Quantification of sweat gland innervation

A clinical–pathologic correlation

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ABSTRACT

Objective: To evaluate a novel method to quantify the density of nerve fibers innervating sweat glands in healthy control and diabetic subjects, to compare the results to an unbiased stereologic technique, and to identify the relationship to standardized physical examination and patient-reported symptom scores.

Methods: Thirty diabetic and 64 healthy subjects had skin biopsies performed at the distal leg and distal and proximal thigh. Nerve fibers innervating sweat glands, stained with PGP 9.5, were imaged by light microscopy. Sweat gland nerve fiber density (SGNFD) was quantified by manual morphometry. As a gold standard, three additional subjects had biopsies analyzed by confocal microscopy using unbiased stereologic quantification. Severity of neuropathy was measured by standardized instruments including the Neuropathy Impairment Score in the Lower Limb (NIS-LL) while symptoms were measured by the Michigan Neuropathy Screening Instrument.

Results: Manual morphometry increased with unbiased stereology ($r = 0.93$, $p < 0.01$). Diabetic subjects had reduced SGNFD compared to controls at the distal leg ($p < 0.001$), distal thigh ($p < 0.01$), and proximal thigh ($p < 0.05$). The SGNFD at the distal leg of diabetic subjects decreased as the NIS-LL worsened ($r = -0.89$, $p < 0.001$) and was concordant with symptoms of reduced sweat production ($p < 0.01$).

Conclusions: We describe a novel method to quantify the density of nerve fibers innervating sweat glands. The technique differentiates groups of patients with mild diabetic neuropathy from healthy control subjects and correlates with both physical examination scores and symptoms relevant to sudomotor dysfunction. This method provides a reliable structural measure of sweat gland innervation that complements the investigation of small fiber neuropathies.

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GLOSSARY

AOI = area of interest; **CI** = confidence interval; **ICC** = intraclass correlation coefficient; **IENFD** = intraepidermal nerve fiber density; **IgG** = immunoglobulin G; **NIS-LL** = Neuropathy Impairment Score in the Lower Limb; **PLP** = paraformaldehyde-lysine-periodate; **QSART** = quantitative sudomotor axon reflex test; **SGNFD** = sweat gland nerve fiber density; **TH** = tyrosine hydroxylase.

Assessment of small myelinated and unmyelinated nerve fibers is a central component of the evaluation of peripheral nerve disease.^{1,2} Most polyneuropathies prominently target small nerve fibers; the subsequent small fiber dysfunction, impaired sensory perception, and sudomotor deficits lead to a predisposition to limb ulceration, infection, and amputation.³ Small sensory fiber function is evaluated with quantitative sensory testing while skin biopsy quantification of intraepidermal nerve fiber density (IENFD) provides a structural measure of small fiber cutaneous innervation.⁴ Functional measures of peripheral postganglionic autonomic sudomotor nerves include the quantitative sudomotor axon reflex test (QSART) and silicone impression

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Medical Devices: BH-2 trinocular head microscope (Olympus, Center Valley, PA); PixeLINK PL-686 (PixeLINK, Ottawa, ON); Zeiss LSM Pascal Exciter (Carl Zeiss, Thornwood, NY).

test. Although reports of autonomic nerve fiber quantitation exist,^{5,6} there is no well-established quantitative, structural measure of the sudomotor system.

We therefore evaluated a new technique to quantify the sweat gland nerve fiber density (SGNFD) using tissue prepared for the standard analysis of IENFD. We examined interobserver and intraobserver reliability, determined the relationship between SGNFD and IENFD, and determined the relationship between SGNFD and several widely used diabetic neuropathy assessment instruments. As a gold standard, we used confocal microscopic Z-stack images through sweat glands in serial sections in combination with unbiased stereologic measurements of nerve fibers.

METHODS Patients. All participating subjects reviewed the protocol and signed an informed consent approved by the Institutional Review Board at Beth Israel Deaconess Medical Center. Thirty patients with diabetes and 64 controls participated in this study. Diabetic subjects were recruited from the clinical practices of the investigators. Control subjects were recruited by local advertisement, had no medical illnesses, were on no medications, and had normal general and neurologic examinations. All diabetic and healthy control subjects had 3-mm punch skin biopsies at the distal leg, distal thigh, and proximal thigh at standard sites.^{7,8} Subjects were assessed using the Neuropathy Impairment Score in the Lower Limb (NIS-LL), the Michigan Diabetic Neuropathy Score (Part 1), and the Toronto Clinical Scoring System.⁹⁻¹² Subjects with NIS-LL scores >8 were excluded to ensure a homogenous group of patients with neuropathy ranging from not clinically evident to mild.

Immunohistochemistry. Skin biopsy specimens were fixed with paraformaldehyde-lysine-periodate (PLP) and cryoprotected.¹³ Tissue blocks were cut by freezing microtome into 50- μ m sections.⁴ Four random sections from each biopsy were evaluated. Four additional sections were analyzed if no sweat glands were identified in the original sections and in 15 random patients with diabetes and 20 random controls to determine the number of sweat glands present in 8 sections. Tissues were stained with PGP 9.5 (ubiquitin hydrolase; Chemicon, Temecula, CA) using standard techniques.^{8,14}

We studied three additional biopsies from the proximal thighs of three healthy control subjects. Tissue sections were fixed with PLP and cut into 50- μ m sections. The biopsies were sectioned exhaustively and costained with PGP 9.5 and tyrosine hydroxylase (TH; a marker of sweat gland neuroendocrine cells).⁹ Briefly, the sections were treated with 0.25% potassium permanganate (Sigma, St. Louis, MO) for 15 minutes and 5% oxalic acid (Sigma) for 2 minutes. The sections were placed in block solution for 1 hour, incubated with rabbit polyclonal PGP 9.5 (Chemicon) and mouse monoclonal TH (Chemicon) antibodies for 1 hour, and moved to 4°C overnight. Samples were incubated with mixtures of donkey antirabbit immunoglobulin G (IgG) conjugated to the fluorophores cyanine 3 (1:500) and biotinylated donkey antimouse IgG (Jackson ImmunoResearch Inc., West Grove, PA; 1:700) for 5 hours, followed by streptavi-

din labeled fluorophores cyanine 2 (Jackson ImmunoResearch Inc. 1:400).¹⁵ Sections were washed with TBS containing 0.5% Triton x100 3 $\times$ 20 minutes and then mounted on coverslips for viewing. Z-stack images of sweat glands were taken in 2- to 5- μ m optical sections acquired at successive levels through the 50- μ m-thick tissue sections (Zeiss LSM Pascal Exciter; Carl Zeiss, Thornwood, NY) shown in figure 1, A–I.

Confocal microscopy and stereology. As a gold standard, Z-stack confocal images of all the sweat glands in three skin biopsies were analyzed by an unbiased stereologic technique using systematic sampling with a cycloid test system to estimate sweat gland nerve fiber length (figure 2, A–F).¹⁵⁻¹⁹

Nerve fiber quantitation: Light microscopy. Biopsies underwent blinded IENFD counting and results were expressed as fibers per millimeter.²⁰ Every sweat gland was digitally captured using a 6-megapixel camera (PixelINK PL-686, Ottawa, ON) mounted on an Olympus BH-2 trinocular head microscope (Center Valley, PA), with images taken at 20 $\times$ magnification. In addition, an out of focus image of each sweat gland was taken to aid in background visual resolution thresholding (Adobe Photoshop CS3 Extended; Adobe, San Jose, CA).

The large variability in sweat gland size requires measurements of SGNFD be normalized by area. Due to high inter-reviewer and intra-reviewer variability in manual outlining, the area of interest (AOI) was selected using the out of focus image of the sweat gland. Counterintuitively, the blurred image creates a more uniform area for selection, thereby reducing variability between reviewers (figure 2H).

Quantification of sweat gland innervation. Sweat gland innervation was evaluated in a blinded fashion. All images were analyzed through the use of Image Pro Plus (Media Cybernetics, Bethesda, MD) using the composite image with the selected AOI around the sweat gland (figure 2H). Superimposed upon the composite image was a standardized grid of circles 10 μ m in diameter spaced 50 μ m apart horizontally, and offset 25 μ m vertically (figure 2, G–K). This created a simple pattern of circles over the sweat gland. Nerve fibers that crossed within the circles were manually counted. Nerve fibers that touched the edge of the circle but did not enter were not counted. Results were expressed as the percent of circles intersected from the total number of circles within the sweat gland (figure 2, G–K). All confocal immunofluorescent images were also analyzed by manual quantification (in addition to the unbiased stereologic approach). Individual confocal images of 2- to 5- μ m thickness and 20- μ m-thick composite confocal images (created to mimic the depth of field seen by light microscopy) were analyzed by manual quantification. The results were compared to the unbiased stereologic estimate of nerve fiber length within the biopsy.

Intra-reviewer and inter-reviewer reliability. Intra-reviewer and inter-reviewer reliability was measured by randomly selecting 48 images (30 control and 18 diabetic) to be remeasured by the primary reviewer. The same 48 images were reviewed by two other observers to evaluate for interobserver reliability.

Statistical analysis. Statistical analysis was performed using SPSS v.15 (Chicago, IL). Results are expressed as mean $\pm$ SD. Pearson correlation coefficients (*r*) were calculated to assess simple relationships between variables. Student *t* test was used to analyze group differences, with Bonferroni adjustments for multiple comparisons. Interclass correlation coefficients were calculated to assess reliability within and between examiners.

Figure 1 Skin biopsy analysis of sweat glands imaged with confocal microscopy

A 3-mm punch skin biopsy was exhaustively sectioned and stained with PGP 9.5 (a pan-axonal marker) and tyrosine hydroxylase (a stain of sweat gland neuroendocrine tissue). Four illustrated sample tissue sections are shown through three sweat glands in the biopsy (A). Representative confocal images through three different sweat glands in the same biopsy are shown (B-I); sections B and I are taken at 10x magnification, sections C-H are at 20x magnification. The confocal images of the sweat glands (B-I) reveal the nerve fibers stained by the pan-axonal marker PGP 9.5 (red) innervating the sweat gland tubules stained by tyrosine hydroxylase (green). Samples from three different sweat glands (B, C-H, I) are shown to highlight sweat glands within the same biopsy. Z-stack confocal images of the same sweat gland are shown to highlight the sweat gland nerve fiber density seen within the same sweat gland in different tissue sections (200 μm apart) and in different planes of view (8 μm apart: C-E, F-H). Scale bar = 100 μm.

Figure 2 The quantification of sudomotor nerve fibers

The unbiased stereologic sampling of sweat glands (A) involves serially sectioning the punch biopsy in a random plane perpendicular to the tissue surface (B), studying a single tissue section (C, D), digitally identifying the nerve fibers (E), and orienting a test grid of cycloids over the sweat glands and counting the intersections between cycloids and nerve fibers in a three-dimensional stepping pattern (F). The estimated length (L_E) of nerve fibers within the sweat gland was calculated from the ratio of tested area to cycloid test length (T_A/T_L), multiplied by the total cycloid intercepts counted (ΣI), divided by the magnification during counting (M), divided by the percent of the sweat gland sampled ($SG_{\%}$). $SG_{\%} = (\text{sampled sections}/\text{total sections}) \times (\text{sampled area}/\text{total area}) \times (\text{sampled depth of field}/\text{total depth of field})$. Thus the estimated length of nerve fibers within a given area is expressed by the following equation: $L_E = (T_A/T_L \times \Sigma I)/(M \times SG_{\%})$.^{16, 17} In this study the $T_A/T_L = 3.05$ cm, $M = 661\times$, magnification and $SG_{\%} = 40\%$ (the biopsies had 50% percent of the tissue sections imaged, 100% of the area sampled, and 80% of the depth of field sampled). The manual quantification of sweat gland nerve fiber density by light microscopy involves taking a digital image of the sweat gland at $20\times$ magnification, shown in G. The same image, taken out of focus, is shown in H, with the selected area of interest highlighted in green. In I, the background staining from the out of focus image (H) is removed from the baseline image (G), providing a composite image (I). The nerve fibers in the composite image (I) have a grid placed over it (J); any circle partially or wholly contained within the AOI is eligible for counting. Nerve fibers that intercept the grid are counted manually (K); nerve fibers that touch but do not enter the circle are not counted. Scale bar = $50\ \mu\text{m}$.

RESULTS Demographics. Diabetic subjects (13 men, 17 women, mean age 36 ± 10 years) and control subjects (34 men, 30 women, mean age 34 ± 9 years) had similar height, weight, and body mass index. Type 1 subjects had diabetes for 11 ± 6.4 years; type 2 subjects had diabetes for 4.5 ± 3.9 years. The average hemoglobin A1c scores (within the prior 3 months) for patients with diabetes were 7.4 ± 1.3 ; control subjects had normal HgA1c levels. In diabetic subjects, the mean NIS-LL was 4.3 ± 2.6 . Control subjects had NIS-LL scores of 0. Detailed anthropomorphic data are provided in table e-1 on

the *Neurology*® Web site at www.neurology.org. A total of 282 biopsies were evaluated, 90 from patients with diabetes (3 biopsies per subject).

Quantitative analysis of sweat gland innervation. A total of 307 sweat glands were identified in control subjects and 144 in diabetic patients with four tissue sections per biopsy; the average number of sweat glands per biopsy was 1.6 ± 0.7 (mean area $0.066 \pm 0.035\ \text{mm}^2$) in control and 1.6 ± 0.5 (mean area $0.073 \pm 0.047\ \text{mm}^2$) in diabetic subjects. No sweat glands were identified in the original sections of four

Figure 3 Sweat gland nerve fiber density (SGNFD) by location in control and diabetic subjects

The SGNFD are shown by site at the distal leg, distal thigh, and proximal thigh. Diabetic subjects are denoted by the clear box plots, healthy controls by the gray box plots. The box plots demonstrate the median value, with first and third quartiles outlined by the box, 10th and 90th percentiles by the whisker lines, and individual outliers shown as solid dots. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

diabetic and seven control subjects (but were present in all when an additional four sections were studied). If eight tissue sections were studied, the number of sweat glands increased to 4.2 ± 1.3 in control and 4.1 ± 1.4 in diabetic subjects.

In diabetic subjects, the SGNFD at the distal leg was $20.8 \pm 12.2\%$, at the distal thigh $28.2 \pm 13.4\%$, and at the proximal thigh $42.5 \pm 9.2\%$. In control subjects, the SGNFD at the distal leg was $40.8 \pm 12.8\%$ ($p < 0.001$ vs diabetic subjects), at the distal thigh $46.6 \pm 13.2\%$ ($p < 0.01$ vs diabetic subjects), and at the proximal thigh $51.3 \pm 11.8\%$ ($p < 0.05$ vs diabetic subjects). These results are shown graphically in figure 3. No significant differences in SGNFD were noted by sex, height, weight, diabetes type, or age in this small group of patients (table e-1).

Neuropathy scores. The SGNFD at the distal leg decreased as neuropathy worsened (by increasing NIS-LL; $r = -0.89$, $p < 0.001$; figure 4A) but to a lesser degree at more proximal sites ($r = -0.52$ distal thigh, $r = -0.42$ proximal thigh). Similar results were seen using the Michigan Diabetic Neuropathy Score Part 1 ($r = -0.87$, $p < 0.001$ distal leg) and the Toronto Clinical Scoring System ($r = -0.91$, $p < 0.001$ distal leg). There was little agreement between SGNFD and the Michigan Neuropathy Screening Instrument; however, subjects who answered “no” to the question “Is the skin on your feet so dry that it cracks open?” had a mean SGNFD at the distal leg of 34 ± 14 , while those who answered “yes” had a SGNFD at the distal leg of 12 ± 9 ($p < 0.01$). Other questions pertaining to small and large fi-

ber neuropathy did not reveal significant differences in SGNFD between those who answered yes or no.

Confocal image analysis and stereology. There were 20 sweat glands in the three biopsies with complete Z-stack images providing a total of 213 individual confocal images. The unbiased estimate of nerve fiber length for each sweat gland with the corresponding manual count result can be seen in figure 4B. The SGNFD assessed using the $20 \mu\text{m}$ extended depth of field increased with the unbiased stereologic estimate of sweat gland nerve fiber length ($r = 0.93$, $p < 0.01$). Manual counting of individual images (no extended depth of field) in a Z-stack resulted in variance of 13%–38% (95% confidence interval [CI]) among sweat gland sections within the same skin biopsy (figure 1, B–I). However, the $20 \mu\text{m}$ extended depth of field confocal images, which have the same depth of field as light microscopy, reduced the variability to 4%–12% (95% CI) among sweat gland sections within the same skin biopsy. The SGNFD from sweat glands that were smaller than $300 \mu\text{m}^2$ did not correlate with SGNFD from other sweat glands of larger areas from the same individual.

Relationship between intraepidermal and sweat gland nerve fiber densities. The SGNFD increased with IENFD at the same site ($r = 0.66$, $p < 0.05$). The IENFD decreased as neuropathy worsened (by increasing NIS-LL; $r = -0.822$, $p < 0.001$; figure 4C).

Intraobserver and interobserver reliability. When studying the composite image with preselected AOI, the intraclass correlation coefficient (ICC) for intra-reviewer reliability for the SGNFD quantification method was $\text{ICC} > 0.975$, $p < 0.001$, and for inter-reviewer reliability was $\text{ICC} > 0.978$, $p < 0.001$. When re-creating the composite image, selecting the AOI and counting the SGNFD, the intra-reviewer $\text{ICC} = 0.886$, $p < 0.001$, and the inter-reviewer $\text{ICC} = 0.892$, $p < 0.001$.

DISCUSSION Sensory and autonomic neuropathies are common in patients with diabetes, and can lead to loss of protective sensation and risk of ulcer development.²¹ Changes in peripheral autonomic nervous system function play a pivotal role in the development of foot ulceration²² and may be the earliest neurophysiologic abnormality in diabetic²³ and other distal small fiber neuropathies.² Although several neurophysiologic tools to study sudomotor function exist, to date, there are no well-established quantitative techniques to study the structure of the sudomotor system. The present data demonstrate that 1) SGNFD can be reliably quantified from standard skin punch biopsies using a manual counting technique in healthy controls and diabetic subjects; 2)

Figure 4 Relationships between SGNFD, IENFD, NIS-LL and unbiased stereology

(A) The relationship between sweat gland nerve fiber density (SGNFD) and the Neuropathy Impairment Score in the Lower Limb (NIS-LL). Each dot represents individual subjects with diabetes. The NIS-LL score is plotted against the SGNFD at the distal leg. The unbroken black line shows the regression ($r = -0.89, p < 0.001$). (B) The relationship between SGNFD and an unbiased stereologic estimate of nerve fiber length. Confocal images with an extended depth of field (20 μm). The results were compared against a gold standard unbiased stereologic estimate of nerve fiber length (expressed as length of nerve fibers in a cubic millimeter of sweat gland tissue) as seen in figure 2. Each dot represents individual sweat glands in the three sample biopsies. The unbroken black line shows the regression ($r = 0.93, p < 0.01$). (C) The relationship between intraepidermal nerve fiber density (IENFD) and the NIS-LL. Each dot represents individual subjects with diabetes. The NIS-LL is plotted against the IENFD at the distal leg. The unbroken black line shows the regression ($r = -0.82, p < 0.001$).

sweat glands are present in skin biopsies in sufficient numbers to enable routine analysis; 3) the SGNFD at the distal leg decreases as the severity of diabetic neuropathy (assessed by standardized instruments) increases; and 4) reduced SGNFD is concordant with symptoms of sudomotor dysfunction. These results support the use of standard skin biopsies to quantify sudomotor innervation in the assessment of neuropathy in patients with diabetes.

A variety of technical challenges have impeded the quantification of sudomotor nerve fibers. These include 1) the three-dimensional structure of the individual sweat glands; 2) variable number and size of sweat glands in each tissue section; 3) high background staining of sweat glands by PGP 9.5; 4) the extended depth of image view (only 20 μm of a 50 μm tissue section can be seen at one time by light microscopy); and 5) the lack of an established gold standard technique with which to compare the results. Our approach to the analysis of SGNFD has resolved these issues and provides a measure that shows concordance with instruments measuring the signs and relevant symptoms of diabetic peripheral neuropathy.

Sweat glands are complex three-dimensional structures enveloped by nerve fibers traveling in multiple planes of view simultaneously. The intricate structure prevents reliable identification and tracking of individual nerve fibers through traditional means of cutaneous nerve fiber quantification. The SGNFD counting technique does not require tracking of individual nerve fibers to calculate the sudomotor innervation density and adapts to the complex structure of sweat glands. An average of one to two sweat glands of varying size was seen in four tissue sections of a typical skin biopsy. Serial sections of three skin biopsies revealed little variability between tissue sections, suggesting adequate sampling can occur despite the relatively low numbers of sweat glands. Although sweat gland size was variable between sections, there was little change in density of sudomotor fibers except when sweat gland areas were smaller than 300 μm^2 . Sweat glands smaller than 300 μm^2 appeared to be at the outer edge or glandular border. These were not representative of innervation in the rest of the sweat glands and should not be included in the analysis.

Nonspecific background staining with PGP 9.5 renders individual nerve fibers and sweat gland margins ambiguous. Furthermore, polyclonal antibodies to PGP 9.5 create variable degrees of background staining in different individuals that limit the use of an algorithm-based preselected thresholding value.^{24,25} Therefore, we use the imaging technique *unsharp mask filter* rather than a specific computer-

Figure 5 Thickened sudomotor nerve fibers in a subject with diabetes

A sweat gland from the distal thigh of a control subject (A) and a subject with diabetes who has an Neuropathy Impairment Score in the Lower Limb of 4 (B). Nerve fibers in the diabetic subject have reduced density and appear thickened (B) compared to the control subject (A). Scale bar = 50 μm .

aided filter for analysis.²⁴ The blurred image paradoxically accentuates the edge of nerve fibers and sweat gland margins. This technique improves interobserver and intraobserver variability in SGNFD assessment and sweat gland margin definition in order to normalize sweat gland innervation in relation to sweat gland size.

A typical skin biopsy section is 50 μm thick but the visual depth of field in a microscope at 20 $\times$ is approximately 20 μm . Selecting the appropriate visual field to study could alter results if SGNFD differed between image views. To address this issue, completely sectioned biopsies were imaged by confocal microscopy, with Z-stack images to study the variability seen in different planes of view. When comparing individual confocal images in a Z-stack (3–5 μm depth of view), there is moderate variability in SGNFD (figure 1). However, images with a 20- μm depth of view reduce the variation in SGNFD because of the larger sampling region. The extended depth of view with confocal images is equivalent to that seen through light microscopy and validates working with bright-field images.

There is no well-established method of SGNFD quantification. We therefore used confocal imaging with unbiased stereology as our gold standard. In three biopsies extensively sectioned and imaged by confocal microscopy, the nerve fibers innervating sweat glands were quantified using unbiased stereology to estimate nerve fiber length and provide a rigorous comparator. Stereology is labor intensive, requiring 30–40 hours to complete the staining, imaging, and nerve quantitation for each biopsy, and is not suitable for routine use. However, data obtained with this technique confirm the validity of assessing

SGNFD from a punch skin biopsy acquired for IENFD analysis.

Our data suggest that evaluation of SGNFD may have clinical utility in patients with diabetic neuropathy. There is a clear decline in SGNFD as the physical examinations of patients with diabetes, scored by the NIS-LL, Michigan Diabetic Neuropathy Score, and Toronto Clinical Scoring System, worsen. In contrast, the correlation between SGNFD and IENFD was not as strong, suggesting that the subtypes of small unmyelinated and lightly myelinated fibers may not degenerate or regenerate at the same rate or time and emphasizing the importance of independent structural measures of sensory and autonomic nerve fibers. While the SGNFD did not correlate well with the items on the Michigan Neuropathy Screening Instrument, which primarily addresses the features of small and large fiber somatosensory neuropathy, there was strong concordance with the item that addresses sudomotor symptoms; diabetic subjects who reported dry cracked skin in the feet had significantly lower SGNFD at the distal leg compared to those who did not. There was no association between SGNFD and age, sex, body size, or diabetes type. Additional studies of greater numbers of control and diabetic subjects are necessary to answer these questions definitively.

In addition to the declining sudomotor density in patients with diabetic neuropathy, we found distinct morphologic changes not seen in healthy control subjects. We observed thickened sudomotor fibers at the distal leg in some patients with mild diabetic neuropathy. The pathophysiology of the thickened nerve fibers (figure 5) is not known, but could suggest a predegenerative process similar to that seen in epidermal nerve fibers in humans with swelling^{26,27} or neuroaxonal dystrophy seen in autonomic ganglia in rodent experimental models of diabetic neuropathy.^{28,29} This observation may be the morphologic correlate of the increase in sweat droplet size and decrease in droplet number seen in silicone impressions of patients with diabetic neuropathy.³⁰ Further studies are required to clarify these clinical observations and morphologic findings.

We have successfully quantified the density of nerve fibers innervating sweat glands using a novel technique. The method shows reliable interexaminer and intraexaminer reproducibility and appears to distinguish a group of patients with mild diabetic neuropathy from a group of healthy control subjects. Quantification of SGNFD requires no additional staining or preparation of tissue other than that performed for standard assessment of intraepidermal nerve fiber density from a punch skin biopsy. Future studies are required to correlate these structural

changes with sudomotor function, to examine diabetic subjects with more severe diabetic neuropathy, and to provide normative data with anthropomorphic variables such as height, weight, and body surface area.

AUTHOR CONTRIBUTIONS

Statistical analysis was conducted by Christopher Gibbons, MD, MMSc.

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