Epidermal nerve fibers

Confidence intervals and continuous measures with nerve conduction

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ABSTRACT

Objectives: Our first objective was to explore the value of estimating 95% confidence intervals (CIs) of epidermal nerve fibers (ENFs)/mm for number of sections to be evaluated and for confidently judging normality or abnormality. Our second objective was to introduce a new continuous measure combining nerve conduction and ENFs/mm.

Methods: The 95% CI studies were performed on 1, 1-2, 1-3 --- 1-10 serial skip sections of 3-mm punch biopsies of leg and thigh of 67 healthy subjects and 23 patients with diabetes mellitus.

Results: Variability of differences of ENFs/mm counts (and 95% CIs) from evaluation of 1, 1-2, 1-3 --- 1-9 compared with 1-10 serial skip sections decreased progressively without a break point with increasing numbers of sections evaluated. Estimating 95% CIs as sections are evaluated can be used to judge how many sections are needed for adequate evaluation, i.e., only a few when counts and 95% CIs are well within the range of normality or abnormality and more when values are borderline. Also provided is a methodology to combine results of nerve conduction and ENFs/mm as continuous measures of normality or abnormality.

Conclusion: Estimating 95% CIs of ENFs/mm is useful to judge how many sections should be evaluated to confidently declare counts to be normal or abnormal. Also introduced is a continuous measure of both large-fiber (nerve conduction) and small-fiber (ENFs/mm) normal structures/functions spanning the range of normality and abnormality for use in therapeutic trials. *Neurology*® 2012;79:2187-2193

GLOSSARY

CI = confidence interval; **DM** = diabetes mellitus; **DSPN** = diabetic sensorimotor polyneuropathy; **ENF** = epidermal nerve fiber; **HS** = healthy subjects; **nd** = normal deviate; **Σ 5 NC nds** = summated standard normal deviates of 5 attributes of nerve conduction.

Enumeration of nerve endings in skin has been used to study differences among species,^{1,2} development, maturation and aging,^{3,4} and disease.^{5,6} Light microscopic analysis of immune-stained intra-epidermal nerve fibers (ENFs) and reference values were introduced to serve as objective and spatial indications of painful and small-fiber sensory polyneuropathy, “possibly useful for therapeutic trials.”⁷⁻⁹ The technique of biopsy was found to be acceptable to patients, and the estimation of ENFs/mm had high intraobserver and interobserver reproducibility.⁸ A somewhat different approach using punch skin biopsy, a blister technique with immunofluorescence and confocal microscopy, was also described (reviewed in Kennedy and Wendelschafer-Crabb¹⁰ and Kennedy et al.¹¹). A series of reports showed the value of ENFs/mm in the diagnosis of painful small-fiber sensory polyneuropathy, especially in patients with few or no neurologic signs or test abnormalities.¹²⁻¹⁹ Decreased ENFs/mm has also been shown to be characteristic of generalized sensorimotor neuropathies of various kinds.^{7,13,15,20-27} Consensus panels have accepted the assessment of ENFs as objective and valid indications of small sensory nerve fiber involvement in disease.^{19,28,29}

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Supplemental Data

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The present study addresses 2 questions: 1) Should confidence intervals (CIs) be added to estimates of ENFs/mm to help decide the number of sections to be evaluated and to confidently judge normality or abnormality? 2) What approach can be used to combine in a continuous measure spanning the range of normality and abnormality ENFs/mm (class I evidence of small sensory fiber involvement) with an objective and quantitative indication of large-fiber polyneuropathy?³⁰

METHODS **Description of the healthy subject and diabetes mellitus cohorts.** To address the 2 issues: 1) the usefulness of 95% CIs, and 2) combining measures of nerve conduction with ENFs/mm, the 3-mm punch biopsied specimens of 32 young adults, 35 elderly healthy subjects (HS), and 23 patients with diabetes mellitus (DM) were studied.

Three-millimeter punch biopsies of leg and thigh, at standard sites, were obtained under local anesthesia after obtaining informed consent and using a protocol of study approved by Mayo Clinic's Institutional Review Board. All HS and patients with DM were clinically evaluated and underwent nerve conduction studies of ulnar, peroneal, tibial, and sural nerves and quantitative sensation tests to establish normality or abnormality. The young HS cohort consisted of 16 men and 16 women, median ages of 31 (range 18–34) and 31 (range 19–34) years, respectively. Comparable numbers of elderly HS were 15 men and 20 women with median ages of 76 (range 70–86) and 79 (range 70–89) years, respectively.

Histologic processing of skip sections for estimation of ENFs/mm. The biopsy specimens were fixed and reacted with protein gene product 9.5 by standard approaches that have been extensively described previously.^{7,8} Fifty-micrometer serial sections were cut (at right angles to the surface of skin) and sections 5, 7, 9 - - - 23 (hereafter referred to as 1, 2, 3 - - - 10 serial skip sections) were available for evaluation of ENFs/mm; the first 4 sections were usually too short and incomplete to be evaluated.

Enumerating ENFs/mm. Evaluation of numbers of ENFs crossing the dermal/epidermal interface was facilitated by a program developed for our Imaging System for Nerve Morphometry.³¹ Using a cursor and a programmed evaluation developed by members of our institution (D.J., R.C., J.D.), the lengths of the dermal/epidermal interface minus openings of glands and hair were measured. A standard reference microscope reticle was used to correct for microscopic and imaging magnification. High-dry microscopy was used for counting ENFs. Counts of ENFs/mm and 95% CIs were based on ENFs crossing the dermal-epidermal boundary. The exact CI for a Poisson random variable was used. Details regarding the CI estimation are provided as a technical appendix (appendix e-1 on the *Neurology*[®] Web site at www.neurology.org).

ENF counts were done by one of us (L.W. or B.H.) and were rechecked by another one of us (J.E.) by checking samples of the sections. Then, to test for reproducibility, counts were retested of every fifth HS and the last 10 of every patient with DM; earlier sections from patients with DM were evaluated even more intensively. For these evaluations, observers were masked to the identity of the specimen. Reproducibility was tested using the intraclass correlation coefficient.

Split cell error. To decide whether corrections should be made for split cell error,^{32,33} assuming that the same unmyelinated nerve fiber may have been cleaved in some cases and seen in different

tissue sections, we considered whether we should correct for split cell error. To do this, we assumed that nerve fibers range in diameter from approximately 0.25 to 1.5 μm , have a section thickness of 50 μm , and that Abercrombie formula applied.³²

Expressing results as ENFs/mm² of surface area. Consideration was also given to expressing ENFs as number/mm² of surface area.

Rationale and approaches used to develop composite scores of nerve conduction and ENFs/mm. Standard normal deviate (nd) scores may be useful for comparing different neurophysiologic and morphometric test results and to fashion composite and continuous measures of severity. Composite scores of nerve tests are especially useful for a condition such as diabetic sensorimotor polyneuropathy (DSPN) because this disease is made up of different symptoms, signs, dysfunctions, and abnormal test results. A composite score of appropriately chosen test results is therefore a better measure of DSPN than a single or individual marker. Composite scores of nerve conduction of leg nerves allow the investigator to combine diverse test attributes that are the most sensitive and representative of a multifaceted condition such as DSPN. To illustrate, summated standard nds of 5 attributes of nerve conduction (Σ 5 NC nds) include fibular motor nerve fiber amplitude, velocity and distal latency, tibial motor distal latency, and sural sensory nerve amplitude, expressing all abnormalities in the same tail of the normal distribution (either upper or lower). All percentile values of attributes are corrected for known applicable variables, e.g., age, gender, and physical characteristics, and are expressed as standard nds. Using this *z* score approach, it is possible to define abnormality at a given percentile, e.g., $\leq 5\text{th}$, $\leq 2.5\text{th}$, or $\leq 1\text{st}$ percentile, avoiding type I error.³⁰ Assuming that reference percentile values for the composite score are available, percentile values can be set for the composite score. We have previously provided reference values for Σ 2 NC, Σ 4 NC, Σ 5 NC, and Σ 6 NC, but other nd composite scores can be derived.³⁴

Attributes of nerve conduction provide an objective and reliable indication of large-fiber function and dysfunction but do not provide direct information about small-fiber function or dysfunction. To provide an objective and quantitative indication of both large and small sensory fiber involvement, we combined a composite measure of large-fiber function, i.e., Σ 5 NC nds, and a measure of small fibers (ENFs/mm nds), and corrected both measures for applicable variables of age, gender, and physical characteristics. The composite score, Σ 5 NC nds and ENFs/mm nds of the 67 HS, was plotted on age to develop 50th and 2.5th percentile lines to serve as measures of normality and abnormality.

RESULTS **Correction for split cell error.** The calculated split cell error using Abercrombie formula was found to be $<1\%$ —therefore not sufficiently large to correct ENF/mm estimates.

Expressing results as ENFs/mm² rather than as ENFs/mm. Although it makes sense to express results as ENFs/mm^{2,3–5,11} this was not done because of previous precedence and because it is such a simple matter to convert ENFs/mm to ENFs/mm², simply by multiplying the former value by 20 (not by squaring the former number).

Effect on numbers of ENFs/mm from evaluation of 1, 1–2, 1–3, 1–4 - - - 1–9 serial skip sections compared with evaluation of 1–10 serial skip sections. In figure 1, we show the difference of estimated ENFs/mm from evaluation of 1, 1–2, 1–3, 1–4 - - - 1–9 serial skip sections compared

Figure 1 Decrease in variability of 95% confidence intervals (CIs) with increased number of sections evaluated

Shown are differences in the assessed number of epidermal nerve fibers (ENFs)/mm from assessment of 1, 1-2, 1-3, 1-4 --- 1-9 serial skip sections compared with 1-10 sections of punch biopsy specimens of distal leg and thigh from 67 healthy subjects (HSs) and 23 patients with diabetes mellitus (DM). Note that this variability decreases progressively without a break point with increased numbers of sections evaluated. In the text, we provide the rationale for expressing estimated ENFs/mm providing 95% CI.

with evaluation of 1–10 serial skip sections. This variability is expressed in absolute numbers and as CIs. For both leg and thigh sites, and for HS and patients with DM, there was a progressive decrease (without a break point) in variability of this difference with increasing number of serial skip sections evaluated (figure 1). The decrease in variability of CIs is also included in figure 1. Note that the decrease in variability of the differences from evaluation of 1–10 sections extends well beyond that for evaluation of 4 sections (the number of sections now frequently evaluated in medical practice).

Intraobserver test-retest reproducibility. Test-retest reproducibility for the first, 1–4, and 1–10 serial skip sections of the sampled HS and DM sections was 0.81, 0.84,

and 0.92 (intraclass correlation [1,1]s)—very high in all cases but increasingly higher with number of sections evaluated.

Illustrating the value of expressing ENFs/mm with 95% CIs.

The HS values of ENFs/mm, shown for illustrative purposes, are similar to those obtained in previously published studies.^{1,35} Also, as found by others,^{7,8,13,36} ENFs/mm were significantly higher in thigh than in leg and decreased density was related to old age and male gender. For illustrative purposes, we show values and regression lines for males and females combined and we place abnormality at the 2.5th percentile value, rather than at the 5th percentile value, ignoring gender differences.

We show the ≤ 2.5 th percentile line to make it the same as we have chosen for $\Sigma 5$ NC nds (see below).

In figure 2, we superimpose estimates of ENFs/mm and 95% CIs of 5 patients (A–E) with DM, based on evaluation of 1, 1–4, and 1–10 serial skip sections. Note that for patients A, B, and C, although estimates of ENFs/mm based on 1, 4, and 10 sections all fall below the 2.5th percentile line (abnormal), 95% CIs from examination of 1 section extend into the range of normal values for all 3 patients. From evaluation of 1–4 sections, 95% CIs extend into the normal range in only 1 patient (patient A), but with examination of 1–10 sections, 95% CIs do not extend into the normal range in any case. Therefore, based on evaluation of 10 skip sections, all 3 patients could confidently be declared as having abnormal ENFs/mm. For patient D, evaluation of 1 section places the measured value in the normal range but the 95% CI extends into the abnormal range. With evaluation of 1–10 sections, the mean value of ENFs/mm and 95% CIs places the ENFs/mm value confidently into the abnormal range. For patient E, even evaluation of 1 section places estimated ENFs/mm and 95% CIs well within the normal range. Considering the results obtained in these examples, 2 inferences seem justified: 1) estimating the ENFs/mm of each section evaluated can be used to help determine the number of sections that need to be evaluated to confidently know whether the obtained estimate is normal or abnormal, and 2) 95% CIs are useful to determine the degree of certainty that can be attributed to a given estimate of ENFs/mm.

Composite nerve conduction ($\Sigma 5$ NC nds) measures in 67 HS and 5 representative patients (A–E) with DM. These data are shown in figure 3. The values of $\Sigma 5$ NC nds of

HS were reasonably distributed around the 50th percentile line based on a previously studied HS cohort.³⁷ None of the HS values were below the 2.5th percentile line. Values for 3 of the patients (A–C) were below the 2.5th percentile line, whereas values for patients D and E were just above the line. The information is provided so that results may be compared with their ENFs/mm (figure 2) and with composite scores of $\Sigma 5$ NC nds + ENFs nds (figure 3).

Composite scoring of $\Sigma 5$ NC nds and ENFs/mm nds in the HS cohort and in illustrated cases of diabetic polyneuropathy. In figure 3, we show the distribution of the composite nd scores of $\Sigma 5$ NC nds and ENFs/mm nds. Note that the values appear to be normally distributed around the 50th percentile line estimated from examination of the 67 HS. A 2.5th percentile line has been fitted for the data. Values for 3 of the illustrated patients with DM are well below the 2.5th line, whereas values for patients D and E are just below the line. Two patients with DM were reclassified as having neuropathy using the nerve conduction and ENFs/mm composite score.

DISCUSSION Counts of ENFs/mm were introduced as an objective and quantitative measure of small sensory fiber abnormality useful for medical practice and research.^{38,39} It provides class I evidence of abnormality of small sensory ENFs.²⁹ As demonstrated here, an attractive feature of the measure is that in addition to providing a dichotomous (normal/abnormal) indication of small-fiber polyneuropathy, it may also be used as a continuous measure spanning the range of normality and abnormality (not sufficiently emphasized previously).

The studies reported herein focus on 2 major issues alluded to in an editorial⁴⁰ but not rigorously tested previously: 1) the influence of CIs on number of sections to be evaluated and the degree of certainty of ENFs/mm estimates in diagnosis, and 2) the approaches and uses that can be made of continuous measures of composite scores of ENFs/mm and $\Sigma 5$ NC nds in cohort studies and in therapeutic trials.

By study of 1, 1–2, 1–3, 1–4 – – – 1–9 serial skip sections in 67 HS and 23 patients with DM as compared with evaluation of 1–10 serial skip sections, we found that the variability of estimated ENFs/mm decreased progressively without a definite break point with increasing number of serial skip sections evaluated. This decrease in variability can be measured by use of the 90% or 95% CI. This information can be used to decide how many sections should be evaluated in a given case and secondly used for the interpretation of results as normal or abnormal. Considering the first point, knowing the ENFs/mm value and its 95% CI of sequentially evaluated sections, i.e., 1, 1–2, 1–3 – – – 1–nth, can be

Figure 2 Epidermal nerve fibers (ENFs)/mm plotted with age in healthy subjects (HSs) (open circles) and selected patients with diabetes mellitus (DM) (A–E)

Plotted are values of ENFs/mm based on the evaluation of 10 serial skip sections of distal leg in 67 HSs (32 young and 35 elderly subjects). The graph is provided to show the 50th and the ≤ 2.5 th percentile line based on evaluation of the studied HSs. For the 5 illustrated patients with DM, observe the influence on the 95% confidence interval (CI) from evaluation of 1, 4, and 10 serial skip sections (discussed in detail in Results). Clearly, when ENFs/mm are near the lower limit of normal (either just abnormal or just normal), a small 95% CI helps the physician determine whether the estimated values of ENFs/mm are indicative of normality or abnormality. *Sections.

Figure 3 Composite nerve conduction [summed standard normal deviates of 5 attributes of nerve conduction ($\Sigma 5$ NC nds)] measures in 67 healthy subjects (HSs) and 5 representative patients (A-E) with diabetes mellitus (DM)

(A) Composite nerve conduction values of $\Sigma 5$ NC nds in HSs (open circles) and selected patients with DM (solid circles). This figure is a plot of the composite score of $\Sigma 5$ NC nds of the 67 HSs and the 5 representative patients with DM whose epidermal nerve fibers (ENFs) are illustrated in figure 2. Note that the distribution of NC values around the 50th percentile line and the ≤ 2.5 th lower limit (from values of a previously studied HS cohort⁵) appears to fit reasonably well with the distribution of values of the presently studied 67 HSs. There is a striking difference between ENFs/mm (figure 2) and $\Sigma 5$ NC nd values for the selected patients with DM (this figure). There are striking differences in the relative position, especially of patients C and E, between these 2 measures. (B) Composite $\Sigma 5$ NC nds + ENFs/mm nds with age in HSs (open circles) and patients with DM (solid symbols). Shown here are plotted values of $\Sigma 5$ NC nds + ENFs/mm on age. Superimposed are the values for the 5 representative patients with DM. As considered in the Discussion section, this graph illustrates normal values of 67 HSs and the 5 representative patients with DM. *Abnormality is in the lower tail. **Based on $n = 330$ Rochester Diabetic Study Healthy Subjects.

used to decide how many sections need to be evaluated in a given case. Thus, if very large or very small numbers of ENFs/mm are found in the first few sections, even with large CIs, the 95% CI values may be well within the range of normality or abnormality so that additional sections need not be evaluated. However, if 95% CIs are either borderline normal or abnormal, additional sections should probably be evaluated, e.g., all 10 serial skip sections. Obviously, derived counts and CIs relate only

to the region of skin evaluated—extrapolation to the more general anatomic region requires other evidence. The following practical approach can be recommended. Cut and react (with protein gene product 9.5) 10 serial skip sections. Obtain ENFs/mm and 95% CI from evaluation of 2 sections, the first chosen at random and the second 5 sections removed. If 95% CIs are well within normal or abnormal ranges, no further evaluation is needed. If 95% CIs are borderline, evaluate all 10 serial skip sections.

ENFs/mm are typically thought of as dichotomous values (i.e., either normal or abnormal), but for some purposes (e.g., epidemiology surveys, cohort studies, and therapeutic trials), they may be used as continuous measures spanning normality and abnormality. To be able to compare them directly with other clinical, physiologic, or morphometric measures, all measurements need to be converted to standard nd (z) scores. We use nd scores for 2 major reasons: 1) to be able to compare disparate end points with each other, and 2) to combine disparate end points into a single composite score. Use of composite scores of neurophysiologic and morphometric test results have considerable value for a condition such as DSPN, which is made up of disparate neurophysiologic tests; morphometric measurement; and clinical dysfunction, impairments, and signs and symptoms. In fashioning such composite scores, we suggest that components be chosen that are representative of the condition and frequently abnormal and unlikely to be abnormal from another cause than the medical condition studied. For DSPN, the composite score $\Sigma 5$ NC nds includes 4 functions, 3 different nerves, and these nerves are in the leg typically affected in diabetic polyneuropathy. Composite nd scores can be used to recognize a neurologic condition as present or absent (dichotomous assessment) or as a continuous measure spanning the range of normality or abnormality—the latter especially useful in therapeutic trials.

In the present study, we have illustrated the approach to combine normal values of $\Sigma 5$ NC nds and ENFs/mm and to set percentile values for 97.5th to 2.5th percentile based on study of 67 HS. We have then illustrated how the composite score was used to determine abnormality in 5 patients with DM. This composite score might find use in therapeutic trials of polyneuropathies with both large- and small-fiber involvement. Polyneuropathies with both large and small sensory fiber involvement include DSPN, trans-thyretin amyloid polyneuropathy, chronic inflammatory sensory polyradiculoneuropathy (Sjögren neuropathy), hereditary sensory and autonomic neuropathies, and a variety of other neuropathies.

AUTHOR CONTRIBUTIONS

JaNean K. Engelstad: acquisition of data and critical revision of the manuscript. Dr. Sean W. Taylor: study concept/design, acquisition of data, and critical revision of the manuscript. Larry V. Witt: acquisition of data

and critical revision of the manuscript. Belinda J. Hoebing: acquisition of data and critical revision of the manuscript. Dr. David N. Hermann: study concept/design, acquisition of data, analysis/interpretation, and critical revision of the manuscript. Dr. P. James B. Dyck: study concept/design, analysis/interpretation, and critical revision of the manuscript. Dr. Christopher J. Klein: study concept/design, analysis/interpretation, and critical revision of the manuscript. David M. Johnson: acquisition of data and critical revision of the manuscript. Jenny L. Davies: acquisition of data, analysis/interpretation, and critical revision of the manuscript. Dr. Rickey E. Carter: study concept/design, analysis and interpretation, and critical revision of the manuscript. Dr. Peter J. Dyck: study concept/design, acquisition of data, analysis/interpretation, critical revision of the manuscript, and study supervision.

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DISCLOSURE

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Effective January 15, 2009, authors submitting Articles or Clinical/Scientific Notes to *Neurology*® that report on clinical therapeutic studies must state the study type, the primary research question(s), and the classification of level of evidence assigned to each question based on the AAN classification scheme requirements. While the authors will initially assign a level of evidence, the final level will be adjudicated by an independent team prior to publication. Ultimately, these levels can be translated into classes of recommendations for clinical care. For more information, please access the articles and the editorial on the use of classification of levels of evidence published in *Neurology*.¹⁻³

1. French J, Gronseth G. Lost in a jungle of evidence: we need a compass. *Neurology* 2008;71:1634–1638.
2. Gronseth G, French J. Practice parameters and technology assessments: what they are, what they are not, and why you should care. *Neurology* 2008;71:1639–1643.
3. Gross RA, Johnston KC. Levels of evidence: taking *Neurology*® to the next level. *Neurology* 2009;72:8–10.