












ORIGINAL RESEARCH

Mitochondrial Complex Abundance, Mitophagy Proteins, and Physical Performance in People With and Without Peripheral Artery Disease

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BACKGROUND: Mitochondrial abnormalities exist in gastrocnemius muscle of people with peripheral artery disease (PAD). Whether abnormalities in mitochondrial biogenesis and autophagy are associated with greater ischemia or walking impairment in PAD is unknown.

METHODS AND RESULTS: Protein markers of mitochondrial biogenesis and autophagy and the abundance of mitochondrial electron transport chain complexes were quantified in gastrocnemius muscle biopsies from people with and without PAD. Their 6-minute walk distance and 4-m gait speed were measured. Sixty-seven participants (mean age 65.0 years [± 6.8], 16 [23.9%] women, 48 [71.6%] Black) were enrolled, including 15 with moderate to severe PAD (ankle brachial index [ABI] < 0.60), 29 with mild PAD (ABI 0.60–0.90), and 23 without PAD (ABI 1.00–1.40). Abundance of all electron transport chain complexes was significantly higher in participants with lower ABI (eg, complex I: 0.66, 0.45, 0.48 arbitrary units [AU], respectively, P trend=0.043). Lower ABI values were associated with a higher LC3A/B II-to-LC3A/B I (microtubule-associated protein 1A/1B-light chain 3) ratio (2.54, 2.31, 2.15 AU, respectively, P trend=0.017) and reduced abundance of the autophagy receptor p62 (0.71, 0.69, 0.80 AU, respectively, P trend=0.033). The abundance of each electron transport chain complex was positively and significantly associated with 6-minute walk distance and 4-m gait speed at usual and fast pace only among participants without PAD (eg, complex I: $r=0.541$, $P=0.008$; $r=0.477$, $P=0.021$; $r=0.628$, $P=0.001$, respectively).

CONCLUSIONS: These results suggest that accumulation of electron transport chain complexes in gastrocnemius muscle of people with PAD may be because of impaired mitophagy in the setting of ischemia. Findings are descriptive, and further study in larger sample sizes is needed.

Key Words: ankle-brachial index ■ electron transport chain ■ mitochondrial biogenesis ■ mtDNA damage ■ muscle

Mitochondrial structural and functional abnormalities have been described in lower extremity muscle biopsies from people with PAD.¹ For example, reduced activity of electron transport

chain (ETC) complexes,^{2,3} possible impairment of mitochondrial autophagy (mitophagy),⁴ increased oxidative stress,^{5–7} and greater mitochondrial DNA (mtDNA) content⁸ and heteroplasmy⁹ have been

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CLINICAL PERSPECTIVE

What Is New?

- Lower ankle-brachial index values were associated with greater abundance of mitochondrial electron transport chain complexes in gastrocnemius muscle biopsies.
- The ratio between nonlipidated (cytosolic) and lipidated (membrane-bound) forms of the macroautophagy marker LC3A/B (microtubule-associated protein 1A/1B) and the protein content of the autophagy receptor p62 were, respectively, higher and lower in participants with lower ankle-brachial index values.
- Abundance of mitochondrial electron transport chain complexes was positively associated with 6-minute walk distance and 4-m gait speed only in participants without peripheral artery disease.

What Are the Clinical Implications?

- These results suggest the possibility that mitophagy is impaired in the setting of ischemia, resulting in accumulation of damaged mitochondrial complexes.
- Findings also suggest that macroautophagy is upregulated in the setting of ischemia, possibly as an attempt to cope with mitophagy impairment.
- Results are cross-sectional, and further study is needed to corroborate these initial findings and determine whether restoring leg blood flow ameliorates mitochondrial turnover in people with peripheral artery disease.

Nonstandard Abbreviations and Acronyms

ETC	electron transport chain
LC3A/B	microtubule-associated protein 1A/1B-light chain 3
mtDNA	mitochondrial DNA
NRF1	nuclear respiratory factor 1
PGC-1α	peroxisome proliferator-activated receptor-gamma coactivator
PINK1	phosphatase and tensin homolog-induced kinase 1
SPPB	short physical performance battery
TFAM	mitochondrial transcription factor A

documented in people with lower extremity ischemia. These alterations have been attributed, at least partly, to overproduction of reactive oxygen species by mitochondria following recurrent cycles of ischemia–reperfusion.^{10,11} However, the relationship

between mitochondrial abnormalities and walking impairment in people with PAD is unclear.^{8,9,12}

Mitochondrial quality control consists of coordinated processes including mitochondrial dynamics (mitochondrial fusion/fission), mitochondrial biogenesis, and mitophagy.¹³ These processes serve to maintain a well-functioning pool of mitochondria within the cell and protect against accumulation of mtDNA mutations and defective organelles.^{14–16} An alteration in any mitochondrial quality control pathways or the loss of their coordination might reduce efficiency of mitochondrial bioenergetics and, hence, tissue function. Whether markers of mitochondrial quality control are altered in muscle of people with PAD and an association of these markers with muscle dysfunction exists in PAD is currently unclear. In the present study, markers of mtDNA damage, mitochondrial biogenesis, including mtDNA copy number and the abundance of ETC complexes, and mitophagy were measured in gastrocnemius muscle biopsies from people with and without PAD. We hypothesized that people with PAD would show increased markers of mitochondrial biogenesis and diminished markers of mitophagy compared with those without PAD. These alterations were expected to be linked with increased levels of mtDNA damage owing to the combination of defective mitophagic removal of mitochondria carrying mutated genomes and their proliferation. We further hypothesized that greater abnormalities in mitochondrial quality control markers would be associated with greater walking impairment in people with PAD.

METHODS

The data that support the findings of this study are available from co-investigator M.M.M. (mdm608@northwestern.edu) upon reasonable request.

Study Participants

This study used gastrocnemius samples collected from people with and without PAD from research studies at Northwestern University (Chicago, IL). Each study had institutional review board approval at Northwestern University and all participants signed written informed consent. Participants were recruited using postcards mailed to people 50 and older living in the Chicago area, advertisements by the Chicago transit authority, letters mailed to patients with PAD at the Northwestern's medical center, a database of participants from prior studies of a co-investigator (M.M.M.), physician referral, newspaper advertisements, and friend referral. Some participants were recruited from among patients at the Jesse Brown Veterans Administration in Chicago. Muscle samples from 2 participants with PAD were matched by age,

sex, and race with a muscle sample from 1 participant without PAD.

Eligibility Criteria

Eligibility criteria were described in detail in prior publications.^{17–20} PAD was defined as ABI <0.90 in either leg.²⁰ PAD was defined as mild if ABI was 0.60 to 0.90 and moderate to severe if ABI was <0.60. Absence of PAD was defined as a resting ABI of 1.00 to 1.40. People with diabetes were excluded. Additional exclusion criteria were presence of foot ulcer or chronic limb threatening ischemia, below-knee or above-knee amputation, wheelchair confinement, use of a walking aid other than a cane, significant visual or hearing impairment, major surgery or lower extremity revascularization in the previous 3 months, and cognitive impairment as documented by a Mini-Mental State Examination²¹ score <23. A muscle biopsy from the medial head of the gastrocnemius was collected as described previously.¹²

General Characteristics, Anthropometry, and Ankle-Brachial Index Measurement

Information on age, sex, race or ethnicity, smoking habits, and medical history were collected through patient report.^{17–20} Body mass index was calculated as the ratio between body mass (kg) and the square of height (m²). The ABI was measured as previously described.²² Briefly, systolic pressures of right and left brachial, dorsalis pedis, and posterior tibial arteries were measured twice using a handheld Doppler probe (Nicolet Vascular Pocket Dop II, Nicolet Biomedical Inc., Golden, CO). The ABI was calculated in each leg as the ratio between the mean of the dorsal pedis and posterior tibial pressures and the mean of 4 brachial pressures.²² The mean systolic pressure in the arm with the higher pressure was used in cases when one brachial pressure was higher than the other side in both measurement sets and the 2 brachial pressures differed by ≥ 10 mmHg in 1 measurement set.²²

Assessment of Physical Performance

Six -Minute Walk

The 6-minute walking test was performed as detailed elsewhere.^{18,23,24} Briefly, participants were asked to walk up and down a 30-m hallway for 6 minute to cover as much distance as possible at their preferred walking speed. The total distance walked (m) was recorded.

Usual and Fast 4-Meter Gait Speed

Gait speed over 4 meters was measured at usual and fast pace.²⁵ For these tests, participants were asked to walk at their normal pace as if they were “walking down the street to go to the store,” and, separately,

to walk as fast as they could over 4 m. Each test was performed twice, and the fastest trial (m/s) was used for the analysis.

Short Physical Performance Battery

The short physical performance battery (SPPB) consists of 3 components: standing balance, gait speed at usual pace over 4 meters, and 5-repetition chair rise.²⁶ For the standing balance test, participants were asked to stand in 3 increasingly difficult positions for 10 seconds each: a side-by-side feet standing position, a semitandem position, and a full-tandem position. Gait speed was measured over a 4-m course at the participant’s usual pace. The faster of 2 trials (m/s) was used for test scoring. For the chair rise test, participants were asked to perform 5 repetitions of standing up and sitting down from a chair with arms folded across their chest as quickly as possible and the performance was timed. Each of the 3 SPPB subtasks was scored on a scale of 0 to 4, with 0 representing inability to do the test and 4 corresponding to the highest level of performance.²⁶ Scores were summed to obtain a total score ranging from 0 to 12, with higher scores indicating better physical performance.²⁶

Biochemical Analyses in Muscle Biopsies

Determination of Selected Protein Markers of Mitochondrial Quality Control by Immunodetection

Protein levels of markers of macroautophagy (LC3A/B [microtubule-associated protein 1A/1B-light chain 3] and p62), mitophagy (PINK1 [phosphatase and tensin homolog-induced kinase 1] and Parkin),²⁷ mitochondrial biogenesis (NRF1 [nuclear respiratory factor 1], PGC-1 α [peroxisome proliferator-activated receptor-gamma coactivator 1 alpha], and TFAM [mitochondrial transcription factor A]), and those of ETC complex I to V were measured in gastrocnemius muscle samples of all participants. The protein content of LC3A/B and ETC complexes I to V was quantified by Western blot, as described previously.²⁸ An automated capillary-based immunoassay was used to measure protein levels of NRF1, p62, Parkin, PGC-1 α , PINK1, and TFAM on a Jess system (ProteinSimple, San Jose, CA).

The protocol followed for the preparation of whole-tissue extracts and the quantification of target proteins as well as technical specifications of primary and secondary antibodies used are detailed in Data S1.

Quantification of mtDNA Copy Number and Damage

mtDNA copy number and damage (ie, mtDNA common deletion, strand breaks, and oxidized purines) were measured in gastrocnemius muscle samples by quantitative real-time polymerase chain reaction using SYBR

Green chemistry on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA), as previously described.¹² Strand breaks were assayed within the following mtDNA regions: ND1/2 (NADH dehydrogenase subunit 1/2), ND4/5 (NADH dehydrogenase subunit 4/5), COII ATPase 6/8 (cytochrome oxidase subunit II and ATPase subunit 6/8), cytochrome B6, and displacement loop. The abundance of mtDNA oxidized purines was quantified within ND4/5, cytochrome B6, and displacement loop regions. All reactions were run in triplicate. mtDNA copy number was calculated as the ratio between amplicons corresponding to ND1 and ND5 relative to nuclear glyceraldehyde 3-phosphate dehydrogenase, as previously described.¹² The abundance of the mtDNA common deletion (mtDNA⁴⁹⁷⁷), strand breaks, and oxidized purines was normalized to mtDNA copy number and differences among groups were calculated according to the Pfaffl mathematical model using the formula $R=2^{\Delta\Delta Ct}$.²⁹

Statistical Analysis

Characteristics of study participants are presented as means and SDs for continuous variables and counts and proportions for categorical variables. Descriptive characteristics of participants with moderate-to-severe PAD (ABI <0.60), mild PAD (ABI 0.60–0.90), and without PAD (ABI 1.0–1.40) were compared by 1-way ANOVA and χ^2 or Fisher's exact tests for continuous and categorical variables, respectively. Linear regressions were used to compare the distributions of markers of mitochondrial quality control, ETC complex abundance, and measures of mtDNA content and damage across participant groups. The independent variable in the model was an ordinal variable with 3 levels, coded as 1, 2 and 3, denoting the 3 ABI categories. Models were adjusted for age, sex, race or ethnicity, and smoking status. Pearson's correlation coefficients were calculated to estimate the associations between biochemical markers and measures of physical performance. Prior work showed differences in markers of mitochondrial biogenesis and bioenergetics or their association with physical performance in people with severe PAD (ie, ABI <0.60) relative to those with mild-to-moderate PAD (ie, ABI 0.60–0.90).^{3,8} Therefore, because ABI values were expected to alter the association between biochemical markers and physical performance measures, separate analyses were conducted for moderate to severe PAD, mild PAD, and non-PAD groups. The 95% CI of the correlation coefficients was estimated, and the null hypothesis of null correlation was tested. The estimated correlations were graphed as heat maps that were generated using R software (RStudio, PBC, Boston, MA). For all tests statistical significance was set at a 2-sided 0.05 level. All statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC).

RESULTS

Study Participants

A total of 67 participants, 15 with moderate to severe PAD, 29 with mild PAD, and 23 without PAD were included. Characteristics of participants according to the presence and severity of PAD are shown in Table 1. Overall mean age was 65.0±6.8 years, 16 (23.9%) were women, and 48 (71.6%) were Black. Age, sex distribution, race or ethnicity, body mass index, prevalence of comorbid conditions, 4-m gait speed at either usual or fast pace, and the SPPB score were not significantly different between participants with and without PAD regardless of disease severity. Participants with PAD, either mild or moderate to severe, had a higher prevalence of current or former smokers and walked a significantly shorter distance on the 6-minute walk test than those without PAD.

Selected Markers of Mitochondrial Quality Control and Damage

Markers of Macroautophagy and Mitophagy

After adjustment for potential confounders (ie, age, sex, race or ethnicity, and smoking status), lower ABI values were associated with a higher LC3A/B II-to-LC3A/B I ratio (P trend=0.017) and lower content of the autophagy receptor p62 (P trend=0.033; Table 2). Lower ABI values were also associated with lower abundance of the mitophagy marker PINK1 (P trend=0.017; Table 2). However, this association was no longer significant after adjustment for potential confounders (P trend=0.070). Protein levels of cleaved PINK1 and Parkin were not significantly different across ABI categories.

Markers of Mitochondrial Biogenesis

The mtDNA copy number and protein expression of markers of mitochondrial biogenesis (NRF1, PGC-1 α , TFAM) were not significantly different across ABI categories (Table 2). Lower ABI values were associated with a significantly higher abundance of all ETC complexes (all P trend values <0.05; Table 2).

mtDNA Damage

There were no statistically significant differences in the abundance of mtDNA⁴⁹⁷⁷ deletion, mtDNA strand breaks, or oxidized purines across ABI categories within any of the assayed regions (Table 3).

Relationship Between Markers of Mitochondrial Quality Control and Measures of Physical Performance

Results of correlation analyses between markers of mitochondrial quality control in gastrocnemius muscle

Table 1. Main Characteristics of Study Participants According to the Presence and Severity of Peripheral Artery Disease

Characteristic	Total	PAD		No PAD	P
	(n=67)	Moderate to severe (ABI <0.60; n=15)	Mild (ABI 0.60–0.90; n=29)	(n=23)	
Characteristic					
Age, y	65.0 (6.8)	64.9 (7.8)	65.4 (6.6)	64.6 (6.6)	0.903
Sex, female	16 (23.9%)	3 (20.0%)	7 (24.1%)	6 (26.1%)	0.911
Race, Black	48 (71.6%)	10 (66.7%)	23 (79.3%)	15 (65.2%)	0.475
Current or former smoking	59 (88.1%)	15 (100.0%)	28 (96.6%)	16 (69.6%)	0.004
Body mass index, kg/m ²	28.2 (5.4)	28.6 (4.6)	28.1 (5.7)	28.2 (5.8)	0.951
ABI	0.83 (0.26)	0.50 (0.08)	0.74 (0.08)	1.14 (0.08)	<0.001
Comorbidities					
Myocardial infarction	8 (12.1%)	2 (13.3%)	4 (13.8%)	2 (9.1%) [†]	0.897
Angina	6 (9.1%)	2 (13.3%)	3 (10.3%)	1 (4.5%) [†]	0.649
Congestive heart failure	5 (7.6%)	0 (0.0%)	4 (13.8%)	1 (4.5%) [†]	0.285
Cancer	9 (13.4%)	4 (26.7%)	3 (10.3%)	2 (8.7%)	0.306
Chronic obstructive pulmonary disease	12 (17.9%)	3 (20.0%)	5 (17.2%)	4 (17.4%)	1.000
Physical performance					
6-minute walk distance, m	367.0 (122.5)	302.7 (95.2)	366.4 (96.3)	409.8 (150.9)	0.029
4-m gait speed at usual pace, m/s	0.85 (0.22)	0.85 (0.17)	0.85 (0.19)*	0.84 (0.28)	0.959
4-m gait speed at fast pace, m/s	1.19 (0.32)	1.20 (0.22)	1.21 (0.28)*	1.15 (0.41)	0.774
Short physical performance battery summary score	9.8 (2.5)	10.7 (1.6)	9.7 (2.3)*	9.2 (3.1) [†]	0.222

Data are mean (SD) or n (%). ABI indicates ankle-brachial index; and PAD, peripheral artery disease.

*n=28.

[†]n=22.

samples and measures of physical performance in participants without PAD and in those with mild or moderate to severe PAD are shown in [Figure 1](#) and [Table S1](#).

In participants without PAD, the abundance of LC3A/B I was negatively associated with 6-minute walk distance ($r=-0.686$; $P<0.001$), 4-m gait speed at both usual ($r=-0.675$; $P<0.001$) and fast pace ($r=-0.575$; $P=0.004$), and the SPPB score ($r=-0.568$; $P=0.006$) ([Figure 1A](#)). A negative association was also observed between the abundance of LC3A/B II and 6-minute walk distance ($r=-0.438$; $P=0.037$) and 4-m gait speed at usual pace ($r=-0.484$; $P=0.019$). A positive association was found between protein levels of cleaved PINK1 and 4-m gait speed at fast pace ($r=0.419$; $P=0.047$). The content of NRF1 and PGC-1 α was negatively and significantly associated with 4-m gait speed at usual pace ($r=-0.525$; $P=0.010$ and $r=-0.451$; $P=0.031$, respectively). Finally, among people without PAD, the abundance of all ETC complexes was positively associated with 6-minute walk distance (complex I: $r=0.541$; complex II: $r=0.623$; complex III: $r=0.530$; complex IV: $r=0.557$; complex V: $r=0.597$; all P values <0.05) and 4-m gait speed at both usual (complex I: $r=0.477$; complex II: $r=0.532$; complex III: $r=0.452$; complex IV: $r=0.510$; complex V: $r=0.500$; all P values <0.05) and fast pace (complex

I: $r=0.628$; complex II: $r=0.640$; complex III: $r=0.554$; complex IV: $r=0.660$; complex V: $r=0.590$; all P values <0.05). The content of complex II was also positively associated with the SPPB score ($r=0.439$; $P=0.041$).

In participants with mild PAD, no significant associations were observed between the protein content of any of the assayed mitochondrial quality control markers and measures of physical performance ([Figure 1B](#)).

In participants with moderate to severe PAD, negative associations were observed between protein abundance of Parkin and PGC-1 α and 4-m gait speed at fast pace ($r=-0.574$; $P=0.025$ and $r=-0.553$; $P=0.033$, respectively); ([Figure 1C](#)). PGC-1 α was also negatively associated with 4-m gait speed at usual pace ($r=-0.646$; $P=0.009$). Positive associations were observed between protein levels of complex II ($r=0.543$; $P=0.037$), III ($r=0.693$; $P=0.004$), and V ($r=0.735$; $P=0.002$) and 4-m gait speed at fast pace. The content of complex III was also positively associated with the SPPB score ($r=0.586$; $P=0.022$).

DISCUSSION

Results from the present study indicate that most biomarkers of mitochondrial quality control were not significantly different across ABI categories in a cohort

Table 2. Protein Levels of Selected Markers of Macroautophagy, Mitophagy, and Mitochondrial Biogenesis, Including mtDNA Copy Number, in Gastrocnemius Muscle Biopsies According to the Presence and Severity of Peripheral Artery Disease

	PAD		No PAD	P trend	
	Moderate to severe (ABI <0.60; n=15)	Mild (ABI 0.60–0.90; n=29)	(n=23)	Unadjusted	Adjusted*
Macroautophagy					
LC3A/B I, cytosolic	0.60 (0.19)	0.53 (0.17)	0.62 (0.25)	0.598	0.590
LC3A/B II, membrane-bound	1.49 (0.56)	1.24 (0.53)	1.32 (0.60)	0.450	0.261
LC3 II/LC3 I	2.54 (0.82)	2.31 (0.61)	2.15 (0.60)	0.079	0.017
p62	0.71 (0.18)	0.69 (0.19)	0.80 (0.23)	0.122	0.033
Mitophagy					
PINK1	0.62 (0.14)	0.79 (0.30)	0.92 (0.52)	0.017	0.070
Cleaved PINK1	0.24 (0.11)	0.21 (0.08)	0.23 (0.09)	0.770	0.566
Parkin	1.18 (0.42)	1.17 (0.49)	1.18 (0.39)	0.990	0.468
Mitochondrial biogenesis					
mtDNA copy number, log-transformed	6.54 (0.43)	6.44 (0.39)	6.37 (0.48)	0.222	0.187
Nuclear respiratory factor 1	0.23 (0.07)	0.26 (0.10)	0.27 (0.14)	0.270	0.287
Peroxisome proliferator-activated receptor gamma coactivator 1 alpha	0.51 (0.26)	0.40 (0.16)	0.49 (0.25)	0.931	0.639
Mitochondrial transcription factor A	0.22 (0.17)	0.21 (0.13)	0.17 (0.11)	0.241	0.113
Complex I	0.66 (0.23)	0.45 (0.23)	0.48 (0.29)	0.085	0.043
Complex II	0.88 (0.24)	0.70 (0.17)	0.72 (0.25)	0.061	0.032
Complex III	0.73 (0.28)	0.49 (0.20)	0.53 (0.25)	0.031	0.022
Complex IV	1.18 (0.43)	0.74 (0.36)	0.84 (0.39)	0.034	0.017
Complex V	0.70 (0.15)	0.57 (0.10)	0.59 (0.13)	0.015	0.018

Data are mean (SD). Spot density values are normalized to the amount of protein loaded except for LC3 II/LC3 I, and are expressed as arbitrary units. ABI indicates ankle brachial index; LC3B, microtubule-associated protein 1A/1B-light chain 3 nonlipidated (I) and lipidated (II) forms; PINK1, phosphatase and tensin homolog-induced kinase 1.

*Adjusted for age, sex, race or ethnicity, and smoking status.

of 67 people with and without PAD. However, lower ABI values were associated with a higher abundance of all 5 ETC complexes. Protein levels of mitochondrial biogenesis transcription factors (NRF1, PGC-1 α , and TFAM) and markers of mitophagy (PINK1, cleaved PINK1, and Parkin) did not significantly differ by ABI. In contrast, lower ABI values were associated with a higher LC3A/B II-to-LC3A/B I ratio and lower content of the autophagy receptor p62, suggesting upregulation of macroautophagy in the setting of ischemia.

The removal of damaged mitochondria and their replenishment with newly synthesized organelles are accomplished via the coordination of mitophagy and mitochondrial biogenesis to maintain a functional pool of mitochondria and meeting cell's energy demands.³⁰ Intracellular signals and environmental factors (eg, nutrient availability, growth factors and hormones, toxins, oxygen fluctuations) operate a spatiotemporal control over mitochondrial biogenesis and mitophagy. Under normal conditions, mitochondrial biogenesis signaling stimulates mitophagy via Parkin, an E3 ubiquitin ligase

that triggers the tagging and clearance of damaged or unnecessary mitochondria.^{31,32} Although higher protein levels of the 5 ETC complexes were associated with lower ABI values, upregulation of mitochondrial biogenesis transcription factors was not observed. The canonical pathway of mitochondrial biogenesis is controlled by the transcriptional regulators PGC-1 α and NRFs.³³ Their activation stimulates the expression of nuclear genes encoding mitochondrial transcription factors, including TFAM.³³ Upon import into mitochondria, TFAM triggers mtDNA transcription and replication. Mitochondria can also upregulate the synthesis of ETC complexes independent of PGC-1 α and NRFs by enhancing the binding of TFAM to mtDNA.³⁴ For instance, mice lacking PGC-1 α retain the ability to upregulate mitochondrial biogenesis in response to wheel-running exercise.³⁵ In the absence of PGC-1 α upregulation, the expression of nuclear encoded ETC subunits may be sustained through alternative signaling pathways involving, for instance, other PGC-1 isoforms (eg, PGC-1 β).³⁶ These observations might explain the greater abundance of ETC complexes

Table 3. Relative Abundance of mtDNA⁴⁹⁷⁷ Deletion, Strand Breaks, and Oxidized Purines in Gastrocnemius Muscle Biopsies According to the Presence and Severity of Peripheral Artery Disease

	PAD		No PAD (n=23)	P trend	
	Moderate to severe (ABI <0.60; n=15)	Mild (ABI 0.60–0.90; n=29)		Unadjusted	Adjusted*
mtDNA ⁴⁹⁷⁷ deletion	1.04 (0.42–7.67)	1.96 (0.56–6.16)	1.20 (0.60–6.03)	0.872	0.7961
Strand breaks					
ND1/2	1.94 (1.49–2.82)	3.12 (2.09–5.32)	2.66 (1.52–4.77)	0.397	0.1798
ND4/5	2.37 (1.88–3.99)	3.40 (2.40–5.47)	3.34 (2.59–5.27)	0.164	0.1042
Cytochrome oxidase subunit II and ATPase subunit 6/8	2.04 (1.79–3.96)	2.88 (2.05–5.14)	2.60 (1.83–4.41)	0.677	0.2374
CytB6	1.83 (0.79–3.17)	2.09 (1.26–4.81)	2.06 (1.11–4.83)	0.406	0.3728
Displacement loop	0.58 (0.45–1.98)	1.95 (0.61–3.99)	0.92 (0.42–2.13)	0.766	0.4531
Oxidized purines					
ND4/5	2.03 (1.58–3.09)	3.61 (1.77–8.40)	4.24 (1.78–5.79)	0.122	0.1194
CytB6	2.84 (0.95–4.28)	4.22 (2.19–30.31)	5.53 (2.29–10.12)	0.184	0.1380
Displacement loop	2.30 (1.17–3.51)	3.35 (2.18–5.86)	4.16 (1.94–6.55)	0.136	0.1496

Data are median (interquartile range). Values are expressed as arbitrary units. ABI indicates ankle brachial index; CytB6, cytochrome B6; ND1/2, NADH dehydrogenase subunit 1/2; and ND4/5, NADH dehydrogenase subunit 4/5.
*Adjusted for age, sex, race or ethnicity, and smoking status.

observed in muscles of participants with moderate to severe PAD without concomitant increases in the expression of PGC-1 α , NRF1, or TFAM. Alternatively, the

accumulation of ETC complexes in participants with moderate-to-severe PAD may have resulted from defective mitochondrial disposal by mitophagy. In either case,

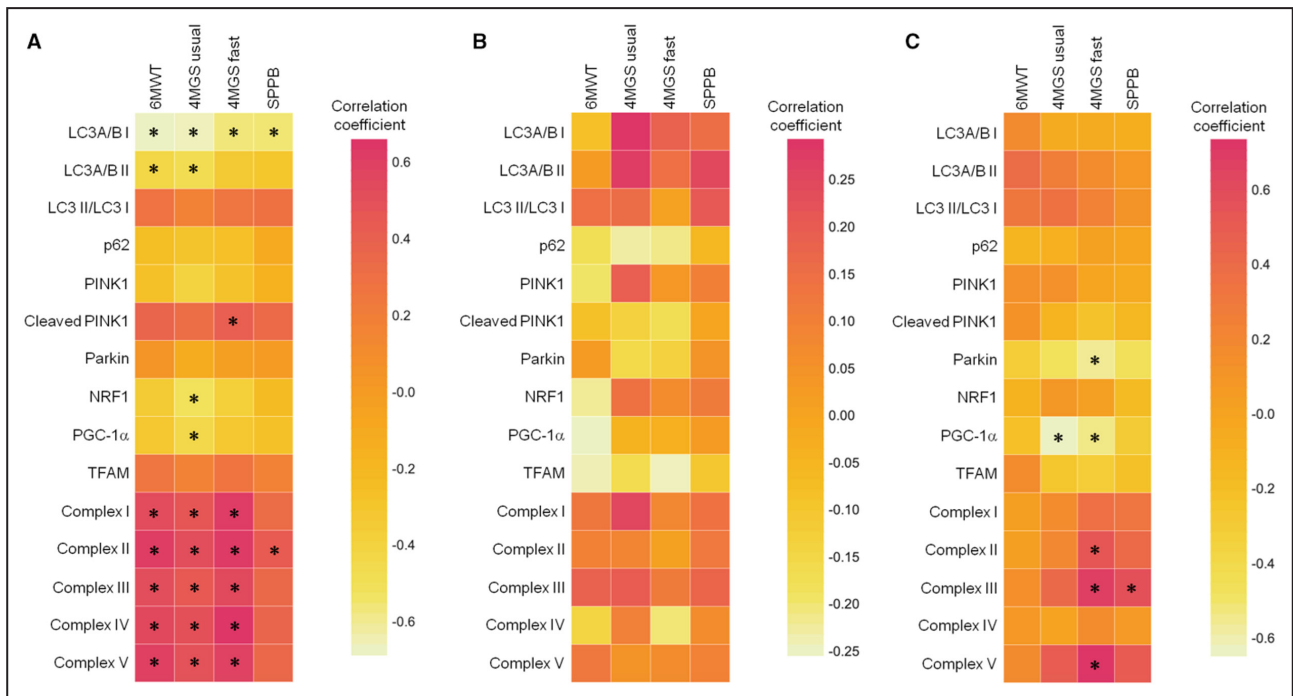


Figure Heat maps of correlation between markers of mitochondrial quality control in gastrocnemius muscle samples and measures of physical performance in participants with and without peripheral artery disease.

A, Participants without peripheral artery disease (PAD); **B**, participants with mild PAD (ankle-brachial index, ABI 0.60–0.90); **C**, participants with moderate to severe PAD (ABI <0.60). *P<0.05. 4MGS fast indicates 4-m gait speed at fast pace; 4MGS usual, 4-m gait speed at usual pace; 6MWT, 6-minute walking test; LC3B, microtubule-associated protein 1A/1B-light chain 3 nonlipidated (I), and lipidated (II) forms; NRF1, nuclear respiratory factor 1; PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PINK1, phosphatase and tensin homolog-induced kinase 1; SPPB, short physical performance battery; and TFAM, mitochondrial transcription factor A.

mitochondrial activity becomes impaired if the extent of damage exceeds the ability to remove dysfunctional mitochondria and generate high-quality organelles.^{6,37} In this scenario, the increase in the LC3A/B II-to-LC3A/B I ratio and the lower protein content of p62 in participants with lower ABI values might be indicative of a compensatory upregulation of macroautophagy in the setting of ischemia.²⁷ However, the accumulation of ETC complexes in the absence of upregulation of mitochondrial biogenesis markers suggests that mitophagy is not stimulated in those with lower ABI values. This view is consistent with the hypothesis of defective mitochondrial removal in PAD and is also in line with previous data showing altered mitophagy signaling in the gastrocnemius muscle of people with PAD.⁴ In this prior work, cavities devoid of mitochondria and with lack of colocalization of LC3 with lysosome-associated membrane protein 2 were identified in approximately 17% of type I muscle fibers of people with PAD.⁴ The main function of mitophagy is to remove dysfunctional, depolarized mitochondria. The loss of mitochondrial membrane potential is induced by a variety of insults, including accumulation of mutated, deleted, or otherwise damaged mtDNA.³⁸ Unexpectedly, the abundance of mtDNA⁴⁹⁷⁷ deletion, mtDNA strand breaks, and oxidized purines was not increased in participants with lower ABI values. The possibility cannot be ruled out that, in our sample, the lack of upregulation of mitophagy might have been compensated by overactivation of mtDNA repair systems. However, the expression or the activity of mtDNA repair enzymes was not measured in this study.

Taken as a whole, data from this study suggest that lower extremity ischemia may induce accumulation of ETC complexes, possibly as a consequence of lack of mitophagy upregulation, as an attempt to sustain mitochondrial bioenergetics in the setting of reduced blood supply. In this scenario, diminished muscle perfusion, rather than accumulation of mitochondrial damage, may be the major factor limiting walking performance. This hypothesis is supported by the lack of an association between the content of any ETC complexes in muscle and the distance walked during 6 minutes in participants with moderate to severe PAD, in spite of higher levels of all 5 complexes relative to both those with mild PAD and participants without PAD (Figure 1, Table 2). This finding is in agreement with the negative association between mtDNA abundance (a proxy for mitochondrial mass) in gastrocnemius muscle and 6-minute walk distance in people with PAD, as described in a prior study.¹²

Limitations

This study has limitations. First, the study was cross-sectional and no causal inferences can be made. This study did not include data from preclinical models that tested specific mechanistic hypotheses. The generation

of such models is warranted to gain mechanistic information on the relationship between mitochondrial quality control and physical performance in PAD and inform the design of future human studies. Second, available muscle specimens were frozen, which did not allow mitochondrial functional assessments, measurement of mitophagy flux, or mitochondrial imaging to be conducted. Third, no markers of mitochondrial dynamics were measured, which precluded obtaining a more comprehensive appraisal of mitochondrial quality control. Fourth, the sample size was relatively small and included multiple comparisons. Some of the statistically significant findings may have been owing to chance. Findings reported here should be replicated in a population with a larger sample size. Fifth, comparisons that did not reach statistical significance may have lacked statistical power.

CONCLUSIONS

In gastrocnemius muscle of people with and without PAD, lower ABI values were associated with greater abundance of ETC complexes and upregulation of macroautophagy. No significant differences in the abundance of mitochondrial biogenesis transcription factors or mitophagy markers were detected across ABI categories. These findings suggest that lower extremity ischemia may interfere with the proper activation of mitophagy, which might result in accumulation of aged mitochondrial proteins. However, the study was cross-sectional and descriptive, and additional research is needed to replicate these findings in a larger sample size. Further study is also needed to determine whether restoring leg blood flow improves muscle mitochondrial turnover in people with PAD.

ARTICLE INFORMATION

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Supplemental Material

Data S1

Table S1

Reference [39,40]

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SUPPLEMENTAL MATERIAL

Data S1. Supplemental Methods

Preparation of muscle tissue extracts and immunodetection of selected markers of mitochondrial quality control

Whole-tissue extracts were prepared from snap-frozen gastrocnemius muscle samples using an extraction buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 1% Triton X-100, 2% glycerol, 50 mM β -glycerophosphate, 1 \times Halt-protease, and phosphatase inhibitor cocktail). Briefly, 20-30 mg of muscle tissue were immersed in a pre-cooled vial containing zirconium beads (\varnothing 3 mm) and extraction buffer (1:20 w/v), placed in a BeadBug homogenizer (Benchmark Scientific, Sayreville, NJ), and homogenized 5 times at setting 4,000 for 30 s each, with intermittent 1-min cooling on ice. Afterwards, the cleared homogenate was sonicated 10 times for \sim 3 s each, followed by centrifugation at 10,000 $\times g$ for 10 min at 4 $^{\circ}C$. The resulting supernatant was collected and total protein content was determined via the Bradford colorimetric assay. Samples were subsequently diluted in Laemmli sample buffer (Bio-Rad, Hercules, CA) supplemented with β -mercaptoethanol (Bio-Rad) for Western blot, or sample buffer and Fluorescent Master Mix (both from ProteinSimple, San Jose, CA) for the Jess system (ProteinSimple), and proteins were denatured at 95 $^{\circ}C$ for 5 min. For Western blot immunodetection, 50 μg of protein were loaded onto a 4–20% Criterion TGX Precast Midi Protein Gel (Bio-Rad), and separated in a Bio-Rad running buffer. Separated proteins were transferred onto a polyvinylidene fluoride or a nitrocellulose membrane (0.2 μm ; Bio-Rad) using a Bio-Rad Trans-Blot Turbo Transfer System and Transfer buffer. The membranes were reversibly stained with Ponceau S (MilliporeSigma, Burlington, MA), imaged with a Bio-Rad ChemiDoc XRS+ imager to visualize total protein, and subsequently blocked for 60 min at room temperature with 5% Blocking-grade Blocker (Bio-Rad) or 5% bovine serum albumin (MilliporeSigma) in Tris-buffered saline (Bio-Rad) with 0.05% tween-

20 (Bio-Rad). Sample protein concentrations of 0.25 and 0.75 $\mu\text{g}/\mu\text{L}$ were loaded on the Jess separation module (12 to 230 kDa; ProteinSimple). The following commercially available primary antibodies and dilutions were used: microtubule-associated protein 1A/1B-light chain 3 (LC3A/B, Cell Signaling, Danvers, MA, #4108; 1:500), p62 (MilliporeSigma, #P0067; 1:600), phosphatase and tensin homolog-induced kinase 1 (PINK1, Novus Biologicals, Centennial, CO, #NB100-644SS; 1:50), Parkin (Abcam, Cambridge, MA, #ab77924; 1:100), nuclear respiratory factor 1 (NRF1, Santa Cruz Biotechnologies, Dallas, TX, #sc-101102; 1:50), PGC-1 α (MilliporeSigma, #516557; 1:25), mitochondrial transcription factor A (TFAM, Novus Biologicals #NBP1-71648SS; 1:400), and ETC complexes I to V (Total OXPHOS antibody cocktail, Abcam #ab110411; 1:500). Secondary antibodies applied in the Jess system were horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG from ProteinSimple. HRP-conjugated secondary antibodies for traditional Western Blot immunodetection were purchased from Cell Signaling (anti-rabbit IgG #7074, 1:5,000; anti-mouse IgG #7076, 1:5,000). All other reagents and supplies used for the automated procedures on the Jess system were purchased from ProteinSimple or from Bio-Rad for traditional Western blots. The optimal dilution of each antibody was determined against a calibration curve of serial homogenate dilutions to ensure linearity of signal quantification. The quantification of electropherograms for the Jess system was accomplished with Compass for SW software (v4.1.0; ProteinSimple) using a Gaussian peak fit or dropped lines distribution for area under the curve. The peak area for each capillary was normalized to total protein using the system's Protein Normalization capability and reagents for the Jess system. For Western blot, protein content was quantified using the Image Lab 6.0 software (Bio-Rad) based on the spot density of target bands acquired with a ChemiDoc XRS+ imager (Bio-Rad). The spot density of target bands was normalized to the amount of protein loaded in each lane as determined by densitometric analysis of the corresponding Ponceau S-stained

membranes.^{39,40}

Table S1. Correlation analysis between markers of mitochondrial quality control in gastrocnemius muscle samples and measures of physical performance in participants with and without peripheral artery disease according to disease severity

	Moderate to severe PAD (ABI <0.60)				Mild PAD (ABI 0.60–0.90)				No PAD			
	6MWT (n=15)	4MGS usual (n=15)	4MGS fast (n=15)	SPPB score (n=15)	6MWT (n=29)	4MGS usual (n=28)	4MGS fast (n=28)	SPPB score (n=28)	6MWT (n=23)	4MGS usual (n=23)	4MGS fast (n=23)	SPPB score (n=22)
LC3A/B I	0.188 <i>P</i> =0.502	-0.057 <i>P</i> =0.841	-0.056 <i>P</i> =0.843	-0.076 <i>P</i> =0.787	-0.086 <i>P</i> =0.659	0.293 <i>P</i> =0.130	0.190 <i>P</i> =0.333	0.156 <i>P</i> =0.427	-0.686 <i>P</i> <0.001	-0.675 <i>P</i> <0.001	-0.575 <i>P</i> =0.004	-0.568 <i>P</i> =0.006
LC3A/B II	0.402 <i>P</i> =0.138	0.272 <i>P</i> =0.326	0.172 <i>P</i> =0.539	0.081 <i>P</i> =0.773	0.024 <i>P</i> =0.902	0.278 <i>P</i> =0.153	0.147 <i>P</i> =0.456	0.252 <i>P</i> =0.196	-0.438 <i>P</i> =0.037	-0.484 <i>P</i> =0.019	-0.340 <i>P</i> =0.112	-0.309 <i>P</i> =0.161
LC3 II/LC3 I	0.325 <i>P</i> =0.238	0.354 <i>P</i> =0.195	0.239 <i>P</i> =0.391	0.114 <i>P</i> =0.687	0.151 <i>P</i> =0.434	0.159 <i>P</i> =0.418	0.0045 <i>P</i> =0.982	0.214 <i>P</i> =0.275	0.286 <i>P</i> =0.187	0.179 <i>P</i> =0.415	0.268 <i>P</i> =0.216	0.289 <i>P</i> =0.192
p62	-0.133 <i>P</i> =0.637	-0.092 <i>P</i> =0.745	0.017 <i>P</i> =0.953	-0.018 <i>P</i> =0.949	-0.179 <i>P</i> =0.352	-0.235 <i>P</i> =0.229	-0.218 <i>P</i> =0.265	-0.058 <i>P</i> =0.769	-0.254 <i>P</i> =0.242	-0.297 <i>P</i> =0.168	-0.270 <i>P</i> =0.214	-0.108 <i>P</i> =0.631
PINK1	0.131 <i>P</i> =0.643	0.133 <i>P</i> =0.637	-0.017 <i>P</i> =0.953	-0.050 <i>P</i> =0.860	-0.199 <i>P</i> =0.300	0.196 <i>P</i> =0.316	0.035 <i>P</i> =0.860	0.103 <i>P</i> =0.602	-0.277 <i>P</i> =0.200	-0.410 <i>P</i> =0.052	-0.284 <i>P</i> =0.190	-0.156 <i>P</i> =0.487
PINK1-cleaved	0.118 <i>P</i> =0.675	-0.123 <i>P</i> =0.662	-0.235 <i>P</i> =0.400	-0.150 <i>P</i> =0.594	-0.089 <i>P</i> =0.647	-0.138 <i>P</i> =0.485	-0.177 <i>P</i> =0.369	-0.0045 <i>P</i> =0.982	0.377 <i>P</i> =0.076	0.321 <i>P</i> =0.135	0.419 <i>P</i> =0.047	0.344 <i>P</i> =0.116

Parkin	-0.320 <i>P</i> =0.245	-0.485 <i>P</i> =0.067	-0.574 <i>P</i> =0.025	-0.474 <i>P</i> =0.074	0.023 <i>P</i> =0.906	-0.160 <i>P</i> =0.415	-0.138 <i>P</i> =0.482	0.048 <i>P</i> =0.808	0.049 <i>P</i> =0.826	-0.121 <i>P</i> =0.581	-0.027 <i>P</i> =0.903	0.001 <i>P</i> =0.998
NRF1	-0.113 <i>P</i> =0.689	0.092 <i>P</i> =0.746	0.024 <i>P</i> =0.932	-0.182 <i>P</i> =0.516	-0.227 <i>P</i> =0.236	0.143 <i>P</i> =0.469	0.066 <i>P</i> =0.738	0.118 <i>P</i> =0.551	-0.351 <i>P</i> =0.101	-0.525 <i>P</i> =0.010	-0.387 <i>P</i> =0.068	-0.235 <i>P</i> =0.293
PGC-1α	-0.219 <i>P</i> =0.432	-0.646 <i>P</i> =0.009	-0.553 <i>P</i> =0.033	-0.309 <i>P</i> =0.262	-0.254 <i>P</i> =0.184	-0.045 <i>P</i> =0.818	-0.034 <i>P</i> =0.866	0.026 <i>P</i> =0.895	-0.323 <i>P</i> =0.133	-0.451 <i>P</i> =0.031	-0.318 <i>P</i> =0.139	-0.277 <i>P</i> =0.211
TFAM	0.169 <i>P</i> =0.548	-0.257 <i>P</i> =0.355	-0.326 <i>P</i> =0.236	-0.232 <i>P</i> =0.406	-0.245 <i>P</i> =0.201	-0.167 <i>P</i> =0.397	-0.250 <i>P</i> =0.200	-0.102 <i>P</i> =0.604	0.264 <i>P</i> =0.223	0.175 <i>P</i> =0.425	0.272 <i>P</i> =0.209	0.178 <i>P</i> =0.429
Complex I	0.032 <i>P</i> =0.911	0.161 <i>P</i> =0.566	0.371 <i>P</i> =0.173	0.331 <i>P</i> =0.228	0.131 <i>P</i> =0.499	0.258 <i>P</i> =0.184	0.079 <i>P</i> =0.688	0.142 <i>P</i> =0.470	0.541 <i>P</i> =0.008	0.477 <i>P</i> =0.021	0.628 <i>P</i> =0.001	0.337 <i>P</i> =0.125
Complex II	0.042 <i>P</i> =0.882	0.198 <i>P</i> =0.479	0.543 <i>P</i> =0.037	0.411 <i>P</i> =0.128	0.101 <i>P</i> =0.603	0.088 <i>P</i> =0.658	0.009 <i>P</i> =0.963	0.125 <i>P</i> =0.526	0.623 <i>P</i> =0.002	0.532 <i>P</i> =0.009	0.640 <i>P</i> =0.001	0.439 <i>P</i> =0.041
Complex III	0.148 <i>P</i> =0.600	0.418 <i>P</i> =0.121	0.693 <i>P</i> =0.004	0.586 <i>P</i> =0.022	0.188 <i>P</i> =0.330	0.200 <i>P</i> =0.307	0.118 <i>P</i> =0.549	0.176 <i>P</i> =0.369	0.530 <i>P</i> =0.009	0.452 <i>P</i> =0.030	0.554 <i>P</i> =0.006	0.352 <i>P</i> =0.108
Complex IV	0.078 <i>P</i> =0.781	0.0001 <i>P</i> =1.000	0.160 <i>P</i> =0.570	0.062 <i>P</i> =0.827	-0.147 <i>P</i> =0.448	0.105 <i>P</i> =0.596	-0.207 <i>P</i> =0.291	0.068 <i>P</i> =0.730	0.557 <i>P</i> =0.006	0.510 <i>P</i> =0.013	0.660 <i>P</i> =0.001	0.379 <i>P</i> =0.082
Complex V	0.178 <i>P</i> =0.525	0.502 <i>P</i> =0.057	0.735 <i>P</i> =0.002	0.509 <i>P</i> =0.053	0.126 <i>P</i> =0.515	0.047 <i>P</i> =0.813	0.068 <i>P</i> =0.731	0.097 <i>P</i> =0.624	0.597 <i>P</i> =0.003	0.500 <i>P</i> =0.015	0.590 <i>P</i> =0.003	0.364 <i>P</i> =0.096

For each variable pair, the Pearson's correlation coefficient and the corresponding P value are shown. 4MGS fast indicates 4-m gait speed at fast pace; 4MGS usual, 4-m gait speed at usual pace; 6MWT, 6-min walking test; ABI, ankle-brachial index; LC3B, microtubule-associated protein 1A/1B-light chain 3 non-lipidated (I), and lipidated (II) forms; NRF1, nuclear respiratory factor 1; PAD, peripheral artery disease; PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PINK1, phosphatase and tensin homolog-induced kinase 1; SPPB, short physical performance battery; and TFAM, mitochondrial transcription factor A.