

# Epidermal Nerve Fiber Density

## Normative Reference Range and Diagnostic Efficiency

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**Background:** The sensitivity of neuron-specific antibodies permit the identification of the small unmyelinated nerve fibers within the skin.

**Objectives:** To develop a reference range of epidermal nerve fiber density in humans, and to evaluate their diagnostic efficiency for sensory neuropathies.

**Methods:** Ninety-eight normal controls (age range, 13-82 years) were examined with both directed neurologic examinations and quantitative sensory testing. The diagnostic utility was examined in 20 patients with sensory neuropathies. Each subject had 2 punch biopsies performed at each site in the thigh and distal part of the leg (total of 392 biopsies). After formalin fixation, 50- $\mu$ m-thick free-floating sections were stained with a polyclonal antibody to neuron-specific ubiquitin hydrolase, anti-protein gene product 9.5. We enumerated intraepidermal nerve fibers per millimeter to derive a "linear density." The linear density technique was validated against a stereological technique that used the fractionator to measure the total length of intraepidermal nerve fibers per 3-mm punch.

**Results:** The biopsy technique was well tolerated, with no notable complications. The linear density quantitation was rapid and had high intraobserver and interobserver reliability. We determined that the density of intraepidermal fibers in normal controls was  $21.1 \pm 10.4$  per millimeter (mean  $\pm$  SD) in the thigh (fifth percentile, 5.2 per millimeter), and was  $13.8 \pm 6.7$  per millimeter at the

distal part of the leg (fifth percentile, 3.8 per millimeter). Significantly higher intraepidermal fiber densities were seen in the youngest group ( $P = .004$ ), and we observed no significant effect of race, sex, height, or weight. The density at the thigh was significantly correlated with that at the distal part of the leg ( $P = .01$ ) and was consistently higher by about 60%, a reflection of the normal proximal-distal gradient. The results obtained with stereology and the linear density correlated significantly ( $P = .001$ ), providing internal validation for the technique. Epidermal nerve fiber density was significantly reduced ( $P = .001$ ) in patients with sensory neuropathies. With a cutoff derived from the fifth percentile of the normative range for the distal part of the leg, the technique had a positive predictive value of 75%, a negative predictive value of 90%, and a diagnostic efficiency of 88%.

**Conclusions:** We have established a reference range for intraepidermal nerve fiber density in normal humans by means of a simple quantitation method based on enumeration of individual intraepidermal nerve fibers on vertical sections of punch skin biopsy specimens stained with the sensitive panaxonal marker anti-protein gene product 9.5. The utility of the density measurement was confirmed for sensory neuropathy with a diagnostic efficiency of 88%. Skin biopsies may be useful to assess the spatial distribution of involvement in peripheral nerve disease and the response to neurotrophic and other restorative therapies.

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**S**ENSORY NEUROPATHY is a relatively common neurologic condition that can complicate, among other disorders, diabetes mellitus, human immunodeficiency virus infection, and cancer chemotherapy, or can occur spontaneously. The frequency of neuropathy in diabetes mellitus is 66%,<sup>1</sup> and in human immunodeficiency virus infection, the annual incidence of sensory neuropathy is 7%.<sup>2</sup> The small-caliber C and Ad nerve fibers that inner-

vate the skin are involved in many of these sensory neuropathies. Diseases of

*For editorial comment  
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these fibers can contribute to neuropathic pain or the loss of protective sensation, with painless injuries. Currently, few objective measures are available for the assessment of small-caliber sensory fibers. The limitations of our current means of assessing these fibers have been an

## SUBJECTS AND METHODS

### CONTROLS

Ninety-eight controls were recruited for biopsy. They consisted of university and hospital employees, volunteers for clinical pharmacology studies, and recruits from a church group. Controls were paid \$100 for their participation. The protocol was approved by our institutional review board, and informed consent was obtained before all biopsies.

The initial screening interview was conducted by telephone and included a review of current and past health status, focusing on potentially neuropathic conditions: diabetes mellitus, cancer chemotherapy, previous acquired or inherited neuropathies, and heavy alcohol abuse or solvent exposure. Positive responses at this stage were a cause of exclusion. The second stage included a directed neurologic examination by a neurologist (J.C.M.) to exclude peripheral neuropathy and other confounding neurologic conditions, such as dementia, cachexia, spinal cord–root disease, and signs of local trauma in the limbs. Two questionnaires were included in the interview: a brief 6-item neuropathy grading scale (the AIDS Clinical Trials Group Subjective Neuropathy Scale) and the Neuropathy Symptom Profile.<sup>20</sup> Quantitative sensory testing was completed by means of the computer-aided sensory evaluator device (CASE IV; WR Medical Electronics, Stillwater, Minn)<sup>21</sup> with a cooling and vibratory algorithm.

Demographic characteristics (by age deciles) of the 98 neurologically normal controls are shown in **Table 1**.

### PATIENTS WITH NEUROPATHY

A group of 20 patients with sensory or sensorimotor neuropathy was included to assess the diagnostic utility of the quantitation technique. These patients were described in greater detail by Holland et al.<sup>22</sup> Three groups were studied: (1) 8 patients seropositive for human immunodeficiency virus (aged 38–57 years), in whom neuropathy developed during treatment with the antiretroviral agents didanosine or zalcitabine, and all of whom had symptomatic human immunodeficiency virus infection or had had

an acquired immunodeficiency syndrome–defining illness, with mean CD4<sup>+</sup> cell count of  $0.03 \times 10^9/L$ ; (2) 10 patients with idiopathic neuropathies with sensory predominance (aged 55–76 years); and (3) 2 patients with diabetes mellitus.

Clinical severity was assessed by a modification of the Total Neuropathy Score.<sup>23,24</sup>

### SKIN BIOPSY TECHNIQUE

Punch biopsies were performed with a 3-mm disposable circular punch (Acupunch, Acuderm, Ft Lauderdale, Fla) after the local injection of 2% lidocaine with epinephrine anesthesia under a sterile technique. No sutures were used. Biopsy specimens were obtained from the following sites: the lateral upper part of the thigh and the distal part of the leg, approximately 10 cm above the lateral malleolus. Details of immunostaining techniques are provided in previous publications.<sup>22,25,26</sup>

### DETERMINATION OF LINEAR DENSITY OF INTRAEPIDERMAL NERVE FIBERS IN VERTICAL SECTIONS

For each biopsy specimen, the numbers of separate intraepidermal fibers in at least 3 sections were counted by a single observer (E.A.S.) blinded to the clinical status of the sample. A priori counting rules were established to count only single intraepidermal fibers and not multiple branches of the same fiber. Note that this paradigm does not enumerate secondary branches within the epidermis. The total length of epidermis in sections was measured by means of a computerized quantitation system instrument (Bio-Quant V, R&M Biometrics, Nashville, Tenn). **Figure 1** illustrates the quantitation system.

Separate intraepidermal fibers were then identified and counted with the use of 3 to 6 sections for each punch. The mean number of intraepidermal fibers per millimeter of length of epidermis was then derived.<sup>27</sup> Results were compared with those of a stereological technique.<sup>25</sup> Nonparametric statistics were used to compare cases and controls by means of a statistical software package (StatView, Abacus Concepts Inc, Berkeley, Calif).

impediment to studies of the natural course and therapeutic responses in sensory neuropathies or in the sensory component of sensorimotor polyneuropathies. As the number of potential therapies increases, especially neurotrophic factors targeted for specific fiber classes, so will the need for improved techniques for the practical structural assessment of the various fiber classes.

The cutaneous innervation has traditionally been considered to consist of a plexus of fibers in the reticular dermis and a more superficial plexus in the papillary dermis, with most sensory endings located in the subpapillary dermis. Intraepidermal nerve terminals have been identified in the basal layers of the epidermis associated with Merkel cells,<sup>3</sup> with cold receptors,<sup>4</sup> and with high-threshold mechanoreceptors.<sup>5</sup> Langerhans<sup>6</sup> first described deeper penetration of intraepidermal nerves, but these fibers have proved difficult to visualize, and penetration beyond the basal layers was thought to be

rare. For example, Ridley<sup>7</sup> identified only “sparse” intraepidermal nerves in a minority of adults, and other investigators have described sensory terminations within the basal epidermis.<sup>8,9</sup> In contrast to these observations, Kruger et al<sup>10</sup> used electron microscopy and substance P staining to demonstrate the deep penetration of sensory axons into the stratum spinosum layer of the epidermis.

Several previous studies have assessed cutaneous nerve fibers in normal individuals and in patients with neuropathy. Dyck and coworkers<sup>11</sup> used cholinesterase stains and Ridley<sup>7,12</sup> used silver stains to investigate Meissner corpuscles. These investigators demonstrated a steep, age-related decline in the density of Meissner corpuscles. However, in these and other studies of Meissner corpuscles, the variability among subjects and between sites was high. In addition, because these sensory end organs are found only in glabrous skin (such

**Table 1. Demographic Characteristics (by Age Deciles) of Neurologically Normal Controls**

	Age, y						
	10-19	20-29	30-39	40-49	50-59	60-69	70-79
No. who underwent biopsy (n = 98)	8	18	20	19	12	9	12
Age, mean ± SD, y	18.3 ± 2.1	25.2 ± 2.2	34.0 ± 3.5	44.8 ± 3.2	53.6 ± 3.1	65.0 ± 2.2	72.9 ± 3.5
Sex, No. (%)							
Male	7 (88)	13 (72)	17 (85)	13 (68)	8 (67)	3 (33)	5 (42)
Female	1 (12)	5 (28)	3 (15)	6 (32)	4 (33)	6 (67)	7 (58)
Race, No. (%)							
White	5 (62)	12 (67)	11 (55)	15 (79)	10 (83)	9 (100)	12 (100)
African American	1 (12)	4 (22)	8 (40)	4 (21)	1 (8)	0 (0)	0 (0)
Other	2 (25)	2 (11)	1 (5)	0 (0)	1 (8)	0 (0)	0 (0)

as the finger or toe pads), the spatial distribution of involvement could not be assessed.

The recent advent of the panaxonal marker anti-protein gene product 9.5 (PGP9.5) has allowed the recognition of intraepidermal fibers by immunocytochemical staining.<sup>6,12-14</sup> These intraepidermal fibers originate from sensory nerves as they express substance P and calcitonin gene-related peptide.<sup>15-17</sup> Kennedy et al<sup>18,19</sup> used PGP9.5 and confocal microscopy to quantify changes in epidermal and sweat gland innervation and recently showed a relationship between clinical severity of peripheral neuropathy and the density of epidermal innervation.

This marker offers a new approach to identify and quantify the unmyelinated intraepidermal nerves, a fiber class frequently affected in length-dependent sensory and sensorimotor neuropathies. We describe a reference range for intraepidermal nerve fiber density in human skin in well-characterized healthy normal controls, and determine the diagnostic utility for classification of cases of sensory neuropathy.

## RESULTS

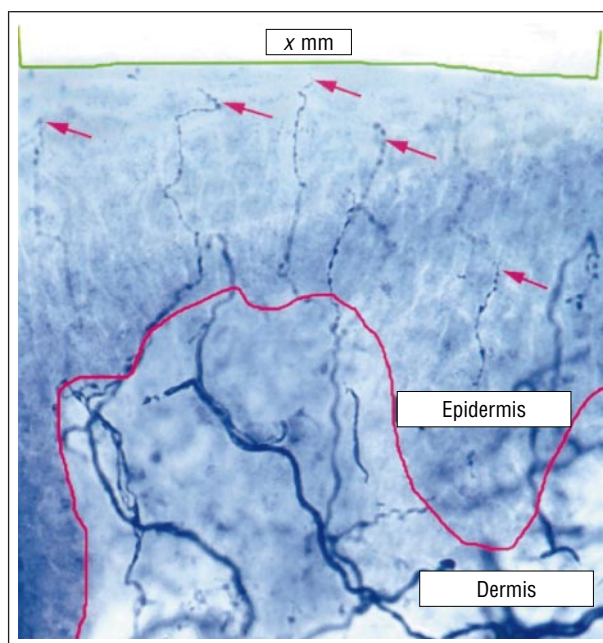
### CUTANEOUS INNERVATION IN CONTROLS

In control subjects, large numbers of nerve fibers and specialized nerve endings were identified by means of PGP9.5 antibodies in 3 locations: the reticular dermis, the subpapillary dermis, and within the epidermis. The morphologic appearance of fibers in the subcutis and the subpapillary dermis conformed to previous descriptions based on silver, methylene blue, and immunocytochemical staining techniques<sup>12,28,29</sup> and have been described in previous publications by Holland et al<sup>22</sup> and McCarthy et al.<sup>26</sup>

#### NORMATIVE DATA: LINEAR DENSITY MEASUREMENTS

##### Method Reliability

As a measure of internal consistency and reliability, we assessed intraobserver and interobserver variability, variability within sections, and variability between adjacent punches at the same anatomic site.



**Figure 1.** Epidermal nerve fiber density measurements illustrated in a 50- $\mu$ m vertical section, immunostained with the panaxonal marker anti-protein gene product 9.5 to demonstrate fine, vertically arrayed unmyelinated nerve fibers within epidermis (arrows). The epidermal-dermal junction is indicated by the red line. Each arrow indicates an individual intraepidermal nerve fiber. The length of epidermis (x mm) as measured by the BioQuant device (R&M Biometrics, Nashville, Tenn) and used to calculate the linear density is indicated by the line above the epidermis.

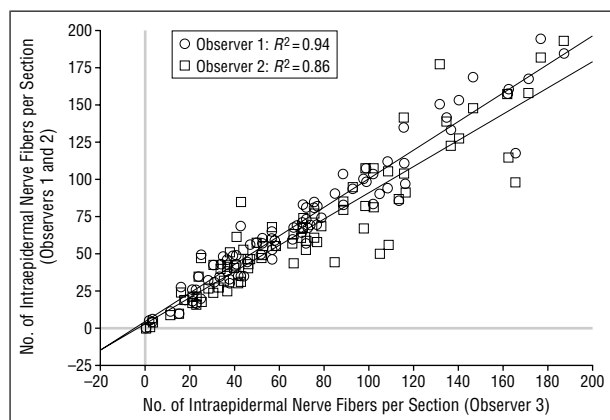
**Intraobserver and Interobserver Reliability.** Intraobserver and interobserver reliability were assessed by blinded comparison of intraepidermal nerve fiber density scores for the same observer (E.A.S.) counting twice, and between 3 observers (including E.A.S.). Multiple controls were counted twice by the same blinded observer (E.A.S.) to assess intraobserver agreement. The correlation coefficients for the separate counts ranged from 0.74 to 0.86 ( $P < .001$ ). Correlation coefficients for interobserver reliability ranged from 0.86 to 0.94 ( $P < .001$ ), indicating a high level of reliability (**Figure 2**).

**Variation Among Adjacent Sections Within Same Site: Intrapunch Variability.** Adjacent sections from the same punch were compared to assess intrapunch variability. The correlation coefficients indicate a high degree of as-

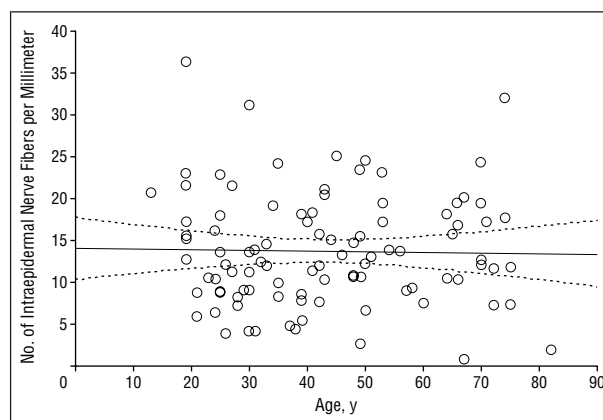
**Table 2. Intraepidermal Nerve Fiber Density by Age Decile**

Skin Biopsy Site	No. of Fibers per Millimeter by Age, y						
	10-19 (n = 8)	20-29 (n = 18)	30-39 (n = 20)	40-49 (n = 19)	50-59 (n = 12)	60-69 (n = 9)	70-79 (n = 12)
Distal part of leg*							
Mean ± SD	20.3 ± 7.4	11.3 ± 5.3	11.9 ± 7.1	14.6 ± 5.6	14.4 ± 5.7	13.3 ± 6.5	14.7 ± 8.2
Range	12.7-36.5	3.8-23.0	4.2-31.2	2.7-25.2	6.7-24.6	0.6-20.2	1.9-32.2
Median	19.1	9.7	10.6	14.8	13.4	15.9	12.4
80th percentile interval	13.5-32.5	6.1-20.6	4.3-21.8	8.8-22.6	8.4-23.6	3.4-20.0	5.7-26.8
Thigh†							
Mean ± SD	31.6 ± 13.2	15.8 ± 5.9	17.8 ± 9.4	22.3 ± 7.4	21.4 ± 14.1	24.0 ± 8.4	23.4 ± 8.3
Range	14.6-50.5	2.9-27.1	4.2-40.6	3.0-32.7	9.7-57.5	16.3-38.1	11.2-39.5
Median	27.3	15.7	17.4	23.6	16.5	20.3	22.9
80th percentile interval	16.6-49.2	8.3-24.0	5.6-29.6	14.5-31.5	10.2-46.4	16.4-37.9	11.9-36.4
Thigh/distal part of leg							
Mean ± SD	1.6 ± 0.6	1.6 ± 0.7	1.8 ± 1.1	1.6 ± 0.7	1.6 ± 0.8	5.1 ± 10.0	2.3 ± 2.2
Range	0.9-2.5	0.3-2.7	0.2-4.5	0.9-3.1	0.7-2.9	0.9-31.8	0.9-9.1
Median	1.4	1.5	1.7	1.4	1.4	1.9	1.6
80th percentile interval	1.0-2.4	0.8-2.4	0.9-3.7	1.0-2.6	0.7-2.9	1.0-20.3	0.9-4.9
P (Mann-Whitney test), distal part of leg vs thigh	.06	.02	.03	<.001	.13	.01	.02

\*P = .03 across age groups (Kruskal-Wallis test).  
†P = .005 across age groups (Kruskal-Wallis test).



**Figure 2.** Interobserver reliability, showing the degree of agreement among 3 independent observers measuring intraepidermal nerve fiber density. Observers 1 and 2 were technicians; observer 3 was one of us (E.A.S).



**Figure 3.** Effects of age on intraepidermal nerve fiber density at the distal part of the leg by age decile for healthy controls.

sociation between density measurements from adjacent sections. The correlation coefficient for sections from the same series of slides was 0.97, and for separate quantitative series of slides from the same punch it was 0.98.

**Variation Between Adjacent Biopsy Specimens at Same Site: Intrasite Variability.** Because several punches were taken from the same site (thigh or distal part of the leg), we were able to compare intrasite variation between 2 punches processed simultaneously. The correlation coefficient ( $r = 0.87$ , distal part of the leg;  $r = 0.86$ , thigh) indicates a high degree of association between the 2 punches at the same anatomical site.

#### Derivation of Reference Range for Normal Epidermal Nerve Fiber Density

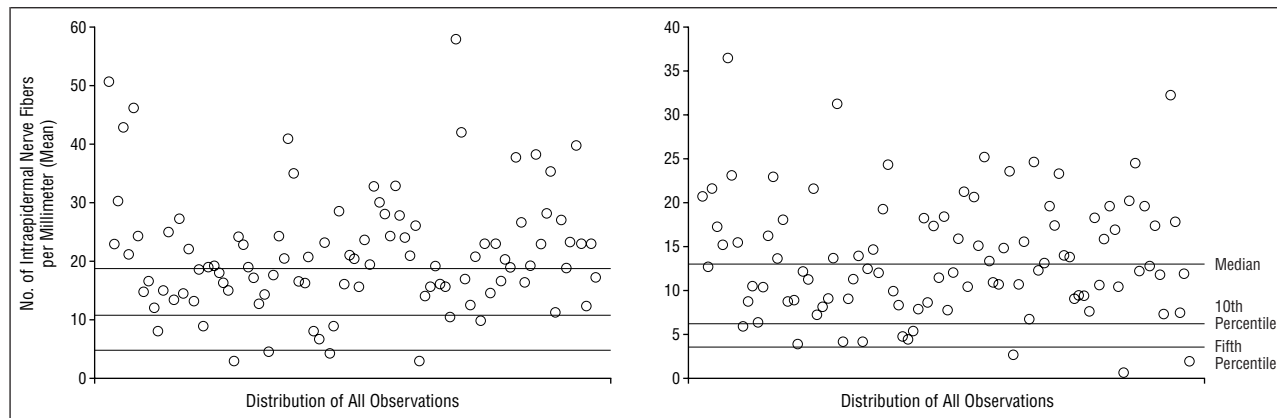
**Table 2** is a summary of the intraepidermal nerve fiber density data for all 98 controls, stratified by age decile, indicating mean values and the distributions. The in-

traepidermal nerve fiber values at the 2 sites were normally distributed (data not shown). The fifth percentile for the thigh was 5.2 fibers per millimeter, for distal part of the leg it was 3.8 per millimeter, and for the ratio of thigh to distal part of the leg it was 0.72 per millimeter. Significantly higher intraepidermal nerve fiber values were noted only for the youngest age decile, but overall there was no significant effect of age for older age groups (**Figure 3**).

**Figure 4** demonstrates the normal distribution of intraepidermal nerve fiber density among the 98 healthy controls. Overall, there was no significant influence of race, height, weight, or sex on intraepidermal nerve fiber density.

#### Variation Between Anatomical Sites: Thigh and Distal Part of the Leg

Epidermal nerve fiber densities at the thigh and distal part of the leg sites were compared to assess whether



**Figure 4.** Epidermal linear density of nerve fibers at the thigh (left) and distal part of the leg (right) for healthy controls.

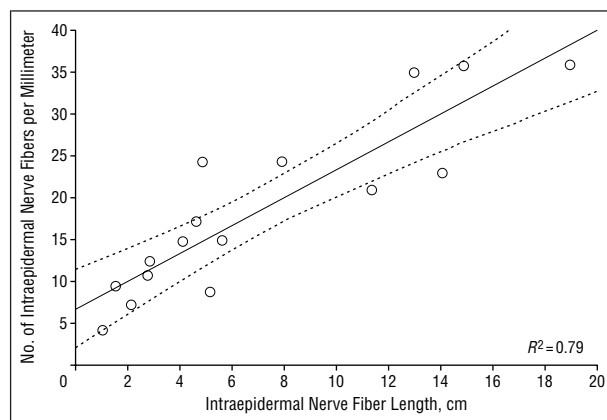
there was a proximal-distal gradient. The correlation coefficient ( $r = 0.56$ ,  $P = .01$ ) indicates a significant relationship between intraepidermal nerve fiber densities for each individual at the 2 sites. The density at thigh sites was, in general, 60% higher than that at the distal part of the leg, confirming a proximal-distal gradient in normal controls.

#### Relationship Between Stereological Quantitation and Linear Density Estimates of Epidermal Innervation

**Stereological Quantitation of Epidermal Nerve Fiber Length.** Stereological estimates of intraepidermal length were determined on a randomly selected subset of the normal controls, and the degree of association with the linear density estimates was determined. This provided a measure of internal consistency and construct validity for the linear density measure.

Full details of the stereological technique and results were described by Stocks et al.<sup>25</sup> Stereology was completed in 8 individuals on a total of 16 punches from thigh and distal part of the leg and related to intraepidermal nerve fiber density determined in the same sections. **Figure 5** shows the relationship between estimated intraepidermal fiber length (stereology) and intraepidermal nerve fiber linear density. The correlation coefficient is 0.79 ( $P = .001$ ), suggesting that the 2 quantitative techniques are internally consistent.

**Cutaneous Nerve Fibers in Neuropathies.** In the individuals with symptomatic sensory neuropathies, we noted an obvious reduction in the numbers of intraepidermal fibers, as Holland et al<sup>22</sup> and McCarthy et al<sup>26</sup> previously reported. In general, there was greater reduction at the distal part of the leg site than in the thigh. In several cases with severe neuropathies, the dermis contained fibers with increased varicosities and segmentation presumably representing degenerating fibers; the epidermis was devoid of PGP9.5-stained fibers. In general, this loss of intraepidermal fibers appeared to parallel the decrement in the number of fibers in the papillary dermal plexus, but in some cases it was more dramatic and out of proportion to the loss of dermal fibers. Significant reductions ( $P = .001$ ) in the density of intraepidermal fibers were



**Figure 5.** Relationship between stereological estimates of total intraepidermal nerve fiber length and intraepidermal nerve fiber density for healthy controls.

found when controls were compared with patients with sensory neuropathy at both the thigh and the distal part of the leg. This difference was most marked at the distal part of the leg, reinforcing the concept that the patients with sensory neuropathy had length-dependent neuropathies.<sup>22</sup> The ratio of densities at the thigh to the distal part of the leg was elevated, confirming that intraepidermal fiber loss is more marked at distal sites.

#### Specificity Analysis

Using the cutoff values for the 10th or fifth percentile for intraepidermal nerve fiber density, we calculated the performance characteristics with the use of the normal controls and the cases of sensory neuropathy. The efficiency (percentage correctly classified) ranged from 82% to 88%, depending on the anatomical site and the cutoff used. Overall, the highest specificity, efficiency, and positive and negative predictive values were obtained with the use of the fifth percentile cutoff at the distal part of the leg (**Table 3**).

#### COMMENT

These studies show that small, unmyelinated fibers can be identified and quantified in a simple manner within

**Table 3. Performance Characteristics of Intraepidermal Nerve Fiber Linear Density Measure\***

Percentile	Sensitivity, %		Specificity, %		Efficiency, %		Positive Predictive Value, %		Negative Predictive Value, %	
	Thigh	Distal Part of Leg	Thigh	Distal Part of Leg	Thigh	Distal Part of Leg	Thigh	Distal Part of Leg	Thigh	Distal Part of Leg
10th	45	60	88	90	82	86	45	55	89	75
5th	25	45	96	97	84	88	56	92	86	90

\*Sensitivity (percentage true positive); specificity (percentage true negative); efficiency (percentage correctly classified); positive predictive value (percentage with abnormal test result, ie, below cutoff point, who truly have neuropathy); and negative predictive value (percentage with normal test result, ie, above cutoff point, who truly do not have neuropathy).

epidermis by means of punch biopsies and immunocytochemical staining techniques. In the normal controls, we were able to visualize deeply penetrating intraepidermal fibers, the dermal plexus of nerve fibers, various sensory transducers, and supporting cells, including Langerhans cells and Schwann cells. The morphologic findings we observed were identical to those described more than 3 decades ago by Arthur and Shelley,<sup>29</sup> Kruger et al,<sup>10</sup> and Ridley.<sup>7</sup> In this study, we developed a normative range for intraepidermal nerve fiber density in the leg and showed that the sectioning and staining technique is robust, that the quantitation system is reliable, and that it correlates well with a separate unbiased sampling method, the stereological estimation of nerve fiber length. Finally, with the use of patients with sensory neuropathy and the intraepidermal nerve fiber normative range, we examined the performance characteristics of the linear density measure and demonstrated its high specificity and acceptable efficiency for distinguishing neuropathic intraepidermal nerve fiber values from normal. The simplicity and repeatability of this technique give it relevance for the study of small-fiber neuropathies. Several groups have recently used the technique to identify and enumerate cutaneous nerve fibers in postherpetic neuralgia<sup>30</sup> and idiopathic small-fiber sensory neuropathy.<sup>31</sup> Recently, Kennedy et al<sup>18</sup> used this marker with confocal microscopy to quantify epidermal and sweat gland innervation and showed that epidermal nerve fiber density correlates with the severity of peripheral neuropathy in diabetic patients.<sup>19</sup>

#### DEVELOPMENT OF NORMATIVE RANGE FOR EPIDERMAL INNERVATION

Previous studies by Dyck et al<sup>11</sup> examining the change in Meissner corpuscles in neuropathies and with age, and by Ridley<sup>7</sup> studying cutaneous nerves, concluded that there was substantial interindividual and anatomical variation. This hindered the development of skin biopsy as a quantitative technique.

The range of normal values in our study is somewhat lower than that recorded by Kennedy et al,<sup>19</sup> who found a range at the calf–distal part of the leg site of 15 to 45 per millimeter compared with our range of up to 35 per millimeter. Erdem et al<sup>32</sup> also reported somewhat higher values than ours. Both studies used confocal microscopy and Zamboni fixative, and counting rules may have differed from our conventional microscopic approach. When a similar technique is used, results are

concordant with ours. For example, using identical staining and sectioning procedures and conventional microscopy, Leland Scott, MD, (written communication, December 1, 1997) found mean values of free nerve endings per linear millimeter of  $5.0 \pm 0.7$  per millimeter at the distal part of the leg and  $15 \pm 1.5$  per millimeter at the thigh.

Despite the wide biological variation in the data, the distribution of intraepidermal nerve fiber densities at the distal part of the leg permits the delineation of a lower limit of normal based on the fifth percentile value for the normal group. With the fifth percentile cutoff value, the percentage correctly classified among the neuropathy cases is 88%. The sensitivity is 45%; specificity, 97%; positive predictive value, 92%; and negative predictive value, 90%. The high specificity of the measure increases the positive predictive value of the test and is therefore ideal for its anticipated use, namely, to verify the presence of a disease for which there may be little clinical or electrophysiologic evidence, or when the clinician must be virtually certain of a diagnosis, for example, before initiating some form of therapy.<sup>33</sup> However, the relatively low sensitivity of the measure implies that a normal intraepidermal nerve fiber density does not rule out the presence of a sensory neuropathy. Potential limitations of our technique include the dependence on observer accuracy in distinguishing intraepidermal nerve fibers from false-positive staining and dermal nerve fibers. The intraobserver and interobserver reliability, however, was excellent, suggesting that the measure has high precision. In widening studies, we are assessing changes in branching patterns, extending the single quantitation system to quantify the degree of branching of intraepidermal nerve fibers. In preliminary observations, it appears that increased branching of intraepidermal nerve fibers can occur before a change in the overall density of intraepidermal nerve fibers (Giuseppe Lauria, MD, written communication, June 7, 1997). Our studies in humans are given additional validity by the experimental work in animals of Hsieh et al<sup>34</sup> and Li et al.<sup>35</sup> Thus, similar patterns of intraepidermal nerve fiber loss were observed in rats by Hsieh et al<sup>34</sup> after sciatic nerve transection and by Li et al<sup>35</sup> after dorsal rhizotomy.

#### CHANGES IN EPIDERMAL NERVE FIBERS WITH AGE

We did not find a significant decrement in intraepidermal nerve fiber density with age, except for higher values in the youngest subjects, aged 10 to 19 years. The

absence of an aging effect in intraepidermal innervation runs counter to intuition, given the well-documented aging changes in cerebral cortex.<sup>36,37</sup> Aging produces several modifications to both neuronal and nonneuronal structures: smoothing of the dermal-epidermal junction,<sup>38</sup> loss of fibers to sweat glands<sup>39</sup> and Meissner corpuscles,<sup>11,40</sup> loss of small myelinated and unmyelinated fibers in the distal sural nerve,<sup>41,42</sup> and appearance of dystrophic axonal swellings in dorsal root ganglia neurons.<sup>43</sup> Joko et al<sup>44</sup> also showed that with increasing age, epidermal branches of the cutaneous nerves begin to degenerate and appear to be thin and sparse compared with those in younger age groups. They also found that in the older individuals, nerve bundles within the dermis were more irregular and thicker than in the younger group. This group used laser scanning confocal microscopy combined with quantitative analysis using NIH Image software (version 1.62, Research Services Branch of the National Institute of Mental Health, National Institutes of Health, Bethesda, Md; public domain software available at: <http://rsb.info.nih.gov/NIH-IMAGE/>) to measure nerve fiber length with PGP9.5 immunostaining. Furthermore, experimental studies reported that older animals show shrinkage of the sensory fields related to small-fiber function, in addition to a decreased collateral sprouting.<sup>45</sup> We remain surprised by these observations and cannot fully explain the absence of age effect on intraepidermal nerve fibers. We have replicated these observations in a separate set of controls. This finding is similar to other investigators' observations. For example, Ridley,<sup>7</sup> using silver staining, did not find any variation in epidermal innervation with aging, and Erdem et al<sup>32</sup> also found little variation except in the oldest individuals.

## CONCLUSIONS

This technique has a number of advantages over conventional nerve biopsy for the study of sensory neuropathies, and is simple, painless, and associated with minimal morbidity. The quantitation technique could be performed by any laboratory without specialized equipment, provided that attention is given to reliability training and accuracy of counting. The small unmyelinated nerve fibers are visualized clearly with light microscopy with the use of commercially available stains, and the linear density measure correlates well with more labor-intensive criterion standard stereological techniques of estimation of intraepidermal nerve fiber density. Finally, the technique may be repeated serially to assess progression of neuropathy and therapeutic response.

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