofiber size, and increased capillary density following exercise interventions in older people without PAD. The finding that gains in skeletal muscle function in response to exercise were lost by 8 weeks after training in older healthy people without PAD is likely to have important implications for older people with PAD, who have particularly low-physical activity levels in the absence of exercise interventions.

## EFFECTS OF TREADMILL WALKING EXERCISE ON SKELETAL MUSCLE MAY DIFFER IN PEOPLE WITH PAD COMPARED WITH OLDER PEOPLE WITHOUT PAD

While aerobic exercise has beneficial effects on lower extremity skeletal muscle in healthy older people

without PAD,<sup>32–37</sup> walking exercise in people with PAD is associated with ischemia and, therefore, may have different effects on lower extremity skeletal muscle compared with those observed in healthy people.<sup>38</sup> Relatively, few studies have performed skeletal muscle biopsy before and after an exercise intervention in people with PAD. Hiatt et al<sup>38</sup> randomized 26 men with PAD (mean age 67 years) to supervised treadmill exercise, lower extremity resistance training, or a nonexercising control group for 12 weeks and performed gastrocnemius muscle biopsies at baseline and after training. Supervised treadmill exercise significantly improved treadmill walking time (from 9.3 $\pm$ 5.6 to 15.1 $\pm$ 6.8 minutes,  $P<0.05$ ) and also increased the proportion of angular muscle fibers (from 7.6 $\pm$ 5.4% to 15.6 $\pm$ 7.5%,  $P<0.05$ ). Angular fibers have been associated with muscle denervation, and it is likely that in this study these changes represented an adaptive response to exercise. No change in the proportion of angular muscle fibers was observed in the control group with PAD that did not exercise (from 9.1 $\pm$ 8.4 to 9.1 $\pm$ 6.5).<sup>38</sup> Participants randomized to supervised exercise also had a statistically significant increase in the proportion of gastrocnemius target myofibers (from 3.0% $\pm$ 3.3 to 7.7% $\pm$ 6.8,  $P<0.05$ ), consistent with reinnervation of myofibers, while the control group had no significant change in the proportion of gastrocnemius target myofibers (from 8.3% $\pm$ 7.6 to 10.6% $\pm$ 5.8).<sup>38</sup> These skeletal muscle fiber changes following treadmill exercise in PAD are consistent with the hypothesis that exercise in patients with PAD induces an adaptive response that involves denervation and innervation of gastrocnemius muscle.<sup>38</sup> Continual denervation and reinnervation of skeletal muscle is also observed in healthy older people and can be an adaptive change in response to aging, but is also associated with myofiber loss, since some muscle fibers that are denervated are not re-innervated, resulting in progressive muscle loss with age.<sup>39</sup> In the trial by Hiatt et al,<sup>38</sup> supervised treadmill walking exercise did not significantly change gastrocnemius muscle citrate synthase or lactate dehydrogenase activity in people with PAD, but phosphofructokinase activity significantly increased by 25% from 15.0 $\pm$ 5.6 to 18.8 $\pm$ 7.5  $\mu$ mol/min-g-nucleosome core particle (NCP) after treadmill exercise ( $P<0.05$ ), suggesting a shift of energetic metabolism toward glycolysis. Calf muscle characteristics did not change significantly among participants with PAD randomized to either resistance training or control.<sup>38</sup> In a separate nonrandomized controlled clinical trial, gastrocnemius muscle biopsies at baseline and 12-week follow-up were collected from 27 patients with PAD and claudication who participated in a 12 week exercise program.<sup>40</sup> A control group of 11 patients with PAD and claudication awaiting lower extremity angioplasty also had gastrocnemius muscle biopsy at baseline and 12-week

follow-up. Treadmill walking distance and myosin heavy chain I increased significantly in the group that exercised (from  $34.3\% \pm 6.8$  to  $45.4\% \pm 4.4$ ) but did not improve in the control group awaiting angioplasty.<sup>40</sup> In the group that exercised, greater increases in myosin heavy chain I expression were significantly correlated with longer time to onset of claudication on the treadmill (correlation: 0.69,  $P < 0.05$ ).<sup>40</sup> In summary, 2 separate walking exercise programs in patients with PAD induced changes in gastrocnemius myofibers consistent with denervation and reinnervation and increased myosin heavy chain I content, respectively.<sup>38,40</sup>

## EFFECTS OF EXERCISE ON REACTIVE OXYGEN SPECIES IN MUSCLE OF PEOPLE WITH AND WITHOUT PAD

Reactive oxygen species (ROS) are unstable oxygen-containing molecules and important cell signaling molecules. In people without PAD, aerobic exercise increases ROS in skeletal muscle, primarily by increasing NADPH oxidase and xanthine oxidase activity and by increasing ROS production by mitochondria.<sup>41,42</sup> Increased ROS abundance has both beneficial and detrimental effects.<sup>43</sup> Exercise-induced increases in skeletal muscle ROS promote an increase in abundance of NF- $\kappa$ B (nuclear factor  $\kappa$ B) and peroxisome PGC-1 $\alpha$  (proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$ ) and in mitogen activated protein kinase (MAPK) activity in skeletal muscle fibers, thereby increasing skeletal muscle antioxidants, mitochondrial biogenesis, and mitophagy.<sup>43-45</sup> Mitophagy facilitates removal of damaged mitochondria and replacement with newer and healthier mitochondria.<sup>46-50</sup> However, excess ROS accumulation, either due to overproduction or to reduced ROS consumption by antioxidants, such as superoxide dismutase and glutathione peroxidase, causes pathological oxidative stress which impairs protein and lipid function, contributes to genomic instability, damages membrane integrity, and inhibits cell function and survival.<sup>43,49</sup> Many aspects of ROS signaling remain unclear, including how ROS facilitates adaptive anabolic responses in skeletal muscle in response to exercise while also promoting catabolic responses in states of inactivity or muscle disuse.<sup>43,49</sup>

People with PAD have a greater abundance of ROS in skeletal muscle, compared with people without PAD.<sup>4,5,14</sup> More severe ischemia, measured by the ABI, is associated with greater ROS.<sup>4</sup> Increased ROS abundance in lower extremity skeletal muscle of people with PAD may be due to effects of repeated ischemia and reperfusion injury during walking activity.<sup>4,5,51</sup> However, extreme inactivity, common in PAD, has also been associated with increased ROS in skeletal muscle.<sup>3,52</sup>

Greater ROS abundance in gastrocnemius muscle is associated with smaller myofibers, greater myofiber

degeneration, and other pathological structure changes in myofibers.<sup>13,14,53</sup> It is unclear whether exercise activity in people with PAD further increases ROS over baseline. One study of 31 patients with PAD and intermittent claudication reported that a single episode of exercise significantly increased plasma levels of thiobarbituric acid-reactive substances and that this increase was eliminated when vitamin C was administered before the bout of treadmill exercise.<sup>54</sup> However, in the subset of participants who underwent muscle biopsy in the LITE randomized clinical trial, high-intensity ischemic symptom inducing walking exercise did not significantly increase nitrotyrosine in gastrocnemius muscle, compared with the control group that did not exercise, despite the fact that the high-intensity walking exercise intervention significantly improved walking endurance compared with control.<sup>25</sup> The specific threshold at which patients with PAD transition from a beneficial abundance of ROS to a detrimental abundance of ROS in skeletal muscle is unknown.

## HINDLIMB ISCHEMIA IN MICE IS ASSOCIATED WITH MITOCHONDRIAL ABNORMALITIES IN SKELETAL MUSCLE

Mitochondrial activity is determined in part by quality and quantity of the 5 mitochondrial complexes, located on the inner mitochondrial membrane. The mitochondrial complexes transfer electrons from electron donors to electron acceptors, via redox reactions, thereby generating energy. The 5 mitochondrial complexes differ with regard to the proteins and enzymes responsible for the electron transfers. Mouse models of hindlimb ischemia demonstrate reduced mitochondrial complex-dependent respiration for complexes I, III, and IV in ischemic muscle along with increased mitochondrial content, consistent with impaired mitophagy.<sup>4</sup> Mouse models of hindlimb ischemia also showed increased oxidative stress and reduced mitochondrial activity in skeletal muscle.<sup>4,53,54</sup>

## MITOCHONDRIAL ABNORMALITIES IN LOWER EXTREMITY SKELETAL MUSCLE OF HUMANS WITH PAD

In humans, mitochondrial activity may be lower in those with PAD, compared with those without PAD, but data are mixed.<sup>1,51,55-57</sup> In one study of 25 patients undergoing lower extremity revascularization for severe PAD and 16 patients undergoing leg operations to treat superficial varicose veins, activity of mitochondrial complexes I, III, and IV were all significantly reduced in people with PAD, compared with those without PAD, after adjusting for mitochondria abundance with citrate synthase.<sup>55</sup> However,

confounding may have contributed to these findings, since the participants with PAD were older ( $63\pm 2$  versus  $60\pm 2$  years), more likely to be current smokers (56% versus 18%), and had a higher prevalence of insulin dependent diabetes (32% versus 0%).<sup>55</sup> The Baltimore Longitudinal Study of Aging measured muscle mitochondrial energy production with the postexercise phosphocreatine recovery rate constant, using phosphorus magnetic resonance spectroscopy of the left thigh, in 363 men and women with ABI values of 0.90 to 1.40.<sup>56</sup> A lower postexercise phosphocreatine recovery rate constant indicates reduced mitochondrial energy production. In this study, participants with mild or borderline PAD (ie, ABI of 0.90 to 1.10) had a significantly lower postexercise phosphocreatine recovery rate constant than those with ABI values of 1.11 to 1.40 ( $19.3$  versus  $20.8$   $\text{ms}^{-1}$ ,  $P=0.015$ ), and this association was not substantially changed after adjusting for age, sex, race, smoking, diabetes, and other confounders.<sup>56</sup> In a study of 30 people with PAD (mean age, 62; mean ABI, 0.55) and 30 sedentary people without PAD (mean age, 65; mean ABI, 1.05), mitochondrial respiration in gastrocnemius muscle was lower in patients with PAD compared with control, for complex I and complex IV.<sup>57</sup> In summary, several small studies have demonstrated poorer mitochondrial activity in people with PAD, compared with those without PAD.

More recent evidence shows increased mitochondrial DNA abundance and greater mitochondrial DNA heteroplasmy in people with PAD, compared with those without PAD.<sup>9,58,59</sup> In 34 people with PAD and 10 without PAD age 65 and older, mitochondrial DNA relative copy number was significantly higher in patients with lower ABI values (ABI <0.60: 914; ABI, 0.61–0.90: 731; ABI, 0.90–1.50: 593;  $P$  trend=0.016), consistent with impaired mitophagy in the setting of lower extremity ischemia.<sup>58</sup> Separately, mitochondrial DNA heteroplasmy, defined as abundance of accumulated mitochondrial DNA mutations, was significantly higher in patients with PAD, compared with those without PAD.<sup>9</sup> However, mitochondrial DNA damage has not been consistently associated with impairment in walking performance in people with PAD.<sup>59</sup> Additional recent biologic findings in lower extremity skeletal muscle of patients with PAD have been significantly greater in people with chronic limb threatening ischemia<sup>26,27</sup> and are beyond the scope of this review.

## EFFECTS OF EXERCISE ON SKELETAL MUSCLE MITOCHONDRIAL ACTIVITY IN PEOPLE WITH PAD

While exercise has favorable effects on mitochondrial oxidative capacity in older adults without PAD, relatively few studies have evaluated the effect of

exercise on mitochondrial function or oxidative capacity in people with PAD. One study of 11 patients with PAD (mean age, 65; mean ABI, 0.65) and 11 healthy older adults without PAD (mean age, 73; mean ABI, 1.10) reported that complex I-mediated oxidative phosphorylation of the electron transport chain in gastrocnemius muscle biopsies was 40% lower in those with PAD at baseline, compared with those without PAD, while mitochondrial complex II activity in the gastrocnemius biopsy was higher at baseline in those with PAD, compared with those without PAD.<sup>60</sup> After one bout of exercise, consisting of continuous calf raises that induced ischemic leg pain in the participants with PAD, mitochondrial complex II activity, but not the remaining mitochondrial complexes, significantly increased in people with PAD. Mitochondrial complex II is the only mitochondrial complex that is entirely encoded by nDNA, while the remaining mitochondrial complexes are encoded by mitochondrial DNA along with nDNA. In contrast, in people without lower extremity ischemia, 100 calf raises significantly increased complex I mitochondrial activity (Table 2).<sup>60</sup> It is important to note that this trial consisted of a single bout of exercise, which may have different effects on muscle than chronic exercise activity in people with PAD.

In a separate trial, Van Schaardenburgh et al<sup>61</sup> randomized 29 people with PAD to either 3× daily home-based calf raise exercises that elicited ischemic leg symptoms ( $n=14$ ; mean age, 66; mean ABI, 0.58) or home-based walking exercise for 30 minutes performed 3× per week ( $n=15$ ; mean age, 70; mean ABI, 0.57) for 8 weeks. At 8-week follow-up, both groups improved pain-free and maximal treadmill walking time, but the improvements in treadmill walking performance were statistically significant only in those randomized to calf raise exercises. Citrate synthase activity, a measure of both mitochondrial abundance and activity, significantly increased in the calf raise exercise group ( $4.74$ – $4.82$   $\mu\text{mol}/\text{min}$  per mg of protein,  $P=0.02$ ) but did not significantly change in the walking exercise group ( $4.84$ – $4.88$   $\mu\text{mol}/\text{min}$  per mg of protein,  $P=0.10$ ).<sup>61</sup> There were no statistically significant improvements in mitochondrial respiration from complex-I activity ( $36.1$  versus  $33.9$  pmol/mg per s,  $P=0.16$ ) or from complex-I+II mediated mitochondrial activity ( $87.6$  versus  $85.2$  pmol/m per s,  $P=0.35$ ).<sup>62</sup> While citrate synthase activity was the only mitochondrial measure that increased following the exercise intervention in this small trial of participants with PAD,<sup>61</sup> the small sample size limited power to detect a statistically significant difference. In addition, the lack of a control group of PAD participants who did not exercise was a significant limitation of the trial of PAD participants,<sup>61</sup> since prior evidence demonstrated that mitochondrial and other skeletal

muscle measures declined in a control group of PAD participants after a follow-up period of six months.<sup>11</sup> In healthy older people with normal blood flow, exercise training significantly increased mitochondrial activity.<sup>62</sup>

In a subsequent analysis of the exercise trial of 29 participants with PAD described above,<sup>61</sup> participants were categorized, regardless of exercise group, according to whether they had the highest improvement in peak treadmill walking time ( $n=8$  responders) or the greatest decline in peak treadmill walking time ( $n=8$  nonresponders).<sup>63</sup> Citrate synthase activity significantly improved among responders (absolute data not shown). In nonresponders, citrate synthase activity did not increase and mitochondrial activity declined.<sup>64</sup> Uncoupling of oxidative phosphorylation from ATP production occurred even in responders.

Murrow et al<sup>64</sup> used near infrared spectroscopy to measure gastrocnemius muscle mitochondrial capacity ( $mVO_2$ max). Near infrared spectroscopy uses spectroscopy to measure mitochondrial capacity by quantifying the recovery rate constant of muscle metabolism after exercise. Murrow et al<sup>64</sup> randomized 36 people with PAD to either traditional walking exercise that elicited ischemic pain or to walking exercise that attained a 15% reduction in skeletal muscle oxygenation for 12 weeks. Both groups exercised 3× weekly. Both exercise groups significantly increased maximal treadmill walking time at 12-week follow-up (from 8.47 to 10.71 per minute and from 8.70 to 10.99 per minute, respectively), but the group that exercised to an intensity that induced ischemic pain improved mitochondrial capacity significantly more than the group that walked for exercise to attain a 15% reduction in skeletal muscle oxygenation (from 0.87 to 1.46 per min versus from 1.07 to 1.31 per min,  $P$  value for interaction = 0.003). These results suggest that exercise induces significant metabolic adaptations in skeletal muscle of patients with PAD only when the threshold for pain is reached. However, only 50% of the randomized cohort completed the trial, which limits validity of the results.<sup>64</sup> A separate study of 64 people with PAD reported that supervised treadmill exercise for three months improved gastrocnemius muscle microvascular flow, oxygen extraction, and gastrocnemius oxidative metabolic capacity, compared with a control group of PAD participants who did not exercise.<sup>65</sup> Compared with the control group that did not exercise, supervised exercise significantly increased peak treadmill walking time (158 versus 53 seconds,  $P<0.005$ ), fiber-optic near infrared spectroscopy measured gastrocnemius muscle microvascular blood flow (0.37 versus  $-0.25$  normalized units,  $P<0.001$ ), oxygen extraction (0.07 versus  $-0.11$  normalized units,  $P<0.001$ ), and gastrocnemius oxidative metabolic capacity (1.05 versus  $-0.50$  normalized units,  $P<0.001$ ). This study suggested that supervised treadmill exercise in patients with PAD may

increase both gastrocnemius mitochondrial activity and microvascular flow to the gastrocnemius muscle.<sup>66</sup> Overall, data on the effects of supervised walking exercise on improvement in mitochondrial oxidative capacity in people with PAD are mixed, perhaps in part due to inconsistent methods of measurement (Table 1).

## EFFECTS OF EXERCISE ON LOWER EXTREMITY SKELETAL MUSCLE CAPILLARY DENSITY IN PAD

Some studies have reported higher capillary density, while others have reported lower capillary density in lower extremity skeletal muscle of people with PAD, compared with people without PAD.<sup>6,15,66–69</sup> White et al. and McGuigan et al reported a higher number of capillaries per fiber in muscle from people with PAD compared with those without PAD.<sup>6,67</sup> However, the study by White et al<sup>6</sup> further reported that greater capillary density did not correlate with better functional performance. While increased capillary density in people with PAD may represent a compensatory phenomenon, similar to increased numbers and size of collateral vessels observed in people with more severe PAD, evidence from a mouse model, in which ischemia was induced in the mouse extensor digitorum longus muscle, showed that adaptive angiogenesis was profoundly impaired in both structure and function.<sup>70</sup> It is possible that adaptive increases in angiogenesis in PAD may be functionally flawed in structure and function.<sup>70</sup>

Currently, there is not evidence that walking exercise programs of 12 weeks or longer significantly improve capillary density in PAD compared with a control group.<sup>71,72</sup> In one randomized trial of 35 participants with PAD randomized to either supervised or home-based exercise training, supervised exercise significantly increased capillary density (from  $216\pm66$  to  $286\pm76$  capillaries per  $mm^2$  of gastrocnemius skeletal muscle) and improved treadmill walking performance within participants randomized to supervised exercise. In contrast, home-based exercise, that did not incorporate effective behavioral methods, did not improve either capillary density (from  $238\pm78$  to  $235\pm91$  capillaries per  $mm^2$  of skeletal muscle) or treadmill walking performance at 12-week follow-up.<sup>71</sup> However, there was no significant difference in change in capillary density between participants randomized to supervised exercise versus home-based exercise. VEGF-A (vascular endothelial growth factor), which promotes angiogenesis, did not significantly increase in gastrocnemius muscle lysates in this trial.<sup>69</sup> Instead, VEGF<sub>165</sub>b, an antiangiogenic factor, increased in gastrocnemius lysate.<sup>69</sup> In contrast, a study of a single bout of either passive extension and flexion of the lower leg or active exercise (eight 3-minute bouts of single leg knee extension exercise at 10

**Table 1. Peripheral Artery Disease, Lower Extremity Skeletal Muscle Pathophysiology, and Effects of Exercise**

Muscle characteristic	Pathophysiologic changes in people with PAD	Effects of exercise	Other considerations
Calf muscle area and mass	Reduced myofiber size Reduced muscle mass. Increased fibrosis. <sup>13,16</sup>	Unclear	Based on evidence from people without PAD, exercise is unlikely to reduce fibrosis.
ROS	People with PAD have increased reactive oxygen species, compared with those without PAD. Greater lower extremity ischemia is associated with increased ROS. <sup>5</sup>	Unclear	ROS levels are high in people with PAD. It is unclear whether walking exercise further increases ROS beyond baseline levels in people with PAD.
Mitochondrial dysfunction	People with PAD may have reduced mitochondrial activity compared with those without PAD. <sup>17,29</sup>	Exercise effects on mitochondrial activity have been mixed. Citrate synthase activity increases in some trials but not others. Some trials showed that exercise increases mitochondrial complex II activity, but not other mitochondrial complexes.	Complex II proteins are the only mitochondrial complex proteins coded entirely by nDNA. The remainder require mitochondrial DNA.
Capillary density	Data are mixed, with some studies demonstrating reduced capillary density and others demonstrating increased capillary density in people with PAD. <sup>12,67</sup>	There is no evidence that exercise significantly increases capillary density in gastrocnemius muscle in people with PAD.	
Plasma nitrite abundance at peak exercise	Plasma nitrite abundance after exercise is reduced in people with PAD, compared with those without PAD. <sup>103</sup>	Plasma nitrite increases at peak exercise after an effective exercise intervention in people with PAD. <sup>102</sup>	Increased nitrite abundance may promote increased perfusion of calf muscle and improved mitochondria biogenesis and activity.

PAD indicates peripheral artery disease; and ROS, reactive oxygen species.

Watts) modestly increased VEGF-A in interstitial fluid of thigh skeletal muscle in people with PAD and in people without PAD.<sup>72</sup> Although lower extremity resistance training has less beneficial effects on walking ability in people with PAD,<sup>23,73</sup> a randomized trial of lower extremity strength training in 20 people with PAD demonstrated that 24 weeks of strength training significantly increased gastrocnemius type I and type II fiber area, capillary density, 6-minute walk distance, and pain-free walk distance in a 6-minute walk test, compared with a control group that did not exercise.<sup>73</sup> In summary, while lower extremity strength training improved capillary density in people with PAD, to our knowledge, there is currently no evidence that walking exercise programs significantly increase angiogenesis in people with PAD, compared with a control group that does not exercise.

### ADDITIONAL CONSIDERATIONS REGARDING THE EFFECTS OF WALKING EXERCISE ON LOWER EXTREMITY SKELETAL MUSCLE CAPILLARY DENSITY IN PAD

Angiogenesis is a complex highly regulated process where various stimuli, such as hypoxia, exercise, and CO<sub>2</sub>, trigger the formation of new vascular structures in the presence of VEGF and other mediators, including inflammatory cytokines. In the skeletal muscle of people with PAD, inadequate oxygen delivery due to arterial stenosis results in hypoxia that is particularly severe during muscle contraction when the oxygen demand rises. Hypoxia triggers the release of angiogenic growth factors that stimulate the de novo formation of new collateral blood vessels (neovascularization) and

new capillaries.<sup>6,15</sup> The precise mechanisms that stimulates angiogenesis is still poorly understood but likely involves the action of VEGF, HIF-1 (hypoxia inducible factor 1), inflammatory cytokines, nitric oxide (NO) as well as the NOTCH and Sonic Hedgehog signaling. The best known of these factors is VEGF, a family of growth factors that includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E. VEGF-A induces the endothelial NO gene synthase and thereby produces NO while VEGF-B is mediated by VEGF-R1 signaling and activates eNOS-related pathways. Several alternative splicing isoforms of VEGF-A protein exist, including the VEGF-A165 variant, which is a particularly powerful angiogenetic factor. It has been shown that despite high VGFA-A165 levels, ischemia is associated with ineffective angiogenesis in a mouse model of extensor digitorum longus muscle ischemia.<sup>71</sup> Furthermore, clinical trials with VEGF-A based gene therapy have not been generally effective.<sup>74</sup>

### EFFECTS OF EXERCISE ON SKELETAL MUSCLE AND NERVE BIOLOGY IN OLDER PEOPLE WITHOUT PAD

Aging is associated with reduced muscle mass, strength and quality, defined as strength per unit of muscle area.<sup>74-76</sup> Microscopically, aged muscle fibers have a small diameter (particularly type 2 fibers) and may be comprised of thin denervated fibers with a cross-sectional triangular shape (angular fibers) and expression of embryonic denervation biomarkers such as neural cell adhesion molecule.<sup>74-76</sup> The process of denervation, common in older people, starts with degeneration of the neuromuscular junction either because of unopposed oxidative stress and defective autophagy,

**Table 2. Selected Randomized Trials of Effects of Exercise on Lower Extremity Skeletal Muscle**

Trial	Participants	Intervention	Main findings	Additional considerations
Hiatt et al <sup>88</sup>	26 men with PAD	Participants were randomized to supervised treadmill exercise, lower extremity resistance training, or a nonexercise control group for 12 weeks.	Supervised treadmill exercise significantly improved treadmill walking time, and also increased the proportion of angular muscle fibers, consistent with denervation. No change in angular muscle fibers was observed in the group that did not exercise. The supervised treadmill exercise group increased the proportion of gastrocnemius target myofibers, consistent with reinnervation of myofibers, but there was no change in this outcome in the control group. The supervised treadmill exercise group did not increase citrate synthase or lactate dehydrogenase but increased phosphofructokinase activity by 25%.	
Duscha et al <sup>71</sup>	35 participants with PAD	Randomized to 12 weeks of supervised exercise or a home-based exercise without effective behavioral methods	In this trial, supervised exercise, but not home-based exercise, improved treadmill walking distance. Differences in change in capillary density did not differ between the 2 groups (216 SD: 66 to 286 SD: 76 capillaries per mm <sup>2</sup> vs 238 SD: 78 to 235 SD: 91 capillaries per mm <sup>2</sup> ).	Capillaries per myofiber did not significantly increase in either group, but peak blood flow significantly improved in the supervised exercise compared with the home-based exercise group.
Van Schaardenburgh et al <sup>60,61</sup>	29 participants with PAD	Three times daily calf raise exercises, eliciting ischemic symptoms vs 3× weekly walking exercise for 30 min/wk. Exercise interventions lasted 8 weeks.	Only the calf raise exercises significantly improved treadmill walking performance. The calf raise exercises, but not treadmill exercise, increased citrate synthase activity but did not improve mitochondrial complex activity.	Participants who responded to exercise, but not nonresponders to exercise, had increases in citrate synthase. The small sample size and relatively short duration of the exercise intervention were study limitations.
Allen et al <sup>103</sup>	28 participants with PAD	Participants were randomized to either supervised treadmill exercise or home-based exercise without behavioral support for 12 weeks.	The supervised treadmill exercise group significantly improved treadmill walking time and also increased plasma nitrite abundance at the end of a treadmill exercise test. The home-based exercise group did not improve either treadmill walking performance or plasma nitrite.	Greater increases in plasma nitrite at the conclusion of an exercise stress test were associated with greater improvement in treadmill walking performance <sup>81</sup>

PAD indicates peripheral artery disease.

the physiological process by which older organelles are replaced with newer organelles. In older people, synaptic areas are reduced in size and presynaptic and postsynaptic structures are fragmented.<sup>77,78</sup> Progressive denervation is followed by sprouting and reinnervation from preferentially type I, functional axons, leading to fewer but larger motor units, and disappearance of neural cell adhesion molecule. In older people, reinnervation is not sufficient, resulting in a gradual decline in innervated fibers per motor neuron.<sup>77–80</sup> Aging is also associated with a decline in satellite cells (muscle stem cells) associated with type 2 muscle fibers, likely contributing to observed reductions in type 2 fiber size and number with age.<sup>81</sup> Age-related changes within skeletal muscle may be further exacerbated by physical inactivity.<sup>82</sup>

In people without PAD, exercise may slow or reverse the decline of skeletal muscle mass and strength with aging and may improve muscle quality.<sup>83,84</sup> In healthy older adults, both acute and chronic exercise increased protein synthesis, muscle fiber size, and capillary density, decreased intramuscular fat accumulation, and increased mitochondrial volume and function.<sup>85–89</sup> These changes contribute to improved endurance performance. Evidence suggests exercise may restore satellite cell numbers and function, thereby restoring the regenerative capacity of aging muscle.<sup>81,90–93</sup> Mackey et al<sup>91</sup> reported an increase in satellite cell number with 12 weeks of resistance training in 13 healthy, elderly men and 16

healthy elderly women (mean age 76 years). Similarly, 12 weeks of resistance training in 13 healthy, elderly men (mean age 72 years) increased type 2 fiber size and satellite cell numbers associated with type 2 fibers.<sup>90</sup> Exercise may also reverse aging associated changes at the neuromuscular junction, but these effects may not change satellite cell abundance.<sup>94–96</sup>

## EFFECTS OF EXERCISE ON NERVE FUNCTION AND THE NEUROMUSCULAR JUNCTION IN PAD

People with PAD have angular and target muscle fibers, characterized by a lack of oxidative enzyme activity within the center of the fiber rimmed by an area of greater than normal enzyme activity within the gastrocnemius muscle (ie, target fibers), indicative of early innervation of denervated muscle fibers.<sup>97,98</sup> In observational studies, severe PAD was associated with lower peroneal and sural nerve conduction velocity.<sup>99,100</sup> Among 413 patients with PAD, lower peroneal nerve conduction was associated with lower calf muscle area and poorer 6-minute walk distance.<sup>100</sup> The association of exercise training with change in nerve conduction velocity and motor unit action potentials was studied in 16 patients with PAD, of whom 9 participated in supervised treadmill exercise training with

or without isometric strength training of leg muscles and 7 received no exercise training.<sup>101</sup> During follow-up periods ranging from 3 to 23 months, both the exercise and control groups declined similarly with regard to the duration of the motor unit action potential, nerve conduction velocity, and the amplitude of long duration motor unit action potentials. This study demonstrated that exercise training in patients with PAD did not prevent or reverse reductions in motor nerve conduction. However, muscle strength declined in the control group but was maintained in the PAD participants who exercised. An image of denervation related changes in a participant with PAD is shown in Figure 1. In summary, the effects of exercise on neuropathy and neuromuscular junction health remain unclear and may not account for the beneficial effects of exercise in people with PAD.

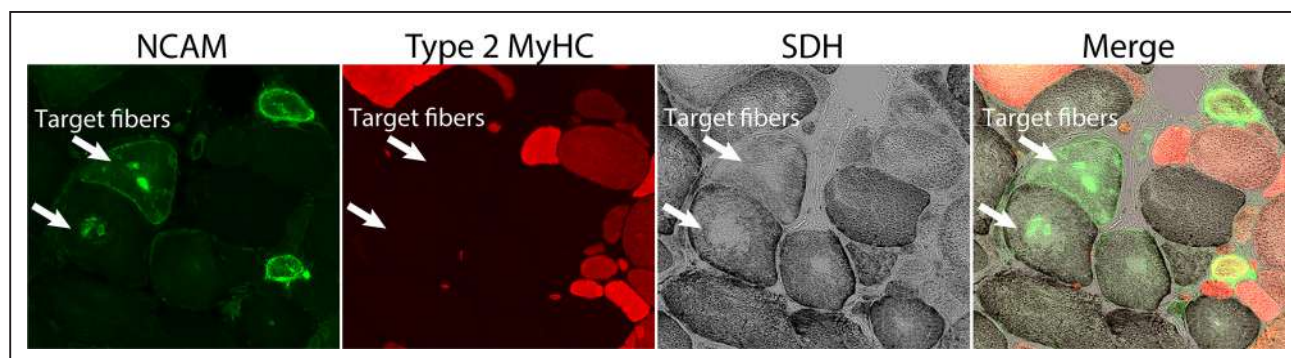
### THE EFFECTS OF SUPERVISED TREADMILL EXERCISE ON NITRITE AND WALKING PERFORMANCE IN PAD

In PAD, exercise may facilitate improved walking ability by improving lower extremity perfusion via increases in nitrite and nitric oxide availability. Increases in nitrite and NO bioavailability could improve vasodilation and perfusion in people with PAD. One randomized trial of 28 participants with PAD randomized to either supervised treadmill exercise or home-based walking exercise without behavioral support demonstrated that 12 weeks of supervised exercise group significantly improved treadmill walking time and simultaneously increased plasma nitrite abundance at the end of a treadmill stress test, compared with baseline, while the home-based exercise did not improve either treadmill walking time or plasma nitrite abundance at the end of the treadmill stress test.<sup>102</sup> Before exercise, plasma nitrite abundance after a treadmill stress test declined in the participants with PAD, while plasma nitrite abundance increased after a treadmill stress test in participants without PAD.<sup>102</sup> In the

participants with PAD, greater improvement in plasma nitrite abundance after a treadmill stress test was associated with greater improvement in treadmill walking performance.<sup>102,103</sup> These data support the hypothesis that exercise may increase bioavailability of plasma nitrite in patients with PAD, potentially improving lower extremity perfusion and potentially improving skeletal muscle health as well. However, randomized trials have not consistently demonstrated benefits of supervised exercise on brachial artery flow-mediated dilation.<sup>21,23</sup>

### CONSIDERATIONS REGARDING AGING, SKELETAL MUSCLE, EXERCISE, AND PAD

In older people with PAD, the effects of aging on muscle may be additive to the detrimental effects of PAD on lower extremity muscle. Aging may also influence response to exercise in older people with PAD. With aging, there is a progressive decline of muscle mass that begins in the third or fourth decade of life and substantially accelerates after age 70 years.<sup>104</sup> Simultaneously, muscle strength declines with age, to a greater degree than the decline in muscle mass, suggesting that aging is associated with substantial changes in muscle quality, defined as the ratio between force and mass. With aging, intermuscular fat (the fat between muscle groups) and intramuscular fat (adipose tissue between myofibers and lipid droplets within myofibers) progressively increase. The presence of fat in skeletal muscle affects strength but is also associated with adverse metabolic effects, including insulin resistance. Aging is also associated with reduced neuromuscular control, with both a central and a peripheral component. Adverse changes in specific regions of the motor brain are longitudinally associated with declines in muscle strength.<sup>105</sup> Autopsy studies from older people who died while relatively healthy have shown that aging is associated with a gradual loss of motor neurons, probably due to an imbalance between denervation and reinnervation.<sup>106</sup> Using



**Figure 1. Immunohistochemistry (IHC) depicting denervated and target fibers in the gastrocnemius muscle from a participant with peripheral artery disease (PAD)**

Representative region of interest depicting the presence of angular muscle fibers expressing neural cell adhesion molecule (N-CAM+), indicative of denervation, and N-CAM+ target fibers (white arrows), associated with early innervation. Left to Right: N-CAM (green), Type 2 myosin heavy chain (MyHC, red), and succinate dehydrogenase (SDH) showing mitochondrial activity (grey). Far right: the merged image showing all markers.

deconvolution of electromyographic signals, McNeil et al<sup>107</sup> demonstrated a gradual reduction in the number of functioning motor units and a decline in neuromuscular firing rate up to the seventh decade, and this decline accounted for the loss of muscle power with aging.<sup>108</sup> Whether mitochondrial mass and number change with aging in skeletal muscle is controversial. Most mitochondrial proteins are significantly underrepresented in older compared with younger persons.<sup>109</sup> Muscle oxidative capacity declines with aging, and this decline is associated with both lower muscle strength and mobility impairment.<sup>110</sup> Evidence suggests that exercise may prevent these adverse changes, including a reduction of muscle fat infiltration<sup>111</sup>; protection of brain health and metabolism assessed by FDG-PET<sup>112</sup>; preservation of motor unit number<sup>113</sup>; and mitochondrial function and mobility.<sup>114,115</sup> It is possible that these beneficial effects of exercise on the aging muscle in people without PAD may contribute to the effectiveness of supervised exercise in people with PAD, who tend to be older.

## EXERCISE, HIF-1, AND AMPK IN PAD

The adaptive response to hypoxia and mitochondrial dysfunction in patients with PAD is affected by 2 master regulators: HIF-1 and AMPK (AMP-activated protein kinase). Because the biological effects of HIF-1 and AMPK are different and even opposite with regard to mitochondrial biogenesis, their interaction is still poorly understood and may change in different clinical phases of PAD severity. Hypoxia stimulates HIF-1 production. The  $\alpha$  subunits of HIF are hydroxylated at conserved proline residues by HIF prolyl-hydroxylases, allowing continuous ubiquitination and proteasome degradation. Hypoxia inhibits HIF-1 degradation resulting in the HIF-1-driven transcription of >100 genes aimed at promoting adaptation and survival. Concurrently, and independent of HIF1-regulation, ischemia triggers the production of REST (RE1-silencing transcription factor) that binds to nDNA and regulates a number of hypoxia-repressed genes, including genes involved in proliferation and cell cycle progression. Thus, HIF-1 and REST act in coordination in the resilience response to hypoxia.<sup>116,117</sup> The main components of this response include upregulating the main glycolytic enzymes, especially lactate dehydrogenase and hexokinase; inhibiting oxidative metabolism by blocking the entry of pyruvate into the tricarboxylic acid cycle, impairing fatty acid oxidation by reducing the DNA binding activity of peroxisome PPAR $\alpha$  (proliferator-activated receptor  $\alpha$ ) and inhibiting PGC-1 $\alpha$ , the main regulator of mitochondrial biogenesis. Additional effects include upregulating mitophagy, which removes and replaces damaged mitochondria, limits ROS production, and removes mitochondrial fragments that trigger inflammation. HIF-1 also promotes angiogenesis mediated by stimulating production of VEGF, although the evidence

for increased capillarization in ischemic muscle has been questioned. Effects may also include reduced cell proliferation and cell cycle progression. Although gene therapy with HIF-1 did not improve functioning in patients with PAD in a randomized trial,<sup>118</sup> the lack of effectiveness may be due to inadequacy of the delivery method and does not rule out a benefit of HIF-1. Furthermore, the HIF-1 signaling pathway is complex because of the presence of multiple HIF-1 isoforms and because of interaction with other signaling molecules. In summary, upregulation of one form of HIF-1 through gene therapy may not be representative of the physiological upregulation of HIF-1 triggered by hypoxia and by exercise. The possibility that exercise exerts its beneficial effects by upregulating HIF-1 cannot be excluded.

With accumulating myofiber damage and a decline in ATP availability, skeletal muscle in patients with PAD may adapt metabolism to optimize energy production and preserve function in the setting of reduced oxygen supply. AMPK is likely critical to this adaptation. AMPK is a protein complex that responds to energy availability by altering the ATP-to-ADP ratio or ATP-to-AMP ratio and by phosphorylating downstream targets that trigger responses aimed at restoring ATP homeostasis. AMPK has effects that include the following: (1) redirecting metabolism towards increased catabolism and decreased anabolism (inhibition of protein, lipid, and glycogen synthesis as well as reduction of cell growth and replication) through the phosphorylation of key proteins in multiple pathways, including mTORC1 (mTOR complex 1); (2) upregulation of autophagy and mitophagy through activation of the kinase ULK1 (unc-51 like autophagy activating kinase 1); (3) promotion of mitochondrial fission (that also facilitates mitophagy) simultaneously promoting mitochondrial biogenesis through PGC-1 $\alpha$ ; and (4) increasing transcription of modulators of autophagy/mitophagy and lysosome biogenesis.<sup>119</sup> When these adaptive mechanisms fail, the energetic stress of inadequate oxygen and energy supply precipitates the cells toward apoptosis, which is found in the skeletal muscle of patients with PAD.<sup>120</sup> Exercise, particularly aerobic exercise, stimulates AMPK and influences increased mitochondrial biogenesis, mitophagy, and angiogenesis via VEGF-A.

The shift of energy production from oxidative phosphorylation to the significantly less efficient anaerobic glycolysis in the muscle of patients with PAD represents an energetic crisis with important biological implications. For example, protein synthesis in response to exercise is energetically expensive. Reduced blood flow reduces energy supply and may limit protein synthesis and turnover resulting in persistence of unrecycled, damaged proteins. However, a recent study in older patients with PAD documented that the synthesis of calf muscle proteins in basal conditions and following a single oral bolus of 15 grams of essential amino-acids EAAs is no different from

age-matched controls.<sup>121</sup> These findings suggest that protein synthesis in the skeletal muscle of PAD may not be inhibited, and that increasing protein synthesis may be a mechanism for the effectiveness of exercise.

### ADDITIONAL CONSIDERATIONS REGARDING HYPOXIA AND LOWER EXTREMITY SKELETAL MUSCLE IN PAD

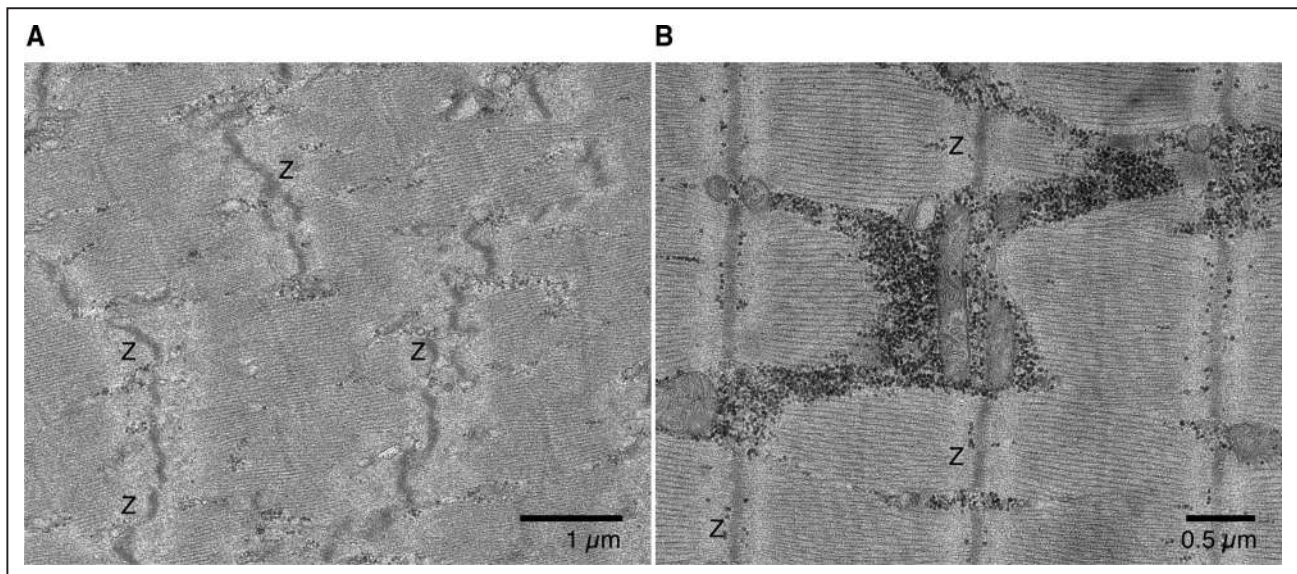
Mechanisms by which chronic hypoxia affect muscle mass and architecture remain unclear but likely involve suppression of protein synthesis and increased fibrosis. It is generally recognized that AMPK activation suppresses mTORC1 signaling, thereby reducing protein translation and translational elongation because of reduced phosphorylation of 70 kDa ribosomal protein S6 K1 (S6 kinase) and eukaryotic initiation factor 4E-BP1 (4E-binding protein 1).<sup>122</sup> Simultaneously, AMPK promotes protein degradation by multiple mechanisms, including the direct activation of FOXO (class O of forkhead box transcription factors) and NF- $\kappa$ B mediated activation of atrogenes (such as MuRF1) that trigger ubiquitination and proteasome degradation<sup>123</sup> and through upregulation of autophagy through direct upregulation of ULK1.<sup>124</sup> Hypoxia induces fibrosis, and increased fibrosis and collagen deposition has been shown in muscle of patients with PAD.<sup>16</sup> While the mechanism of fibrosis is not clear, hypoxic signaling through HIF-1 together with TGF- $\beta$  signaling induces the expression of CCN2 (connective tissue growth factor) by vascular endothelial cells leading

to collagen deposition.<sup>125</sup> Structural changes in a myofiber of a patient with PAD is shown in Figure 2.

### ADDITIONAL CONSIDERATIONS REGARDING EXERCISE AND LOWER EXTREMITY SKELETAL MUSCLE IN PEOPLE WITH PAD

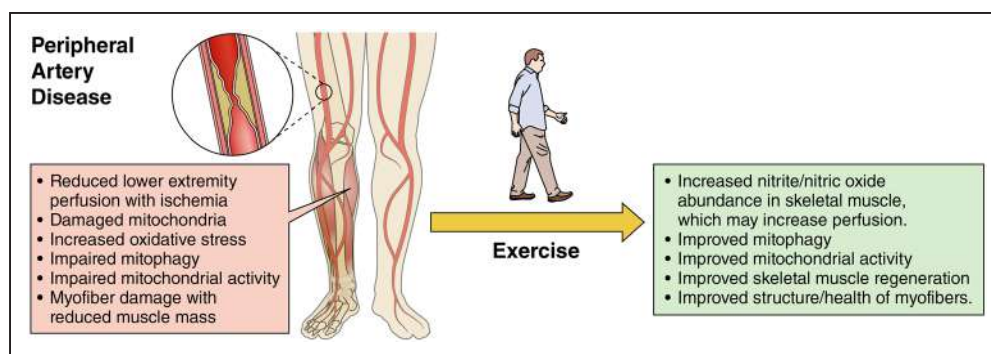
Saltin et al<sup>126</sup> demonstrated that in healthy individuals, one-leg endurance training increased peak oxygen uptake from the trained leg more than the untrained leg, suggesting that exercise affects muscle physiology at the local level. This is important because the fixed arterial obstructions in PAD make it unlikely that changes in central hemodynamics are fully responsible for the beneficial effects of exercise in people with PAD. In patients with PAD, mechanisms that increase oxygen extraction and utilization may be important and may contribute to functional improvements. Importantly, the peripheral mechanisms by which supervised exercise improves walking performance in PAD are not fully understood but likely include changes in muscle cell composition, increased collateral flow, improved perfusion, and endothelial function and improved mitochondria oxidative phosphorylation (Figure 3).

For most people without PAD, maximum exercise capacity is greater than the exercise capacity required to maintain mobility during daily life. In people with PAD, the presence of atherosclerotic lesions in the femoral artery impair the capacity to increase blood flow and the



**Figure 2. Transmission electron micrographs of gastrocnemius muscle biopsies from a person with peripheral artery disease (PAD; A) and control (B).**

Note the z disc (Z) offset in the section through affected muscle of a participant with PAD (A) compare to the fully organized allaying in the control (B). Glycogen granules (small dark particles) and mitochondria are present in both, though the distribution has changed. Interfibrillary (IF) mitochondria are typically located along the A band as seen in B. Samples were surgically removed, chemically fixed and processed for classic room temperature resin embedding. Whether the architectural derangement is reversible by exercise or other interventions is unknown.



**Figure 3. Proposed model for biologic processes in skeletal muscle before and after a walking exercise intervention in people with peripheral artery disease.**

Illustration credit: Patrick Lane.

delivery of oxygen and oxidative substrate with increasing metabolic demand which may be just sufficient to allow normal walking. Poor mobility leads to a sedentary state that can impair maximum exercise capacity over time, contributing to further mobility loss in PAD.<sup>127</sup> Therefore in people with PAD, any increase in exercise capacity, regardless of the mechanism, may improve walking ability. Whether exercise improves mobility in people with PAD via the same biologic pathways that it improves skeletal muscle characteristics and mobility in people without PAD remains unclear. Understanding biologic responses to hypoxia is important for formulating hypotheses about the potential mechanism by which exercise affects skeletal muscle in PAD.

## FUTURE DIRECTIONS

Walking exercise that induces lower extremity ischemia may be necessary to improve walking performance in people with PAD,<sup>25</sup> but the effects of walking exercise on lower extremity skeletal muscle remain unclear. Randomized trials of the effects of walking exercise on lower extremity skeletal muscle have been small and have not consistently included control groups that do not exercise. Currently available evidence suggests that walking exercise may preferentially improve nitrite abundance and perfusion, while several studies showed no effects of walking exercise on mitochondrial activity. There is no evidence that exercise significantly increases capillary function in PAD. It is unlikely that exercise would reverse the fibrosis induced by chronic ischemia.<sup>7</sup> Future studies of the effects of walking exercise in lower extremity skeletal muscle should include larger sample sizes of participants with PAD and a control group that does not exercise, since evidence shows that skeletal muscle characteristics in people with PAD who are not treated can worsen over a 6 month time period.<sup>11</sup> Serial biopsies may best facilitate characterization of the natural history of lower extremity ischemia and the specific effects of

treatment on lower extremity skeletal muscle in PAD. In addition, biologic factors associated with nonresponse to exercise in people with PAD should be investigated.

In summary, in people with PAD, exercise improves walking performance, but the effects of exercise on lower extremity skeletal muscle remain unclear. Considerations of the effects of exercise on skeletal muscle in PAD should include that many people with PAD are older and that age influences both skeletal muscle pathology in PAD and response to exercise. Understanding the biologic strategies by which exercise improves walking in people with PAD remains an unresolved question.

## ARTICLE INFORMATION

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