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EFNS/PNS SKIN BIOPSY GUIDELINE

European Federation of Neurological Societies/Peripheral Nerve Society Guideline on the use of skin biopsy in the diagnosis of small fiber neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society

Joint Task Force of the EFNS and the PNS[†]

Abstract Revision of the guidelines on the use of skin biopsy in the diagnosis of peripheral neuropathy, published in 2005, has become appropriate due to publication of more relevant papers. Most of the new studies focused on small fiber neuropathy (SFN), a subtype of neuropathy for which the diagnosis was first developed through skin biopsy examination. This revision focuses on the use of this technique to diagnose SFN. Task force members searched the Medline database from 2005, the year of the publication of the first EFNS guideline, to June 30th, 2009. All pertinent papers were rated according to the EFNS and PNS guidance. After a consensus meeting, the task force members created a manuscript that was subsequently revised by two experts (JML and JVS) in the field of peripheral neuropathy and clinical neurophysiology, who were not previously involved in the use of skin biopsy. Distal leg skin biopsy with quantification of the linear density of intraepidermal nerve fibers (IENF), using generally agreed upon counting rules, is a reliable and efficient technique to assess the diagnosis of SFN (level A recommendation). Normative reference values are available for bright-field immunohistochemistry (level A recommendation) but not yet for confocal immunofluorescence or the blister technique. The morphometric analysis of IENF density, either performed with bright-field or immunofluorescence microscopy, should always refer to normative values matched for age (level A recommendation). Newly established laboratories should undergo adequate training in a well established skin biopsy laboratory and provide their own stratified age and

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gender-matched normative values, intra- and interobserver reliability, and interlaboratory agreement. Quality control of the procedure at all levels is mandatory (Good Practice Point). Procedures to quantify subepidermal nerve fibers and autonomic innervated structures, including erector pili muscles, and skin vessels are under development but need to be confirmed by further studies. Sweat gland innervation can be examined using an unbiased stereologic technique recently proposed (level B recommendation). A reduced IENF density is associated with the risk of developing neuropathic pain (level B recommendation), but it does not correlate with its intensity. Serial skin biopsies might be useful for detecting early changes of IENF density, which predict the progression of neuropathy, and to assess degeneration and regeneration of IENF (level C recommendation). However, further studies are warranted to confirm the potential usefulness of skin biopsy with measurement of IENF density as an outcome measure in clinical practice and research. Skin biopsy has not so far been useful for identifying the etiology of SFN. Finally, we emphasize that 3-mm skin biopsy at the ankle is a safe procedure based on the experience of 10 laboratories reporting absence of serious side effects in approximately 35,000 biopsies and a mere 0.19% incidence of non-serious side effects in about 15 years of practice (Good Practice Point).

Key words: autonomic, evoked potentials, guidelines, immunohistochemistry, morphometry, nerve conduction study, nerve fibres, neuropathology, neuropathy, pain, protein gene product 9.5, quantitative sensory testing, skin biopsy

Objectives

This document is written under the auspices of the European Federation of Neurological Societies (EFNS; *www.efns.org*) and the Peripheral Nerve Society (PNS; *www.pnsociety.com*). The purpose is to revise the guidelines on the use of skin biopsy in the diagnosis of peripheral neuropathy *(Lauria et al., 2005)*. In the last 4 years, a considerable number of new papers have been published. These are mainly focused on small fiber neuropathy (SFN). The role of skin biopsy as a diagnostic tool was analyzed in the evidence-based review of the American Academy of Neurology, American Association of Neuromuscular and Electrodiagnostic Medicine, and American Academy of Physical Medicine and Rehabilitation *(England et al., 2009)*. Since skin biopsy retains a particular interest in clinical practice for the diagnosis of SFN, we focused the present guidelines on this specific subtype of neuropathy.

The revision includes recommendations on: (i) methods; (ii) safety; (iii) normative reference values; (iv) diagnostic yield; (v) correlation with other measures of neuropathy; (vi) use as outcome measure; (vii) EFNS/PNS standards; (viii) new studies to address unresolved issues.

Search Strategy

The Task Force systematically searched the Medline database from 2005, the year when the first ENFS guidelines were published *(Lauria et al., 2005)* to June 30th, 2009. For each specific issue, we stored all the articles published in English sorted by the Medline search using the following keywords: skin biopsy, punch biopsy, small fiber neuropathy, painful neuropathy, normative values, intraepidermal nerve fibers, cutaneous innervation, and skin nerves. We omitted those articles that were not pertinent, read and rated the remaining articles according to the guidance for EFNS guidelines *(Brainin et al., 2004)* and objectives of the current paper. In some cases, investigators were asked for original data and methodological details.

Method for Reaching Consensus

Data extraction was carried out and compared among each member of the Task Force. A first draft of the manuscript was prepared and diffused among the members of the task force. After revision, a second draft was prepared. Discrepancies on each topic were further discussed and settled during a consensus meeting held in Würzburg on July 7th, 2009. The third draft of the manuscript prepared after the consensus meeting was revised by an expert member of the task force (W.R. Kennedy) and two experts in the field of peripheral neuropathy (J.M. Leger) and clinical neurophysiology (J. Valls-Solé) not directly involved in the use of skin biopsy. The final version of the guideline is presented here.

Definition of Small Fiber Neuropathy

Definitions of SFN have been proposed and used by various authors *(Holland et al., 1998; Lacomis, 2002; Mendell and Sahenk, 2003; Said, 2003; Sommer, 2003; Hoitsma et al., 2004; Herrmann et al., 2004a; Lauria, 2005; Sommer and Lauria, 2006; Goodman 2007; Devigili et al., 2008; Bakkers et al., 2009; Nebuchennykh et al., 2009; Tavee and Zhou, 2009)*, but conclusive diagnostic criteria are not yet available. However, the most recent papers focusing on the clinical applications of skin biopsy in SFN used similar inclusion criteria for patients, based on normal sural NCS, clinical symptoms and signs considered suggestive and/or altered QST findings. The authors provided data on sensitivity and sensibility, from which we derived our level of recommendations.

Methological Issues

How to perform skin biopsy and choice of biopsy location

Skin biopsy is most commonly performed by means of a 3-mm disposable circular punch under sterile technique, after topical anaesthesia with lidocaine. No suture is required. A shallow biopsy (3-4 mm) is adequate to study epidermal nerve fibers, whereas a deeper biopsy (6-8 mm) is required to include sweat glands, hair follicles, artero-venous anastomosis. To optimise the sampling of such structures and of myelinated fibers in hairy skin, particular attention should be paid in order to include a hair in the specimen *(Provitera et al., 2007)*.

Earlier studies were performed in healthy subjects *(Dalsgaard et al., 1989)* and in patients with leprosy and diabetic neuropathy *(Karanth et al., 1989; Levy et al., 1989)*. The current technique was developed at the Karolinska Institute *(Wang et al., 1990)*, and later standardized at the University of Minnesota *(Kennedy and Wendelschafer-Crabb, 1993)* and at the Johns Hopkins University *(McCarthy et al., 1995)*.

A less invasive sampling method is the removal of the epidermis alone by applying a suction capsule to the skin. With this method there is no bleeding and local anaesthesia is not needed. However, the method does not provide information on dermal and sweat gland nerve fibers. Moreover, thus far it has not been systematically used to investigate patients with small fiber neuropathy. This technique was developed at the University of Minnesota *(Kennedy et al., 1999)*.

In most studies, hairy skin biopsies were obtained from the distal part of the leg (10 cm above the lateral malleolus), in some from the calf and the paraspinal region, and in many of them also from the upper lateral aspect of the thigh (20 cm below the anterior iliac spine) or other proximal locations where normal values are available. These locations were chosen to detect the length dependent loss of nerve fibers, which is typical of axonal polyneuropathy. These sites may also be sampled in the case of a non-length-dependent ganglionopathy. To evaluate receptors and myelinated fibers, glabrous skin biopsies are usually obtained from the tip or lateral aspect of the index or middle finger. Skin biopsy can be performed in other body sites to reveal a uni-lateral neural process. However, a control sample may be needed from the contralateral non-affected side for comparison of the innervation density *(Lauria and Lombardi, 2007)*.

Recommendations

For diagnostic purposes in length-dependent SFN, we recommend that a 3-mm punch skin biopsy be performed at the distal leg (10 cm above the lateral malleolus) for quantification of IENF density (level A recommendation). An additional biopsy from the proximal thigh may provide information about both length-dependent and non length-dependent processes (level C recommendation). When biopsy is taken from other body sites for evaluation of a uni-lateral process, a control biopsy from a similar non-affected region should be taken (Good Practice Point).

Tissue preparation

In neurology, punch skin biopsy has been primarily developed to evaluate both qualitatively and quantitatively IENF immunostained by the cytoplasmatic neuronal marker PGP 9.5, an ubiquitin carboxyl-terminal hydrolase. Antibodies against specific cytoskeletal (i.e., tubules and microtubules) *(Lauria et al., 2004)* and axonal membrane (i.e., Ga0) epitopes *(Polydefkis et al., 2004)* label the same number of PGP 9.5-positive IENF, suggesting that targeted markers could be used to investigate sensory endings. Antibodies against substance P and calcitonin-gene related protein have been also used *(Schulze et al., 1997)*.

After the biopsy is performed, the specimen is immediately fixed in cold fixative for approximately 24 h at 4°C, then kept in a cryoprotective solution for one night, and serially cut with a freezing microtome or a cryostat. Each biopsy yields about 50 vertical 50-μm sections. However, the first and the last few sections should not be used for nerve examination because of possible artefacts. Most studies for bright-field microscopy used 2% paraformaldehyde-lysineperiodate (2% PLP), whereas most studies for indirect immunofluorescence with or without confocal microscopy used Zamboni's (2% paraformaldehyde, picric acid) fixative. Formalin fixation should be avoided

for possible artefacts causing a more fragmented appearance of nerve fibers *(McCarthy et al., 1995; McArthur et al., 1998)*, though it did not affect the measurement of the innervation density *(Herrmann et al., 1999; Lauria et al., 1999)*.

Either bright-field immunohistochemistry or immunofluorescence with or without confocal microscopy have been used, but the technique does not affect the reliability of skin biopsy in assessing IENF loss in SFN *(Lauria et al., 2005)*. However, no study has been designed yet to compare the two techniques. In most studies using bright-field immunohistochemistry and immunofluorescence without confocal microscopy, at least three sections of 50 μm thickness from each biopsy were examined. In confocal microscopy studies, usually sections of 50–100 μm thickness were immunostained. Confocal microscopy allows analysing double, triple, and even quadruple stained sections. PGP 9.5 and collagen IV double stained sections were used to visualize axons and basement membrane in order to trace IENF from the site where they penetrate the basement membrane to their endings.

IENF morphometry and measurement reliability

Quantification of IENF density using bright-field immunohistochemistry was mostly based on the assessment of the number of fibers per linear measurement. Significant correlation with a stereologic technique *(Stocks et al., 1996)* supported the reliability of linear IENF density *(McArthur et al., 1998)*. IENF are counted either under the light microscope at high magnification (i.e., 40x objective) or using software for image analysis. The length of the epidermal surface is measured using software for biological measures (a freely available software is available at http://rsb.info.nih.gov/nih-image/index.html). The density is calculated in at least 3 sections as the number of IENF per length of the section (IENF/mm). Other studies reported the IENF density per skin surface area *(Koskinen et al., 2005; Panoutsopoulou et al., 2009)*.

Quantification of IENF density using confocal immunofluorescence technique is usually performed on images based on the stack of consecutive 2 μm optical sections (usually 16 sections) for a standard linear length of epidermis. The thickness of skin sections varies from 32 to 60 μm. Four epidermal areas are selected for confocal image acquisition, two images on each of two different sections excluding areas containing hair follicles and sweat ducts. For quantitative analysis, IENF are counted at high magnification, (i.e., 40x objective) for light microscope or (20x) for epifluorescence microscope using software for image analysis (e.g., Neurolucida, Microbrightfield) on digitised confocal images. Other semi-quantitative methods of IENF density estimation have been previously suggested *(Lauria et al., 2005)*. In both bright-field and immunofluorescence methods, single IENF crossing the dermal–epidermal junction are counted, whereas secondary branching is excluded from quantification. No study provided information on the rules for counting IENF fragments, which have been comprehensively reviewed by Kennedy and colleagues *(Kennedy et al., 2005)*.

Intra- and interobserver variability on IENF counts has been examined in several studies. Gøransson and colleagues *(Goransson et al., 2004)* assessed blindly the intraobserver (number of sections, 100) and interobserver (number of sections, 58) reliability. The mean difference in IENF by intraobserver analysis was 0.2 ± 1.2 IENF/mm. The 95% of the difference between paired counts was expected to lie within standard deviation, which is defined as the limit of agreement. For intraobserver variability, this limit of agreement was −2.2 to 2.6 IENF/mm. The interobserver variability was higher than the intraobserver variability, with a mean difference in IENF of 0.4 ± 1.5 fibers/mm. The limit of agreement was −3.4 to 2.6 IENF/mm. Most recently, Bakkers and colleagues *(Bakkers et al., 2009)* provided a blind assessment of intra- and interobserver variability (50 slides, 150 sections) within the Maastricht (NL) laboratory and the interlaboratory variability (30 slides, 90 sections) between the skin biopsy laboratories of Maastricht, Ferrara (I), and Milan (I) laboratories. Test-retest reliability was determined using the weighted kappa-statistic measures *(Cohen, 1968)*. The authors reported good intraobserver reliability values (weighted kappa = 0.95 and 0.90) and interobserver scores (0.94). The interlaboratory agreement values (weighted kappa) between three laboratories were: Maastricht-Milan = 0.78; Maastricht-Ferrara = 0.83; Milan-Ferrara = 0.91.

Wõpking and colleagues *(Wopking et al., 2009)* found an unexpected significant difference in IENF density between three observers, which is not in line with the high interobserver reliability reported before. There was no significant difference in IENF density between observers for biopsies with a well-defined basement membrane. The authors concluded that skin biopsies with an inexactly defined basement membrane should not be used diagnostically for the determination of IENF density.

The ''skin blister'' is an alternative technique to assess the epidermal innervation density *(Kennedy et al., 1999)*. This technique allows quantifying IENF in an area several times larger than the surface of a single 3 mm section and offers a horizontal perspective that makes immediately apparent an uneven distribution of nerve fibers and permits detailed analyses of IENF. However, this method does not allow evaluation of dermal structures and the time required

for blister formation (47-158 min) can be a limitation. Blisters are obtained by applying to the skin surface a suction capsule with single or multiple 2 or 3 mm holes depending upon the number and size of samples desired. A negative pressure induces the epidermis to separate at the dermal–epidermal junction without damaging the basement membrane and the underlying capillary loops. The application of a 3 mm tape disk (3M Tegaderm) on the area to be blistered prevents the blister roof from overstretching, facilitates its removal and flattens it for processing. After removing the capsule, the blister roof is excised, fixed, and immunostained. Panoutsopoulou and colleagues *(Panoutsopoulou et al., 2009)* compared the reliability of IENF quantification per area (IENF/mm^2) using confocal immunofluorescence with the blister method. IENF density in blister roofs from foot and calf correlated with IENF density in skin biopsies from adjacent areas in 25 healthy subjects ($r = 0.64$ and $r = 0.57$, respectively) showing no systematic differences between skin blisters and biopsies ($p = 0.29$) or between pairs of blisters from the same location ($p = 0.15$).

Recommendations

For diagnostic purposes, we recommend bright-field immunohistochemistry or immunofluorescence with rabbit polyclonal anti-PGP 9.5 antibodies in 2% PLP or Zamboni's fixed sections of 50-μm thickness. For methodological issues on bright-field immunohistochemistry we refer to McCarthy *et al. (1995)*, on immunofluorescence to Wang *et al. (1990)*, and on confocal microscopy to Kennedy and Weldelschafer-Crabb *(1993)*. IENF should be counted at high magnification in at least three sections per biopsy. We emphasize that only single IENF crossing the dermal–epidermal junction should be counted, excluding secondary branching from quantification. The length of the section should be measured in order to calculate the exact linear epidermal innervation density (IENF/mm) (level A recommendation). Further studies are warranted to establish the reliability of the ''blister technique'' (level C recommendation) for quantification of IENF density in SFN.

Quantification of sweat gland innervation

The quantification of sudomotor nerve fibers is technically challenging because of the complex three-dimensional structure of the sweat glands. Different methods have been proposed but none has been standardized *(Lauria et al., 2005)*. A novel method using an unbiased stereologic technique has been recently proposed *(Gibbons et al., 2009a)*. The authors examined blindly 30 diabetic neuropathy patients and 64 healthy subjects finding a significant difference between groups. The density of sweat gland nerve fibers at the distal leg of diabetic patients decreased as the Neuropathy Impairment Score in the Lower limbs worsened ($p < 0.001$) and was concordant with symptoms of reduced sweat production ($p < 0.01$). In a further work, the authors reported a significant correlation between the stereologic unbiased method and a new automated technique for quantification of sudomotor nerve fibers, and showed that the descriptive semiquantitative approach has a poor inter- and intraobserved reliability *(Gibbons et al., 2010)*.

Recommendations

Morphometric data on sweat gland innervation density in healthy subjects and in patients with SFN are limited and further studies are warranted. The descriptive semi-quantitative approach should not be used to quantify sweat gland innervation (level B recommendation). The unbiased stereologic technique recently proposed could be a helpful tool (level B recommendation).

Safety

No side effects have been reported in published studies but no study focused on safety was performed. The approximate number of biopsies performed with 3-mm disposable punch and side effects recorded (in parentheses) in healthy subjects and patients with neuropathy of different etiology in the 10 laboratories participating in this guideline are: Milan 1,600 (2); Telese Terme 2,000 (2); Taiwan 1,700 (1); Maastricht 300 (0); Utah 2,000 (3); Stockholm 1,000 (0); Minneapolis 10,000 (3); Würzburg 800 (10); Tromsø: 600 (1); Johns Hopkins University: 15,000 (44). The most common side effect was a mild infection due to improper wound management recovering with topical antibiotic therapy. The only other complication reported was excessive bleeding which did not need suture. The estimated frequency of side effects is 1.9:1,000. However, this figure may be underestimated because patients with a milder infection could have it treated by a general practitioner or treat themselves without reporting to the centre. Healing occurs within 7–10 days.

Recommendations

Skin biopsy performed with 3-mm disposable punch is a safe and minimally invasive procedure based on the experience of the 10 established laboratories

reported here. It requires training and is safe as long as sterile procedures and haemostasis are correctly performed (Good Practice Point).

Normative Reference Values

Bright-field immunohistochemistry

After the publication of the first guidelines on skin biopsy, three further large studies *(Umapathi et al., 2006; Devigili et al., 2008; Bakkers et al., 2009)* estimated the density of IENF at the distal leg (10 cm above the lateral malleolus) in healthy subjects. Overall, including all previously cited papers *(Lauria et al., 2005)*, values ranged from 13.8 ± 6.7/mm (mean ± SD) to 9.8 ± 3.6/mm (mean ± SD).

The largest normative study *(Bakkers et al., 2009)* included 188 healthy subjects from three different laboratories (Maastricht, Ferrara, Milan) and stratified the study population *per* age and gender, providing normative values *per* decade. The authors reported that IENF density at the distal leg is lower in males than females, that weight and height do not have any significant impact, and that values decline with age (Table 1), thus confirming previous observations *(Chien et al., 2001; Pan et al., 2001; Goransson et al., 2004)*.

Immunofluorescence technique

No study specifically assessed the normative range of IENF density at the distal leg using indirect immunofluorescence with or without confocal microscopy. Overall, values obtained with confocal microscopy were higher than those found using light microscopy technique. Normal values were reviewed by Kennedy and co-authors *(Kennedy et al., 2005)*. Data from 267 healthy subjects included in 17 studies performed with and without confocal microscopy *(Periquet et al., 1999; Nolano et al., 2001; 2006; 2008; Hoitsma et al., 2002; Pittenger et al., 2004; Boucek et al., 2005; 2008; Davis et al., 2006; Sorensen et al., 2006; Walk et al., 2007; Obermann et al., 2008; Uluc et al., 2008b; Vlckova-Moravcova et al., 2008b; Panoutsopoulou et al., 2009; Scherens et al., 2009; Wopking et al., 2009)*, ranged between 7.6 ± 3.1 and 33 ± 7.9/mm (mean ± SD) in subjects aged 20–59 years. Density of 20.1 ± 5/mm (mean ± SD) was found in subjects over 60 years (lower 5th percentile = 11.8).

Blister technique

Panoutsopoulou and colleagues *(Panoutsopoulou et al., 2009)* reported the normative values of IENF density (expressed by number of IENF per epidermal surface area) using both blister technique and punch biopsy with confocal immunofluorescence microscopy at foot and calf in 25 healthy subjects (age 35-62 years). Mean IENF density on the foot was 174 IENF/mm^2 for the punch biopsy and 162 IENF/mm^2 for the blister method. Mean IENF density on the calf was 158 IENF/mm^2 for the punch biopsy and 143 IENF/mm^2 for the blister method. Intra- and interblister variability was less than intrabiopsy variability. The authors found a significant correlation between the two techniques.

Recommendations

Normative reference values must consider that IENF density at the distal leg (10 cm above the lateral malleolus) declines with age (level A recommendation) and may be lower in males than in females. However, it is not influenced by weight and height. Normative reference values are available for bright-field immunohistochemistry (level A recommendation) but not yet for confocal immunofluorescence or blister technique.

Diagnostic yield of skin biopsy

In the first guideline paper *(Lauria et al., 2005)*, we reported specificity and sensitivity of skin biopsy for the diagnosis of SFN based on an unpublished meta-analysis of 161 patients from 9 studies, two of them performed with confocal microscope technique. The same year, Koskinen and colleagues *(Koskinen*

Table 1. Intraepidermal nerve fibre (IENF) density at the ankle: normative values for clinical use (reproduced from Bakkers *et al.*, Neurology, with permission).

	Females (n = 97)		Males (n = 91)	
Age (years)	0.05 quantile values per age span	Median values per age span	0.05 quantile values per age span	Median values per age span
20–29	6.7	11.2	5.4	9.0
30–39	6.1	10.7	4.7	8.4
40–49	5.2	9.9	4.0	7.8
50–59	4.1	8.7	3.2	7.1
60–69	3.3	7.9	2.4	6.3
≥70	2.7	7.2	2.0	5.9

et al., 2005) reported similar values for idiopathic or secondary SFN.

In the last few years, other studies indicated that the reduction of IENF density is a useful approach for investigating patients with SFN. In two studies including 185 patients with symptoms or signs suggestive of SFN, 76 patients had reduced IENF densities at the distal leg, and the reduction of IENF was more severe in patients with large-fiber nerve involvement *(Herrmann et al., 2004a; Loseth et al., 2006)*. In 14 out of 17 patients with non-length dependent SFN/ganglionopathy and early involvement of the face, trunk or proximal limbs, skin denervation was equal to or more prominent in the thigh than in the calf *(Gorson et al., 2008)*, confirming previous findings *(Sghirlanzoni et al., 2005)*. In patients with diabetes mellitus but without clinical evidence of neuropathy, the IENF density at the distal leg was significantly lower compared to controls *(Umapathi et al., 2007; Loseth et al., 2008)*. Among 101 patients of advanced HIV infection with CD4+ cell count less than 300 cells/mm^3 and exposure to nucleoside analogue antiretroviral therapy for $\geq$15 consecutive weeks in the past, 15.5% of patients had mild denervation at the distal leg and 23.2% had severe denervation at both distal and proximal thigh *(Zhou et al., 2007)*. In SFN associated to vasculitis, eosinophilia, and celiac disease, reduced IENF density was found in 100%, 83.3% and 62.5% of patients, respectively *(Brannagan et al., 2005; Lee et al., 2005; Chao et al., 2007)*. Tseng and colleagues *(Tseng et al., 2006)* investigated 45 consecutive patients with systemic lupus erythematosus (21 with active lupus and 20 with neuropsychiatric syndrome) finding that 82.2% of patients had reduced IENF densities, and that IENF density inversely correlated with the activity of the disease and with small fiber sensory symptoms and signs. Based on clinical and skin biopsy findings, three cross-section studies investigated SFN in systemic lupus erythematosus, primary Sjögren syndrome, and rheumatic arthritis reporting abnormal IENF densities in 13%, 3%, and 4% of patients, respectively *(Goransson et al., 2006a; 2006b; 2006c)*. Laaksonen and colleagues *(Laaksonen et al., 2008)* compared women carrying Fabry's disease mutation (83.3% symptomatic) with healthy controls and found that 27.3% of them had abnormal IENF densities.

Two studies focused on the analysis of the diagnostic yield of skin biopsy in SFN using the receiver operating characteristic (ROC) approach that graphically describes the discrimination threshold of sensitivity *vs.* specificity or true positives *vs.* false positives. Vlckova-Moravcova and colleagues *(Vlckova-Moravcova et al., 2008b)* studied a group of 58 patients with pure SFN and 37 healthy subjects. They reported that the cut-off IENF density of $\leq$8.8/mm was associated with a sensitivity of 77.2% and a specificity of 79.6% (maximum likelihood estimates 88% [78–93]). In the same group of SFN patients, the cut-off density of 7.25% subepidermal nerve fibers (expressed as percent of the subepidermal area of the size 200 $\times$ 50 μm adjacent to the dermal-epidermal junction) had a sensitivity of 78.2% and a specificity of 79%. Taking both IENF and subepidermal nerve fiber densities together, the diagnostic sensitivity in pure SFN was further improved to 86%.

Devigili and colleagues *(Devigili et al., 2008)* investigated 67 patients with pure SFN (from a cohort of 124 patients with sensory neuropathy) diagnosed by the presence of at least two abnormal results on clinical examinations, quantitative sensory testing (QST), and skin biopsy. The authors reported sensitivity of 88%, specificity of 88.8%, positive predictive value of 89.4%, and negative predictive value of 87.5%. The cut-off of 7.63 IENF/mm at the distal leg was associated with specificity of 90% and sensitivity of 82.8% comparing 110 patients with painful neuropathy and 47 healthy subjects. The authors found a normal IENF density in 16 patients with symptoms mimicking a possible SFN (psychiatric illness, lumbar stenosis, venous insufficiency).

Nebuchennykh and colleagues *(Nebuchennykh et al., 2009)* compared the diagnostic yield of skin biopsy for diagnosing SFN (45 patients and 134 healthy subjects) using three statistical methods: 1) Z-scores, calculated from multiple regression analysis, which cut-off values were estimated for each patient and adjusted for age and gender; 2) fifth percentile, which cut-off value was 6.7 IENF/mm; and 3) ROC analysis, which cut-off value was 10.3 IENF/mm. Highest specificity was obtained with Z-scores (98%) and fifth percentile (95%), which had lower sensitivity (31% and 35%, respectively) compared to the ROC analysis that showed specificity of 64% and sensitivity of 78%. The authors emphasized that the diagnostic yield of skin biopsy depends on how the reference and cut-off values have been assessed.

In most studies, skin biopsy has been used to investigate patients with SFN either idiopathic or associated to different conditions, including diabetes, infectious diseases, systemic connective tissue disorders, and genetic diseases. However, no study was designed to demonstrate whether skin biopsy can be useful to identify the etiology of SFN. Therefore, no data on this issue are yet available.

Assessment of morphological changes

Two studies analyzed the morphological changes in patients with SFN and reported that IENF and dermal

nerve fiber swellings, weaker axonal immunoreactivity, and a unique type of IENF (crawler) were common findings *(Wendelschafer-Crabb et al., 2006; Ebenezer et al., 2007)*. However, some of these findings were also common to a lesser extent in normal individuals *(Wendelschafer-Crabb et al., 2006)*. In three other studies evaluating SFN of different etiologies, isolated morphological abnormality with normal IENF densities were noted in 29.1%, 20%, and 25% of cases *(Herrmann et al., 2004b; Brannagan et al., 2005; Chai et al., 2005)*. Similar results were reported in 62 patients with sensory neuropathy, 29% of whom had abnormal morphology but normal IENF density *(De Sousa et al., 2006)*. One further study provided a blind assessment of IENF swellings in 28 patients with pure SFN *(Gibbons et al., 2006)*. The amount of axonal swelling was semi-quantitatively scored as large, medium, or small. Repeated biopsy showed that patients with large swellings had a significant reduction in IENF density compared to those without swellings. However, the retrospective design of the study, the variability in timing between biopsies, and the absence of a control group limited the interpretation of the results.

Recommendations

Skin biopsy with linear quantification of IENF density is a reliable and efficient technique to confirm the clinical diagnosis of SFN (level A recommendation). This conclusion derives from the examination of studies involving homogeneous groups of patients with possible SFN. However, since the definition of SFN varied in the different studies, we could not provide the range of sensitivity and sensibility values.

Immunohistochemical technique does not seem to influence the diagnostic efficiency in diagnosing SFN. However, data from comparative studies using the two techniques in homogeneous groups of SFN patients are not available yet and are warranted.

For diagnostic purposes we recommend quantitative assessment of IENF density with appropriate quality controls, which include all the steps of the procedure, in particular the aspect of intra- and inter-observer ratings. The diagnosis of SFN with skin biopsy should be based on the comparison with normative reference values adjusted by age (level A recommendation) and possibly gender (level B recommendation). Diffuse IENF swellings, especially if large, may have a predictive value to the progression of neuropathy (level C recommendation). Further studies to investigate the ability of skin biopsy in differentiating patients with symptoms mimicking SFN are warranted.

Correlation between IENF density and other measures of neuropathy

Correlation with clinical measures

In the last 4 years, a number of studies investigating the correlation between skin biopsy and clinical scales have been published. However, there are no definite diagnostic criteria nor validated scales for SFN. Therefore, we report here available comparative data between skin biopsy, clinical findings, and various neuropathy scales.

IENF density was closely related to, and predicted, pin sensation loss in 106 subjects with idiopathic SFN *(Walk et al., 2007)*. Among subjects with diabetic neuropathy, IENF density progressively declined with increased severity of clinical neuropathy, measured using the Neurological Disability Score *(Quattrini et al., 2007; 2008; Vlckova-Moravcova et al., 2008a)*. Another study in diabetic subjects with normal nerve conduction studies found a negative correlation between IENF density at the lower leg and the Neuropathy Impairment Score *(Loseth et al., 2008)*. A recent study *(Bakkers et al., 2009)* investigated 3 groups of patients with sarcoidosis: 1) patients without SFN symptoms (n = 14), 2) patients with SFN complaints and normal IENF density findings (n = 39), and 3) patients with SFN complaints and abnormal IENF density values (n = 19). The authors found that significantly more SFN related symptoms (as reported by a SFN-related symptoms inventory questionnaire) were present in patients with abnormal IENF density, with a gradual transition between the three subgroups. In 2 studies investigating patients with systemic lupus erythematosus, IENF density negatively correlated with cutaneous vasculitis and disease activity *(Tseng et al., 2006; Chao et al., 2007)*. Conversely, in HIV associated neuropathy there was no correlation between distal IENF density and Total Neuropathy Score *(Zhou et al., 2007)*, although a baseline reduction in IENF density predicted the risk of developing neuropathy symptoms over a 2.9 year period, which was 14-fold higher in patients with IENF density of less than 10 fibers/mm *(Herrmann et al., 2006)*. Another study failed to demonstrate a relationship with the Neuropathy Symptoms Score *(Nebuchennykh et al., 2009)*. IENF density was lower in diabetic neuropathy patients with pain compared to those without *(Sorensen et al., 2006; Quattrini et al., 2007; Vlckova-Moravcova et al., 2008b)*, whereas no correlation was previously found in another study *(Pittenger et al., 2004)*. In HIV-associated sensory neuropathy, IENF density inversely correlated with pain severity assessed with both VAS and the Gracely Pain Score *(Zhou et al., 2007)*. Conversely, a previous study found a correlation only with patient's and doctor's evaluation

scores *(Polydefkis et al., 2002)* and another *(Herrmann et al., 2004b)* showed that assessment of IENF density could not differentiate between symptomatic or asymptomatic HIV neuropathy patients. In patients with pure SFN of mixed aetiology, IENF density was lower in those with pure spontaneous pain than those with pure evoked pain, but it did not correlate with its intensity *(Devigili et al., 2008)*.

Correlation with sensory nerve conduction studies

Concordance between sural sensory nerve action potential (SNAP) amplitude and IENF density was investigated in several studies with different results. This is likely in keeping with the different types of neuropathy examined (i.e., large or mixed fiber *vs* small fiber) with most studies focusing on SFN. We have previously reported *(Lauria et al., 2005)* that concordance between sural SNAP amplitude and IENF density was found in patients with clinical impairment of large nerve fibers, whereas skin biopsy appeared more sensitive than sural sensory nerve conduction study (NCS) in diagnosing SFN.

Recent studies strengthened this assumption. In 67 patients with pure SFN, sensory NCS were normal and IENF density at distal leg was reduced in 88% of cases *(Devigili et al., 2008)*. However, a recent study *(Uluc et al., 2008a)* confirmed the previously observed linear correlation between medial plantar SNAP amplitude and IENF density in patients with SFN *(Herrmann et al., 2004a)* and found a correlation with digital plantar near-nerve needle sensory NSC at the multivariate analysis. These findings suggest that large sensory fibers can be impaired in distal segments in some patients with clinical picture of pure SFN. Therefore, clinically pure SFN can be part of a mixed sensory neuropathy.

Patients with mixed sensory neuropathy associated to diabetes or HIV showed a significant direct correlation between sural SNAP amplitude and IENF density *(Quattrini et al., 2007; Zhou et al., 2007)*. A similar relationship was found in a large study involving 210 patients with neuropathy of different aetiology *(Nebuchennykh et al., 2009)*. One study found no correlation between IENF density and sural nerve SNAP amplitude, but a good correlation of SNAP amplitude with the density of the subepidermal nerve fiber *(Vlckova-Moravcova et al., 2008b)*. In the prospective study of IGT-associated neuropathy, the authors found a positive correlation between the degree of IENF regeneration and the increase in sural SNAP amplitude, although there was no baseline relationship between the two *(Smith et al., 2006)*.

Correlation with small fiber related evoked potentials

Few studies have examined the relationship between skin biopsy and neurophysiological tests for assessing small fiber function, and most of the available data come from single case reports *(Nolano et al., 2000; Perretti et al., 2003; Donadio et al., 2005; 2008)*. In a subgroup of 10 patients with pure SFN and decreased IENF at the distal leg, laser evoked potentials did not differ compared with 18 healthy subjects *(Devigili et al., 2008)*. Two studies examined contact heat evoked potentials (CHEP) in 66 neuropathy patients. Both found a positive correlation between IENF density and the amplitude of Aδ-related potential *(Atherton et al., 2007; Chao et al., 2008a)*. One study showed a correlation between IENF density and pain-related electrically evoked potential (PREP) amplitudes and latencies *(Obermann et al., 2008)*.

Correlation with quantitative sensory testing (QST) and autonomic nervous system testing

Psychophysical assessment of thermal, heat-pain, and vibratory thresholds provides information on A∂ and C, and Aβ fibers, respectively. However, the correlation between QST and IENF density remains controversial. IENF density inversely correlated with both cold and warm/heat detection thresholds *(Quattrini et al., 2007; Devigili et al., 2008; Loseth et al., 2008)*, whereas other studies showed a closer correlation with warm and heat-pain thresholds *(Pan et al., 2001; 2003; Chiang et al., 2002; Pittenger et al., 2004; Shun et al., 2004; Tseng et al., 2006; Vlckova-Moravcova et al., 2008b; Scherens et al., 2009)* than with cooling threshold *(Holland et al., 1997; Periquet et al., 1999; Novak et al., 2001)*. Moreover, two studies suggested a closer correlation with cold than heat detection *(Sorensen et al., 2006; Zhou et al., 2007)*. A correlation with vibratory detection threshold was more likely when patients have clinical and electrophysiological evidence of large fiber neuropathy *(Sorensen et al., 2006; Zhou et al., 2007)*.

Although IENF have somatic functions, several studies investigated their relationship with autonomic dysfunction in neuropathy of different aetiology. Clinical signs of dysautonomia and abnormal veno-arteriolar reflex and vasodilatation induced by local heating, reflecting impaired skin axonal reflexes carried by somatic C-fibers, were found in about 70% of patients with pure SFN *(Devigili et al., 2008)*. Another study did not find any correlation between IENF density and measures of autonomic function in SFN *(Vlckova-Moravcova et al., 2008b)*.

Correlation with sural nerve biopsy

In the last 4 years, no further study investigated the correlation between skin and nerve biopsy. Therefore, we refer to the recommendations proposed in the first EFNS skin biopsy guidelines *(Lauria et al., 2005)*.

Recommendations

Decreased IENF density reliably indicates the presence of SFN (level A recommendation). However, correlation between IENF density, validated measures of neuropathy severity, and clinical disability needs further evaluation in patients with neuropathy of specific etiologies (level C recommendation).

The relationship between IENF density and neuropathic pain is more complex than a simple inverse correlation. Lower IENF density may be associated with the presence of neuropathic pain, especially in pure SFN (level B recommendation), but it does not correlate with the intensity of pain.

Quantification of IENF density can better assess the diagnosis of SFN than sural NCS and sural nerve biopsy (level A recommendation). Concordance between IENF quantification and medial plantar SNAP amplitude in patients with normal sural NCS suggests that distal sensory nerve recording might be more sensitive than sural NCS in the diagnosis of sensory neuropathy (level C recommendation).

IENF density correlates with psychophysical examination of small fiber dysfunction using thermal and nociceptive detection thresholds (level A recommendation), but correlation with specific sensation (e.g., cooling, warm, heat-pain) remains uncertain (level C recommendation). Correlation with autonomic dysfunction needs more extensive validation (level C recommendation).

Further studies are required to determine the relative diagnostic utility of non-conventional neurophysiological methods to investigate small fiber function (e.g., LEPs, CHEPs, and PREPs) and their correlation with IENF density.

Skin biopsy as a measure of outcome

Several prospective studies and case reports have investigated the relationship between skin innervation and outcome. In patients of asymptomatic HIV infection with or without neurological signs, a lower IENF density was associated with a higher risk of progression to symptomatic HIV neuropathy *(Herrmann et al., 2006)*. A progressive reduction in IENF densities at the foot dorsum correlated with the severity of the neuropathy in diabetic patients *(Quattrini et al., 2007; 2008)*. Boucek and colleagues *(Boucek et al., 2005; 2008)* investigated skin innervation in 18 patients with type 1 diabetes before and after pancreas/kidney transplantation, but did not find significant changes. In a 1-year study of diet and exercise in patients with impaired glucose tolerance (IGT) and neuropathy, skin reinnervation (increased IENF density at the proximal thigh) positively correlated with decreased neuropathic pain intensity *(Smith et al., 2006)*. Studies measuring the rate of IENF regeneration following capsaicin chemical denervation showed that it is slower in patients with diabetes or HIV without signs or symptoms of neuropathy compared to healthy subjects *(Polydefkis et al., 2004; Hahn et al., 2007)*. In case reports of toxic-related painful neuropathy caused by linezolid *(Chao et al., 2008b)* or hypothyroidism *(Penza et al., 2009)*, the authors showed that the regeneration of IENF was associated with recovery of thermal threshold and neuropathic pain.

Recommendations

In SFN, the reduction of IENF density over time can be used as an index of progression of neuropathy (level A recommendation). In HIV patients without neurological symptoms, skin biopsy with quantification of the IENF densities may predict the risk of progression to symptomatic HIV neuropathy (level B recommendation). Regeneration of IENF may be associated with recovery of neuropathic pain and sensory symptoms (level C recommendation). Skin biopsy may be considered as an endpoint in future neuro-protective neuropathy trials (level B recommendation).

EFNS/PNS standards

Skin biopsy with quantification of IENF density is a reliable technique to diagnose SFN. For diagnostic purposes, we endorse 3-mm punch skin biopsy at the distal leg (10 cm above the lateral malleolus), and quantification of linear IENF density in at least three 50-μm-thick sections per biopsy, fixed in 2% PLP or Zamboni's solution, by immunohistochemistry using rabbit polyclonal anti-PGP 9.5 antibodies, using either bright-field microscopy or immunofluorescence with or without confocal microscopy. Appropriate normative data from healthy subjects matched for age and gender should be always used.

We strongly recommend training in an established cutaneous nerve laboratory before performing and processing skin biopsies in the diagnosis of SFN. Quality control should include all the steps of the procedure,

in particular the aspect of intra- and inter-observer ratings for qualitative assessments and for quantitative analysis of epidermal densities.

Proposal for new studies

Normative reference studies reporting age and gender matched values of IENF density at proximal and distal sites using indirect immunofluorescence technique with and without confocal microscopy are warranted. These studies should be collaborative and designed in order to compare the diagnostic yield of this technique with that of bright-field microscopy in patients with SFN.

A clinimetric approach should be used to assess the correlation between skin innervation and the clinical symptoms and signs of SFN. Such studies should include patients whose clinical picture mimics that of SFN, in order to definitely assess specificity and sensitivity of skin biopsy in the diagnosis of this type of neuropathy.

A consensus definition of SFN is needed in order to plan new studies that will determine the sensitivity and specificity of skin biopsy and other potential diagnostic strategies.

The reliability of already tested or new methods to quantify the density of nerve fibers in the sub-epidermal dermis and autonomic structures (e.g., sweat gland nerve, erector pili muscle, and vessels) should be confirmed by further studies in patients with homogeneous types of peripheral neuropathy, including SFN. Correlative studies between skin biopsy, autonomic tests, and non-conventional neurophysiologic tools are also warranted.

Lastly, further studies should focus on the ability of skin biopsy to detect early changes of nerve fibers that predict the progression of neuropathy and that assist in assessing nerve degeneration and regeneration rates over time, in order to confirm the potential usefulness of the technique as an outcome measure in clinical practice and research.

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