Morphometric analysis of gastrocnemius muscle biopsies from patients with peripheral arterial disease: objective grading of muscle degeneration

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1Bioengineering, Wichita State University, Wichita, Kansas; 2Department of Surgery University of Nebraska Medical Center Division of General Surgery, Nebraska Medical Center, Omaha, Nebraska; 3Biological Systems Engineering, University of Nebraska, Lincoln, Nebraska; 4Computer Science and Engineering, University of Nebraska, Lincoln, Nebraska; and 5Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha Nebraska

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Cluff K, Miserlis D, Naganathan GK, Pipinos II, Koutakis P, Samal A, McComb RD, Subbiah J, Casale GP. Morphometric analysis of gastrocnemius muscle biopsies from patients with peripheral arterial disease: objective grading of muscle degeneration. Am J Physiol Regul Integr Comp Physiol 305: R291–R299, 2013. First published May 29, 2013; doi:10.1152/ajpregu.00525.2012.—Peripheral arterial disease (PAD), which affects ~10 million Americans, is characterized by atherosclerosis of the noncoronary arteries. PAD produces a progressive accumulation of ischemic injury to the legs, manifested as a gradual degradation of gastrocnemius histology. In this study, we evaluated the hypothesis that quantitative morphological parameters of gastrocnemius myofibers change in a consistent manner during the progression of PAD, provide an objective grading of muscle degeneration in the ischemic limb, and correlate to a clinical stage of PAD. Biopsies were collected with a Bergström needle from PAD patients with claudication (n = 18) and critical limb ischemia (CLI; n = 19) and control patients (n = 19). Myofiber sarcolemmas and myosin heavy chains were labeled for fluorescence detection and quantitative analysis of morphometric variables, including area, roundness, perimeter, equivalent diameter, major and minor axes, solidity, and fiber density. The muscle specimens were separated into training and validation data sets for development of a discriminant model for categorizing muscle samples on the basis of disease severity. The parameters for this model included standard deviation of roundness, standard deviation of solidity of myofibers, and fiber density. For the validation data set, the discriminant model accurately identified control (80.0% accuracy), claudication (77.7% accuracy), and CLI (88.8% accuracy) patients, with an overall classification accuracy of 82.1%. Myofiber morphometry provided a discriminant model that establishes a correlation between PAD progression and advancing muscle degeneration. This model effectively separated PAD and control patients and provided a grading of muscle degeneration within clinical stages of PAD.

myofiber morphology; fluorescence microscopy; imaging; linear discriminant analysis

PERIPHERAL ARTERIAL DISEASE (PAD) is a vascular disease characterized by stenosis of the noncoronary arteries caused by fatty deposits and plaque buildup (11). Although PAD affects a large range of arterial beds, most occlusions occur in the arteries of the legs, pelvis, and abdominal aorta causing reduced blood flow to the lower extremities (11). PAD is a slowly progressive disease that develops over many years. It is estimated that PAD affects ~8–10 million Americans (10, 19). The disease becomes more common as one gets older, and the number of affected Americans is expected to increase as the population ages (1, 6).

Intermittent claudication, walking impairment induced by simple exercise, is an early manifestation of PAD (Fontaine stage 2) (8, 19) and an indicator of the damage to leg muscles (20, 22). More advanced disease is characterized by pain, while at rest (Fontaine stage 3) and nonhealing foot ulcers and gangrene (Fontaine stage 4). The pathophysiology of PAD is complex and involves changes occurring on multiple levels, such as reduced blood flow, altered metabolic processes, oxidative damage, and degeneration of nerves and muscle (23). Currently, PAD is diagnosed primarily on the basis of patient complaints about pain, nonhealing wounds in the legs, and reduced limb hemodynamics, such as the Ankle/Brachial Index (ABI; the ratio of systolic pressure in the ankle to that in the arm). Stenoses and occlusions of the arteries supplying the legs produce low ABIs, the hemodynamic hallmark of PAD.

PAD produces a progressive accumulation of ischemic injury to muscles, nerves, blood vessels, skin, and subcutaneous tissues of the leg (3, 20, 21). At the level of the skeletal muscle, this ischemic injury is manifested as a gradual and characteristic degeneration of muscle histology (9, 15, 17, 22). We hypothesize that quantitative morphometric parameters of gastrocnemius myofibers change in a consistent manner during the progression of PAD, provide an objective grading of muscle degeneration in the ischemic limb, and correlate to the clinical stage of PAD. Thus, the specific objectives of this study are 1) to quantify morphological parameters of myofibers in gastrocnemius biopsies, 2) to develop and validate a discriminant model using morphological parameters that classify muscles as control, claudicating, and CLI, and 3) to grade the disease particularly in its early stage (Fontaine stage 2) on the basis of morphological measurements.

MATERIALS AND METHODS

Tissue sample collection. The experimental protocol was approved by the Institutional Review Board of the VA Nebraska and Western Iowa Medical Center, and all subjects gave informed consent. We recruited 18 patients with clinical diagnosis of claudication, 19 patients with clinical diagnosis of critical limb ischemia (CLI), and 19 controls who did not have PAD (Table 1). Patients recruited in this study came from a patient population treated at the VA hospital in Omaha, Nebraska. Patient demographics are given in Table 1. Muscle samples obtained from controls, claudicating, and CLI patients are designated as control, claudicating, and CLI, respectively throughout...
Table 1. Demographics of patients with peripheral arterial disease and control patients

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Claudication</th>
<th>CLI</th>
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<tbody>
<tr>
<td>Number of subjects</td>
<td>19</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>Mean age, yr</td>
<td>64.6 ± 2.6</td>
<td>60.2 ± 1.9</td>
<td>64.0 ± 2.1</td>
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<tr>
<td>Sex (male/female)</td>
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<td>18/1</td>
</tr>
<tr>
<td>Smoking</td>
<td>41.2%</td>
<td>64.7%</td>
<td>88.2%</td>
</tr>
<tr>
<td>Obesity</td>
<td>29.4%</td>
<td>23.5%</td>
<td>10.5%</td>
</tr>
<tr>
<td>Hypertension</td>
<td>64.7%</td>
<td>88.2%</td>
<td>78.9%</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
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<td>29.4%</td>
<td>57.9%</td>
</tr>
<tr>
<td>ABI</td>
<td>1.08 ± 0.02</td>
<td>0.35 ± 0.06</td>
<td>0.27 ± 0.05</td>
</tr>
</tbody>
</table>

CLL, critical limb ischemia; ABI, ankle brachial index.

the article. Patients presenting with rest pain or tissue loss were grouped into the CLI category. Biopsies of the gastrocnemius were acquired with a Bergström needle, fixed in methacarn and embedded in paraffin. Two 4-μm cross sections, taken ~40 μm apart, were mounted on glass slides for fluorescence labeling.

Fluorescence labeling of tissue sections. In preparation for labeling sarcolemma and myosin types I and II, the slide specimens were deparaffinized with xylene and rehydrated via a series of ethanol washes and distilled water. The rehydrated slide specimens were heated at 94°C in Tris buffer (pH 9.0) for 30 min and then allowed to cool for 20 min. The slide specimens were soaked in SuperSensitive Wash buffer (SS; Biogenex Laboratories, Fremont, CA) for 30 min at room temperature, with stirring. A pap pen was used to apply a lipid barrier on dried areas around the tissue specimens. While applying the lipid barrier, care was taken to ensure that the tissue specimens were kept moist.

A programmable autostainer (BioGenex i6000; BioGenex Laboratories, Fremont, CA) was used to label sarcolemma and myosin heavy chains. The slide specimens were washed 10 times with SS buffer (4 min per wash) and then blocked with 10% goat serum for 20 min. Subsequently, the slides were treated with a mixture of anti-type I myosin (Sigma-Aldrich, St. Louis, MO; M8421, clone NOQ7.5.4D) and anti-type II myosin (Sigma-Aldrich; M4276, clone MY-32) antibodies, for 1 h. The slide specimens were washed 2 times with SS buffer (4 min per wash) and then treated for 1 h with a mixture of wheat germ agglutinin-Alexa Fluor 350 to label the sarcolemma (Life Technologies-Molecular Probes, Fremont, CA) and goat anti-mouse IgG-Alexa Fluor 488 (Life Technologies-Molecular Probes; A11004). Treated slides were washed three times with SS buffer. Finally, ProLong Gold antifade medium (Life Technologies-Molecular Probes; P36930) was applied to the labeled specimens and covered with a coverslip. The slides were stored at room temperature for 24 h and then kept at 5°C for subsequent fluorescence imaging.

Fluorescence image acquisition. Gray-scale (12-bit) images (1,344 × 1,044 pixels) of gastrocnemius myofibers were acquired with a CCD camera (Orca ER C4742-95; Hamamatsu Photonics, Bridgewater, NJ), a Leica microscope (DMRXA2; North Central Instruments, Plymouth, MN) (10× objective; 0.5128 μm/pixel), and Image-Pro Plus software. Excitation energy was delivered with a 150 W HgXe lamp (Hamamatsu Photonics).

Fluorescence images were collected from a total of five different locations (frames) on each of two slides per patient. At each location, images were collected in fluorescence channels corresponding to each fluorophore: 1) excitation at 350 nm and emission at 460 nm (Alexa Fluor 350) and 2) excitation 480 nm and emission at 535 nm (Alexa Fluor 488). The images of the sarcolemma (Fig. 1A) were collected in the 460-nm channel, and the images of myosin (Fig. 1B) were collected in the 535-nm channel. In total, 20 images were collected for each patient, i.e., 10 images of the myosin signal and 10 images of the corresponding sarcolemma signal.

Image processing. To extract morphometric parameters for each myofiber, the individual myofibers were isolated by segmentation of the fluorescence image. Image segmentation was done with a custom algorithm written in MatLab (R2009a; Mathworks, Natick, MA), using thresholding, edge detection, erosion, dilation, and a set of heuristics. Labeled sarcolemma (Fig. 1A) provided an outline of each myofiber, while labeled myosin (Fig. 1B) confirmed that a given outline encompassed a myofiber, as opposed to a fat cell or any other element of the intermyofiber space. Myofibers on the edge of the image were omitted from the final binary segmented image, as accurate morphometric parameters could not be extracted from only part of the fibers. The final binary segmented image preserved the geometry of individual myofibers, as displayed in Fig. 1C.

Morphometric measures. Multiple morphometric parameters of each myofiber were measured as variables that may discriminate PAD and control muscle and provide an objective grading of the disease. It was expected that precise myofiber morphometrics would offer insight into the process of muscle degeneration in the ischemic limb and could be related to underlying biological mechanisms. The morphometric parameters included 1) myofiber cross-sectional area, calculated as the number of pixels enclosed within a segmented myofiber, 2) lengths of major and minor axes of the myofiber, 3) myofiber perimeter, measured as the number of pixels on the boundary of the myofiber, and 4) equivalent diameter, defined as the diameter of a circle that has the same area as the segmented myofiber region and calculated using Eq. 1.

\[
\text{Equivalent Diameter} = \sqrt{\frac{4 \times \text{(Actual Fiber Area)}}{\pi}}
\]

Additional morphometric parameters of each myofiber included three measures of roundness given in Eqs. 2–4. Multiple measures of roundness were employed to capture the irregularities and variations of shape and geometry among myofibers.

![Image](image_url)

Fig. 1. A: sarcolemma labeling provided an outline of each myofiber. B: myosin labeling served as a confirmation that a given sarcolemma outline encompassed a myofiber. C: final binary segmented image contained the geometry of each myofiber. Note that myofibers on the edge of the image were removed, as accurate morphometric parameters could not be extracted from only part of the myofibers.
Roundness1 = \frac{\text{Minor Axis}}{\text{Major Axis}} \quad (2)

Roundness2 = \frac{\text{Equivalent Diameter} \times \pi}{\text{Actual Perimeter}} \quad (3)

Roundness3 = \frac{4\pi \times (\text{Actual Area})}{(\text{Actual Perimeter})^2} \quad (4)

Myofiber solidity, defined as the area of the myofiber divided by the area of a fitted convex hull, given in Eq. 5, was measured as well. A fitted convex hull is the smallest convex polygon that can encompass the myofiber. A polygon is convex if it satisfies the criterion that any line segment connecting any two points inside the polygon lie entirely within the polygon. We selected this measure to capture the concavities or lack of concavities in the myofibers.

\text{Solidity} = \frac{\text{Area of Myofiber}}{\text{Area of fitted Convex Hull}} \quad (5)

Fiber density was defined as the area occupied by the myofibers divided by the area occupied by the myofibers plus interstitial tissue, given in Eq. 6. In terms of biological relevance, we expect that these mathematical equations will provide objective measures that quantitatively reflect pathological changes in the muscle, including myofiber atrophy, necrosis, or apoptosis (myofiber cross-sectional area, perimeter, equivalent diameter, roundness, and solidity), loss of the normal polygonal myofiber shape (myofiber roundness and solidity), and replacement of muscle by fibrous and/or adipose tissues (myofiber density).

\text{Fiber Density} = \frac{\text{Area of Fibers}}{\text{Area of Fibers} + \text{Interstitial tissue}} \quad (6)

Finally, a quartile analysis was performed on each tissue specimen, by which the myofibers were separated into three groups based on cross-sectional fiber area, the lower quartile (small fibers) denoted as \textit{LQ}, the middle two quartiles (medium-sized fibers) denoted as \textit{M50\%}, and the upper quartile (larger fibers) denoted as \textit{UQ}. Quartile analysis was performed so that morphometric parameters within the lower and upper quartiles would not be lost in the average of the global morphometric measurements. The mean and standard deviation of the nine morphometric parameters for all myofibers and for myofibers in quartiles of cross-sectional area (72 variables), in addition to fiber density (one variable), were used to represent each patient. Thus, a total of 73 variables for each of the 56 patients were used to build a classification model that discriminates PAD and control muscle, and provides an objective grading of disease.

\text{Discriminant model development}. Control, claudicating, and CLI patients were assigned randomly to training (9, 9, and 10 patients, respectively) and validation (10, 9, and 9 patients, respectively) sets. Variable contribution analysis and model development were implemented using the training data set. A data-preprocessing step was performed to check and verify discriminant analysis assumptions. In addition, the multivariate data were standardized to remove units and put each variable on the same scale. Variable contribution was analyzed using a stepwise selection procedure (12, 14) in SAS 9.2 (The SAS Institute, Cary, NC). Stepwise selection uses both forward selection and backward elimination procedures while evaluating the contribution of parameters to the discriminatory power of the model (4).

Linear discriminant analysis was used to create rules that discriminate PAD patients and controls on the basis of the computed morphometric variables. The discriminant analysis was divided into two stages corresponding to two models. The first model (\textit{model 1}) was designed to discriminate patients with CLI from the group of control and claudicating (control/claudicating) patients. The second model (\textit{model 2}) was designed to discriminate control patients from the claudicating patients.

Six morphometric variables were selected for discriminant \textit{model 1}, namely, solidity of the lower quartile (\textit{LQ Solidity}), standard deviation of the major axis from the lower quartile (\textit{LQ Stdev. Major Axis}), standard deviation of roundness2 from the global measurements (Global Stdev. Roundness2), the standard deviation of roundness3 from the middle two quartiles (\textit{M50\% Stdev. Roundness3}), standard deviation of solidity from the middle two quartiles (\textit{M50\% Stdev. Solidity}), and fiber density.

The training of discriminant \textit{model 1} consisted of 10 CLI patients and 18 control/claudicating patients, each from the training data set. Note that in the training of \textit{model 1}, the controls and claudicating patients were treated as a single group. All patients that were classified into the control/claudication group by \textit{model 1} were then subsequently passed on to the next stage for analysis by \textit{model 2}.

The training of discriminant \textit{model 2} consisted of nine controls and nine claudicating patients from the training data set. Three morphometric variables were selected for \textit{model 2}, namely, the standard deviation of roundness3 (\textit{M50\% Stdev. Roundness3}) from the middle two quartiles, roundness1 from the global measurements (Global Roundness1), and the standard deviation of solidity from the upper quartile (\textit{UQ Stdev. Solidity}).

\text{RESULTS}

Muscle degeneration in PAD exhibits multiple features that include combinations of myofiber atrophy, loss of the normal polygonal myofiber shape, nuclear clumps, increased numbers of internal nuclei fiber vacuolization, target lesions, myofiber regeneration, myofiber necrosis, and fibrosis and replacement of muscle by adipose tissue. Changes from mild (Fig. 2B) to moderate (Fig. 2C), to severe (Fig. 2D) degeneration represent a continuous progression in the extent and severity of these features. Our goal is to use linear discriminant analysis of morphometric parameters of gastrocnemius specimens to discriminate control (Fig. 2A), claudicating, and CLI patients and to provide objective grading of disease. Linear discriminant analysis is a multivariate statistical model that seeks to establish a mathematical rule to separate two or more classes from each other (2, 7, 13). Once a mathematical rule is derived, it can be used to optimally assign new observations into a given classification (13).

\text{Model 1}. Linear discriminant \textit{model 1} is given in Eq. 7.

\begin{equation}
\begin{aligned}
\chi \in &\quad \{\text{CLI if} \quad -5.13 (\text{Fiber Density}) + 4.42 (M50\% \text{ Stdev. Solidity}) \\
&\quad \text{Control or} \quad -4.01 (\text{Global Stdev. Roundness2}) - 2.85 (\text{LQ Solidity}) \\
&\quad \text{C}lau\text{dication} \quad + 2.19 (\text{LQ Stdev. Major Axis}) + 1.35 (M50\% \text{ Stdev. Roundness3}) \geq 0 \\
\end{aligned}
\end{equation}
The model discriminated CLI patients and control/claudication patients with 100% accuracy in the training data set (n = 28). While validating the discriminant rule, with the validation data set (n = 28), model 1 was able to accurately classify eight of nine CLI patients (88.8%) and correctly classified 19 of 19 control/claudicating patients (100%). Further, this model provides a grading index for progression of muscle degeneration among both claudicating and CLI patients (Fig. 3). The possible biological relevance of the variables used in the model is further discussed in the DISCUSSION section.

An independent t-test was used to test for significant differences in group means of the standardized variables used in model 1 (Fig. 4), within the training data, where a P value < 0.05 was considered significant. Fiber density was significantly lower (P = 0.0005) in CLI patients (n = 10; $\bar{x} = -0.81; \pm SE = 0.38$) compared with controls/claudicating patients (n = 18; $\bar{x} = 0.45; \pm SE = 0.10$). The ratio of fiber area to the area of "fibers plus interstitial tissue", was lower in CLI compared with control/claudication myofibers, indicating that the interstitial tissue was thicker in CLI. An example of the fiber density of a control muscle tissue sample, is given in Fig. 5, A–C, while Fig. 5, D–F presents an example of the fiber density of a CLI muscle sample. This variable mathematically quantifies the thickening interstitial tissue or fibrosis and adipose deposition as the disease progresses.

Standard deviation of Solidity among the middle two quartiles of the fibers was significantly lower (P < 0.0001) for the controls/claudicating patients (n = 18; $\bar{x} = -0.52; \pm SE = 0.13$) compared with the CLI patients (n = 10; $\bar{x} = 0.93; \pm SE = 0.31$). Additionally, the Solidity of the lower quartile of fibers was significantly higher (P = 0.0021) for the controls/claudicating patients (n = 18; $\bar{x} = 0.40; \pm SE = 0.19$)
compared with the CLI patients \( n = 10; \bar{x} = -0.73; \pm SE = 0.28 \). Both of these measures of solidity indicated that control/claudicating patients had myofibers with fewer concavities and more uniform solidity compared with CLI patients, as shown in Fig. 6.

The standard deviation of global roundness2 was significantly lower \((P < 0.0001)\) for control/claudicating patients \((n = 18; \bar{x} = -0.50; \pm SE = 0.15)\) compared with CLI patients \((n = 10; \bar{x} = 0.91; \pm SE = 0.26)\). Similarly, the standard deviation of Roundness3 from the middle two quartiles of fibers was significantly lower \((P < 0.0001)\) for control/claudicating patients \((n = 18; \bar{x} = -0.54; \pm SE = 0.13)\) compared with CLI patients \((n = 10; \bar{x} = 0.97; \pm SE = 0.27)\). Both of these measures of roundness indicated that CLI patients had larger variation in roundness of their myofibers.

**Model 2.** Linear discriminant model 2 is given in Eq. 8.

\[
x \in \begin{cases} 
7.75(M50\% \text{ Stdev. Roundness3}) & \text{Claudication if} \\
3.30(\text{Global Roundness1}) & -0.07(UQ \text{ Stdev. Solidity}) \geq 0 \\
-3.22 & \text{Control} \\
\end{cases}
\]

The model separated controls and claudicating patients with 94.4% accuracy in the training data set \((n = 18)\), in which eight of nine controls (88.0%) were correctly classified and nine of nine claudicating patients (100.0%) were correctly classified. In validating \((n = 19)\) discriminant model 2, eight of 10 controls (80.0%) were correctly classified, and seven of nine claudication patients (77.7%) were correctly classified, giving an overall accuracy of 78.9%.

In model 2, the standard deviation of Roundness3 in the middle two quartiles of fibers was significantly lower \((P = 0.0078)\) for controls \((n = 9; \bar{x} = -0.58; \pm SE = 0.30)\) compared with claudicating patients \((n = 9; \bar{x} = 0.58; \pm SE = 0.23)\), shown in Fig. 7. This measure of roundness indicated that claudicating patients had more variation in roundness compared with control patients.

Combining the results from models 1 and 2 yielded an overall accuracy of 96.4%, for the training set, shown in Table 2 and 82.1%, for the validation set, as presented in Table 3.

**DISCUSSION**

We have established that there are consistent and quantifiable changes in myofiber morphology that reflect the progression of PAD muscle degeneration and offer the possibility of an objective index for grading severity of disease within clinical classifications, i.e., Fontaine Stage. Myofiber morphometrics were determined precisely with mathematical equations that incorporated multiple, objectively defined parameters, including fiber density, roundness, minor and major axes, and solidity. To the present time, morphological studies of PAD muscle have been limited to qualitative histopathological

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**Fig. 3.** On the basis of morphometric parameters of their gastrocnemius myofibers, patient scores from model 1 (A) discriminate control and claudicating patients from CLI patients, while scores from model 2 (B) discriminate control from claudicating patients. In model 1, control and claudicating patients with scores closer to zero have myofiber morphology that is approaching or is more similar to the myofiber morphology of CLI patients.
assessments and measurements of myofiber cross-sectional area (5, 9, 15).

Currently, PAD is diagnosed and treated primarily on the basis of the patient's clinical presentation and the results of hemodynamic and imaging evaluation of the arterial system of the lower limbs (19). However, a simple histological evaluation of skeletal muscle from PAD limbs reveals damage and degeneration and suggests that the pathophys-

Fig. 5. Fiber density was defined as the area occupied by the myofibers divided by the area occupied by the myofibers plus interstitial tissue. A and D: myosin images, with myofiber segmentation overlaid in green. B: fiber density (0.86) of a muscle tissue sample from a control subject. C: fiber density (0.70) of a muscle tissue sample from a patient with critical limb ischemia (CLI) and severe myopathic changes. C and F: sarcolemma image, with myofiber segmentation overlaid in green.

Fig. 6. Binary images of segmented myofibers. A: control. B: critical limb ischemia (CLI). C: control middle 50% based on fiber area (M50% Solidity) and fitted convex hulls (red). D: CLI middle 50% based on fiber area and fitted convex hulls (red). M50% CLI fibers exhibit higher variation in solidity (i.e., more concavities) compared with M50% control fibers. Arrows identify myofibers with reduced solidity.
Table 2. Combining models 1 and 2 training results, classification table

<table>
<thead>
<tr>
<th>Predicted Membership</th>
<th>Controls</th>
<th>Claudication</th>
<th>CLI</th>
<th>Total</th>
<th>Accuracy %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actual Membership</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>Controls</td>
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<tr>
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<td>9</td>
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<tr>
<td>Total</td>
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<td>18</td>
<td>33</td>
<td>82.1%</td>
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Table 3. Combining models 1 and 2 validation results, classification table

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<tr>
<th>Predicted Membership</th>
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<td>15</td>
<td>10</td>
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<td>33</td>
<td>82.1%</td>
</tr>
</tbody>
</table>

Fig. 7. Means of standardized selected parameters for model 2, used to discriminate between controls and claudicating patients. *Group means were significantly different using a t-test: M50% Stdev. Roundness3 (P = 0.0078) shows middle 50%, i.e., medium-sized fibers; and UQ Stdev. Solidity shows upper quartile, i.e., large fibers.

...
classify PAD muscle, as a guide for specific treatment of individual patients. It may be necessary to incorporate another level of measurement to account for patients that have normal morphometric indices but are still CLI.

Our study has limitations. Our data are observational and identify a correlation between discriminant indices of muscle degeneration and disease stage (claudication and CLI). However, the data do not establish that the advancing muscle degeneration within a clinical stage (claudication or CLI) represents progression of disease. Second, the sample set is not large enough to allow determination of the potential contributions of individual patient characteristics, such as comorbid conditions and activity levels, to the degree of muscle degeneration. Approximately 6%, 29%, 57% of the control, claudicating, and CLI patients had diabetes mellitus, respectively (Table 1). Ideally, this study would exclude diabetic patients to ensure that the discriminant model is discriminating muscle damage due to PAD only. However, in as much as diabetes mellitus is reflected in the tissue samples as a covariate, the multivariate nature of discriminant analysis will implicitly incorporate the relative contribution of diabetes mellitus in the PAD tissue samples in the variance covariance matrix. Third, the control subjects are not precisely matched with PAD patients on the basis of comorbidities, medications, and activity levels. We selected a control group of similar age and sex composition to the PAD group and included patients undergoing lower extremity operations for indications other than PAD. All controls were selected to have normal ABIs at rest and after stress, and all led sedentary lifestyles.

PAD injury could be more precisely classified with the addition of other parameters, such as, myofiber oxidative damage, inflammatory cytokines, and mitochondrial dysfunction. Development and validation of such a panel of tissue-based biomarkers may produce a powerful classification tool which can 1) more effectively guide the treatment of individual patients, with the patients having the most advanced injury being treated more aggressively, and 2) allow objective determination, at the tissue level, of the outcomes produced by pharmacological and surgical interventions. In future studies, these models, which provide scores representing a continuum of myofiber damage, may be evaluated on the basis of their ability to predict objective, biomechanical indicators of limb function.

**Perspectives and Significance**

Morphological damage to myofibers can be quantified with small gastrocnemius samples obtained with minimally invasive needle biopsy, using discriminant modeling. A discriminant model based on myofiber morphometrics has established a correlation between advancing muscle degeneration and PAD progression. This model separated PAD from control patients and provided a grading of muscle degeneration within clinical stages of PAD. The long-term goal of our work is to develop a comprehensive profile of quantitative morphometric changes in PAD muscle, which will serve as a guide for more extensive mechanisms and intervention studies essential for much needed improvement of diagnosis, staging, and treatment of PAD.

**ACKNOWLEDGMENTS**

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


