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EFNS TASK FORCE/CME ARTICLE

EFNS guidelines on the use of skin biopsy in the diagnosis of peripheral neuropathy

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Skin biopsy has become a widely used tool to investigate small calibre sensory nerves including somatic unmyelinated intraepidermal nerve fibres (IENF), dermal myelinated nerve fibres, and autonomic nerve fibres in peripheral neuropathies and other conditions. Different techniques for tissue processing and nerve fibre evaluation have been used. In March 2004, a Task Force was set up under the auspices of the European Federation of Neurological Societies (EFNS) with the aim of developing guidelines on the use of skin biopsy in the diagnosis of peripheral neuropathies. We searched the Medline database from 1989, the year of the first publication describing the innervation of human skin using immunostaining with anti-protein-gene-product 9.5 (PGP 9.5) antibodies, to 31 March 2005. All pertinent papers were rated according to the EFNS guidance. The final version of the guidelines was elaborated after consensus amongst members of the Task Force was reached. For diagnostic purposes in peripheral neuropathies, we recommend performing a 3-mm punch skin biopsy at the distal leg and quantifying the linear density of IENF in at least three 50-μm thick sections per biopsy, fixed in 2% PLP or Zamboni's solution, by bright-field immunohistochemistry or immunofluorescence with anti-PGP 9.5 antibodies (level A recommendation). Quantification of IENF density closely correlated with warm and heat-pain threshold, and appeared more sensitive than sensory nerve conduction study and sural nerve biopsy in diagnosing small-fibre sensory neuropathy. Diagnostic efficiency and predictive values of this technique were very high (level A recommendation). Confocal microscopy may be particularly useful to investigate myelinated nerve fibres, dermal receptors and dermal annex innervation. In future, the diagnostic yield of dermal myelinated nerve fibre quantification and of sweat gland innervation should be addressed. Longitudinal studies of IENF density and regeneration rate are warranted to correlate neuropathological changes with progression of neuropathy and to assess the potential usefulness of skin biopsy as an outcome measure in peripheral neuropathy trials (level B recommendation). In conclusion, punch skin biopsy is a safe and reliable technique (level A recommendation). Training in an established cutaneous nerve laboratory is recommended before using skin biopsy as a diagnostic tool in peripheral neuropathies. Quality control at all levels is mandatory.

Objectives

In the last decade skin biopsy has gained widespread use as a method to investigate small-diameter nerve fibres in human epidermis and dermis. In particular, this technique may be used to evaluate either qualitatively or quantitatively somatic unmyelinated intraepidermal nerve fibres (IENF). Skin biopsy can be used to evaluate abnormalities in cutaneous innervation for diagnosis of neuropathy including those with so-called

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'pure' small fibre sensory neuropathy (SFSN), at different stages of neuropathy, and in different types of peripheral neuropathies, including autonomic and demyelinating neuropathies.

A growing number of laboratories in Europe and the United States have been using skin biopsy in the diagnostic evaluation of patients with peripheral neuropathy. However, different techniques for tissue processing and nerve fibre evaluation have been developed.

The objectives of our Task Force were to: (i) evaluate the techniques for performing skin biopsy and the choice of biopsy location; (ii) evaluate the methods for tissue processing and for quantification of IENF; (iii) assess the diagnostic performance of skin biopsy in peripheral neuropathies; (iv) compare skin biopsy with clinical, neurophysiological, psychophysical, autonomic and sural nerve biopsy examination; (v) recommend EU standards; and (vi) propose, if needed, new studies to address unresolved issues.

Search strategy

The Task Force systematically searched the Medline database from 1989, the year when the first papers reporting immunostaining of human skin with anti-protein-gene-product 9.5 (PGP 9.5) antibodies were published (Dalsgaard *et al.*, 1989; Wang *et al.*, 1990), to 31 March 2005. For each specific issue, we stored all the articles sorted by the Medline search, omitted those that were not pertinent, read and rated the remaining articles according to the guidelines of the European Federation of Neurological Societies (EFNS) (Brainin *et al.*, 2004). In some cases, investigators were asked for original data and methodological details.

Method for reaching consensus

Data extraction was carried out and compared amongst each member of the Task Force. Discrepancies in each topic were discussed and settled during a consensus meeting held in Milan on 8 January 2005. The revised and final version of the guidelines is presented here.

Results

Methods to perform skin biopsy and choice of biopsy location

Skin biopsy was most commonly performed by means of a 3-mm disposable circular punch under sterile technique, after topical anaesthesia with lidocaine. No suture was required, and no side effects were reported. Healing was reported to occur within 7–10 days. Epidermis and superficial dermis, including sweat glands, are taken. This technique was first developed at the Karolinska Institute (Wang *et al.*, 1990), and later standardized at the University of Minnesota (Kennedy and Weldelschafer-Crabb, 1993) and at the Johns Hopkins University (McCarthy *et al.*, 1995).

A less invasive sampling method is 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 fibres. Moreover, thus far it has not been systematically used to investigate patients with peripheral neuropathy. This technique was developed at the University of Minnesota (Kennedy and Wendelschafer-Crabb, 1996).

In most studies of peripheral neuropathies, skin biopsies were obtained from the distal part of the leg (10 cm above the external malleolus), in some from the calf, and in many of them also from the upper lateral aspect of the thigh (20 cm below the anterior iliac spine). These locations were chosen to detect the length-dependent loss of cutaneous nerve fibres, which is typical of axonal polyneuropathy.

Recommendations

We emphasize that the 3 mm punch skin biopsy is a minimally invasive technique. It requires training and is safe as long as sterile procedures and haemostasis are correctly performed. For diagnostic purposes in peripheral neuropathies, we recommend performance of a 3-mm punch skin biopsy. In polyneuropathies, we recommend performing skin biopsy at the distal leg for quantification of epidermal innervation density. An additional biopsy from the proximal thigh may provide information about a length-dependent process (level A recommendation).

Methods to process tissue and to quantify IENF

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. $G_{\alpha 0}$) 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 in peripheral neuropathies.

After the biopsy is performed, the specimen is immediately fixed in cold fixative for up to 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 55 vertical 50-μm sections. However, the first and the last few sections should not

be used for cutaneous nerve examination because of possible artefacts.

In most studies, either 2% paraformaldehyde-lysine-periodate (2% PLP) or Zamboni's (2% paraformaldehyde, picric acid) fixative were used. In early studies (McCarthy *et al*., 1995; McArthur *et al*., 1998), tissue was fixed in formalin, which produced a more fragmented appearance of nerve fibres when compared with PLP, without affecting innervation density (Herrmann *et al*., 1999; Lauria *et al*., 1999). No study systematically compared IENF evaluations in peripheral neuropathies using different fixatives, although Ljungberg and Johansson (1993) studied the influence of the immunohistochemical method, including the choice of fixative, on the procedure for visualizing neuronal markers in human skin.

Two immunostaining methods have been used: bright-field immunohistochemistry (Wang *et al*., 1990; Hilliges *et al*., 1995; McCarthy *et al*., 1995; Holland *et al*., 1997, 1998; Karanth *et al*., 1989; Lauria *et al*., 1998, 1999, 2001, 2003; McArthur *et al*., 1998, 2000; Herrmann *et al*., 1999, 2004a,b; Hilliges and Johansson, 1999; Johansson *et al*., 1999; Scott *et al*., 1999; Hirai *et al*., 2000; Polydefkis *et al*., 2000, 2002, 2004;; Chien *et al*., 2001; Pan *et al*., 2001, 2003; Smith *et al*., 2001; Omdal *et al*., 2002; Chiang *et al*., 2002; Nodera *et al*., 2003; Rajan *et al*., 2003; Sumner *et al*., 2003; Gøransson *et al*., 2004; Shun *et al*., 2004; Singer *et al*., 2004; Koskinen *et al*., 2005; Li *et al*., 2005) and indirect immunofluorescence with or without confocal microscopy (Kennedy *et al*., 1996, 1999; Kawakami *et al*., 2001; Reilly *et al*., 1997; Facer *et al*., 1998; Periquet *et al*., 1999; Nolano *et al*., 2001, 2003; Novak *et al*., 2001; Hoitsma *et al*., 2002; Besnè *et al*., 2002; Sommer *et al*., 2002; Perretti *et al*., 2003; Hilz *et al*., 2004; Moura *et al*., 2004; Pittenger *et al*., 2004).

In most studies using bright-field immunohistochemistry, at least three sections of 50 μm thickness from each biopsy were examined (Fig. 1). In confocal microscopy studies, usually sections of 80–100 μm thickness were immunostained. Confocal microscopy allows analysing double, triple and even quadruple-stained sections, for example, with antibodies against PGP 9.5 and collagen IV to visualize axons and basement membrane in order to trace IENF from the site where they penetrate the basement membrane to their endings (Fig. 2). Quantification of IENF density was performed on images based on the stack of consecutive 2 μm optical sections (usually 16 sections) for a standard linear length of epidermis (usually 1–3 mm). IENF may be evaluated either qualitatively or quantitatively.

For quantitative analysis, IENF are counted either under the light microscope at high magnification, i.e. 40×, or using software for image analysis. In both 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. The length of the section is measured with computerized software (freely available at http://rsb.info.nih.gov/nih-image/index.html) and the linear epidermal innervation density is therefore calculated. IENF density is

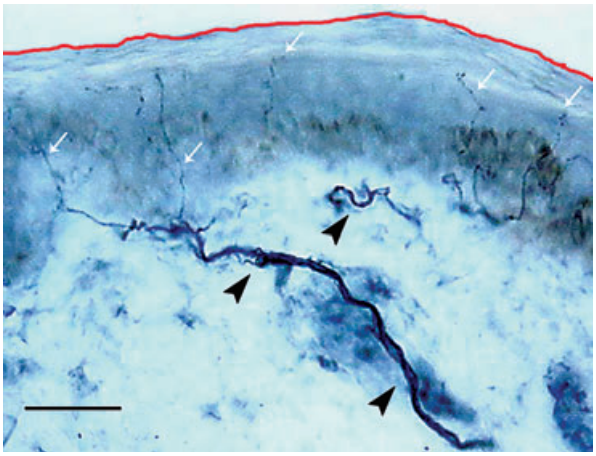

Figure 1 Quantification of intraepidermal nerve fibre (IENF) density using bright-field immunohistochemistry with anti-protein-gene-product 9.5 antibodies. Arrows indicate IENF and arrowheads indicate dermal nerve bundles. The red line marks the length of the section. Linear IENF density (IENF/mm) is obtained dividing the number of IENF by the length of the section. Bar = 30 μm.

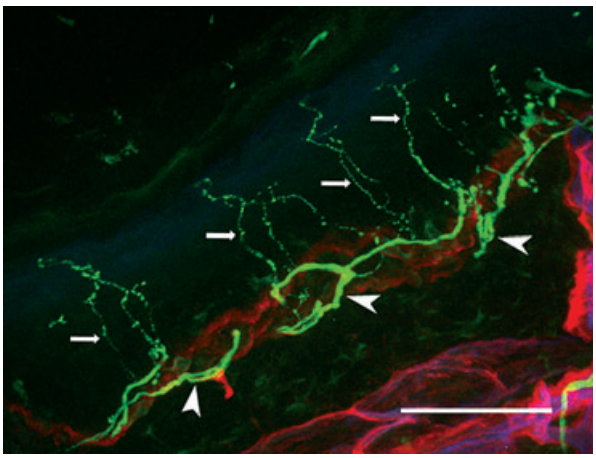

Figure 2 Projection of a stack of 16 optical sections of 2 μm thickness obtained by confocal microscopy from a triple-stained skin section. Nerve fibres are stained in green (protein-gene-product 9.5), basement membrane and the blood vessels are stained in red (collagen IV), and endothelium and epidermis are stained in blue (ULEX-Europaeus agglutinin I). Arrows indicate IENF and arrowheads indicate dermal nerve bundles. The quantification is performed in 3D on the stack of optical sections using Neurolucida software. Bar = 50 μm.

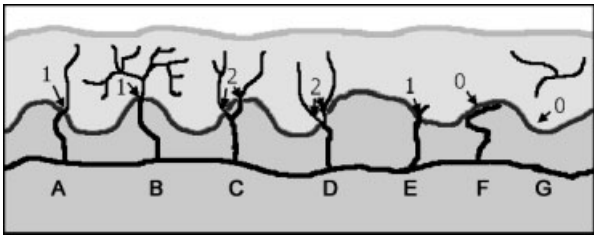

Figure 3 Intraepidermal nerve fibre counting rules. Diagram of skin innervation: nerves (black), basement membrane (dark grey), dermis (medium grey) and epidermis (light grey). (A) Count nerve as it crosses the basement membrane of the epidermis. (B) Nerves that branch after crossing the basement membrane are counted as a single unit. (C) Nerves that split below the basement membrane are counted as two units. (D) Nerves that appear to branch within the basement membrane are counted as two units. (E) Nerve fragments that do cross the basement membrane are counted. (F) Nerve fibres that approach the basement membrane but do not cross it are not counted. (G) Nerve fragments in the epidermis that do not cross the basement membrane in the section are not counted.

reported as IENF/mm. A comprehensive review on methods and rules for IENF counting is available (Kennedy *et al*., 2005) (Fig. 3).

Significant correlation with a stereologic technique (Stocks *et al*., 1996) supported the reliability of linear IENF density quantification under light microscopy (McArthur *et al*., 1998). No systematic study comparing light and confocal microscope method has been carried out. However, a recent meta-analysis (N. Rosenberg, personal communication) emphasized that sensitivity and specificity of IENF quantification in patients with SFSN was not influenced by different microscopy techniques and suggested that, for diagnostic purposes, confocal microscopy that is more complicated, expensive, and time consuming is not required.

An alternative estimation method based on simple 'counting and calculating', with no aid of an image analysis system, has been used under light microscopy (Chien *et al*., 2001; Pan *et al*., 2001; Herrmann *et al*., 2004b). This is based on the hypothesis that the epidermal length of specifically defined sections (i.e. the 19th, 25th and 31st) is close to the maximum diameter of the skin punch, namely 3 mm. The 'ocular IENF density' in the section is therefore calculated dividing the number of IENF by 3 mm. This method significantly correlated with the conventional quantification of epidermal innervation density obtained by measuring the length of the section (Chien *et al*., 2001).

Hilliges and Johansson (1999) compared three methods to quantify IENF density per projected area (IENF/mm^2) in 14 μm thick sections in 45 biopsies from healthy subjects: (i) the unbiased nerve fibre profile and nerve fibre fragment estimation methods, (ii) the traditional method of counting whole nerve fibres and (iii) the nerve fibre estimation method. Comparative analysis showed a good correlation ($R > 0.96$) between the three numerical methods. It was emphasized that section thickness and nerve fibre shape could affect the count, and therefore always need to be separately analysed and corrected for in a pilot run before commencing any larger comparative study. This study is one of the very few examples where an unbiased, correct, and efficient counting rule on vertical sections by design-based stereology and with a fixed reference space was utilized within this specific area of interest. Hilliges and Johansson (1999) concluded that reporting nerve fibre profiles counted per projected surface area is very useful for comparing results from different laboratories regardless of section thickness, shape or form.

Koskinen *et al*. (2005) compared the epidermal innervation density at the distal leg estimated per epidermal length with that calculated per epidermal area using a volume-corrected mitotic index that might correct the variations caused by optic factors, such as different high-power microscopic fields between microscopes. The two methods showed a significant correlation coefficient.

The 'skin blister' method has also been used to quantify the innervation of the epidermis (Kennedy *et al*., 1999). 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. After removing the capsule, the blister roof is excised, fixed and immunostained. Epidermal nerve fibre density is calculated in sampling areas (IENF/mm^2) using a grid to reduce the chance of double counting IENF. Counting includes secondary branching of fibres. This technique allows quantifying IENF in an area several times larger (up to 7 mm^2) than the surface of a single 3 mm section and offers a horizontal perspective that makes immediately apparent an uneven distribution of nerve fibres. The 'skin blister' method has been used in a limited number of patients and controls.

Recommendations

For diagnostic purposes in peripheral neuropathies, we recommend bright-field immunohistochemistry or immunofluorescence with 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 (i.e. 40×) 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 'ocular' method (level B recommendation) and the 'blister technique' (level C recommendation) for quantification of IENF density in peripheral neuropathies.

Diagnostic performances of skin biopsy

Different normative range and cut-off values of IENF density in neuropathy patients have been reported using either bright-field immunohistochemistry or confocal microscope technique (Kennedy *et al*., 2005). No systematic study comparing the two methods has been carried out. Therefore, data are presented separately. Skin biopsy was also used to investigate dermal myelinated nerve fibres in healthy subjects, immune-mediated neuropathy (Lombardi *et al*., 2005) and in inherited neuropathies (Li *et al*., 2005).

Bright-field immunohistochemistry

Normative data. Three large studies estimated the density of IENF in 98 (McArthur *et al*., 1998), 106 (Gøransson *et al*., 2004) and 55 (Pan *et al*., 2001) healthy subjects ranging from 13 to 92 years. It ranged from 13.8 ± 6.7/mm (mean ± SD; lower 5th percentile 3.8) (McArthur *et al*., 1998) to 12.4 ± 4.6/mm (mean ± SD) (Gøransson *et al*., 2004) and 12.9 ± 5.3/mm (mean ± SD) (Pan *et al*., 2001). Increasing age and male gender was independently associated with decreasing IENF density at the distal leg on multivariate analysis (Gøransson *et al*., 2004). Similarly, Chien *et al*. (2001), Pan *et al*. (2001) and Shun *et al*. (2004) reported different normative values between subjects aged <60 years [11.1 ± 3.7/mm (mean ± SD); lower 5th percentile 5.8/mm] and >60 years [7.6 ± 3.0/mm (mean ± SD); lower 5th percentile 2.5/mm]. Conversely, McArthur *et al*. (1998) found no sex or age effect (except for higher values in the youngest subjects aged 10–19 years).

Mean IENF density at the proximal thigh, estimated in one of these normative studies (McArthur *et al*., 1998), was 21.1 ± 10.4/mm (mean ± SD; lower 5th percentile 5.2/mm). This value did not differ from that found in smaller series (Holland *et al*., 1997; Lauria *et al*., 1999, 2003; Scott *et al*., 1999; Smith *et al*., 2001), which confirmed the presence of a decreasing gradient of epidermal innervation in the lower limb, with a density approximately 60% higher at the proximal thigh than at the distal leg. All these studies used the same method to estimate the linear epidermal innervation density, namely count of single IENF and measurement of exact section length. Overall, interobserver and intraobserver agreement was generally high and supported the reliability of the method, as recently confirmed by an interlaboratory study (Smith *et al*., 2005).

Diagnostic yield. Two studies (McArthur *et al*., 1998; Chien *et al*., 2001) were specifically designed to assess the diagnostic performances of skin biopsy in peripheral neuropathies of different aetiology. Moreover, several smaller studies (McCarthy *et al*., 1995; Holland *et al*., 1997; Herrmann *et al*., 1999; Lauria *et al*., 1999, 2001, 2003; Scott *et al*., 1999; Smith *et al*., 2001; Chiang *et al*., 2002; Omdal *et al*., 2002; Polydefkis *et al*., 2002; Pan *et al*., 2003; Sumner *et al*., 2003; Herrmann *et al*., 2004a,b; Shun *et al*., 2004) investigated the density of IENF in 537 patients with peripheral neuropathies using the same technique. Most of them described a length-dependent loss of IENF with significantly lower density at the distal leg, reflecting the dying-back process typical of axonal neuropathies. A non-length pattern of skin denervation was found in sensory ganglionopathies (Lauria *et al*., 2001).

McArthur *et al*. (1998) compared 98 healthy subjects (age 13–82 years) and 20 patients with neuropathy (diagnosis was based on a composite measure using the Total Neuropathy Score (Cornblath *et al*., 1999), whereas Chien *et al*. (2001) investigated 55 healthy subjects (age 25–73 years) and 35 patients with SFSN (diagnosis was based on clinical grounds and elevated sensory thresholds to warm and cold stimuli). Density of IENF below the lower 5th percentile was considered abnormal. Specificity (percentage of true negative) did not differ between the two studies (97 and 95% respectively), whereas sensitivity (percentage of true positive) was higher in the study of Chien *et al*. (80%) than in the study of McArthur *et al*. (45%). The lower sensitivity in the study of McArthur *et al*. (1998) might be due to the non-homogeneous group of patients in the study. The high specificity suggests that quantification of IENF density is a good tool to verify the presence of a neuropathy. This may apply to pure SFSN, in which clinical and electrophysiological examinations can be normal. Conversely, normal IENF density does not rule out the presence of sensory neuropathy.

A recent meta-analysis (N. Rosenberg, personal communication) focused on patients with possible SFSN and normal electrophysiological examination, including 161 patients from nine studies (two of them based on confocal microscope technique). Using the cut-off values of the different studies, the sensitivity of

IENF density assessment for the diagnosis of SFSN ranged from 69 to 82% with a specificity of 97%.

Koskinen *et al.* (2005) reported a diagnostic efficiency of 93% for idiopathic or secondary (diabetic, cytotoxic or amyloid) SFN (sensitivity of both methods was 90%, specificity 95%, positive predictive value 95% and negative predictive value 91%).

Immunofluorescence technique

Normative data. No study was specifically designed to assess the normative range of epidermal innervation density by indirect immunofluorescence with or without confocal microscopy. Overall, values were higher than those found using light microscopy technique. Normal values have been reviewed by Kennedy *et al.* (2005) in the new Dyck and Thomas textbook on peripheral neuropathies.

Density of IENF at the distal leg, quantified in 81 healthy subjects included in five studies (Kennedy *et al.*, 1996; Periquet *et al.*, 1999; Nolano *et al.*, 2001; Hoitsma *et al.*, 2002; Pittenger *et al.*, 2004), ranged between 17.4 ± 7.4 to 33.0 ± 7.9/mm (mean ± SD) in subjects with age 20–59 years (lower 5th percentile 20.0) and was 20.1 ± 5.0/mm (mean ± SD) in subjects over 60 years (lower 5th percentile 11.8). The thickness of skin sections analysed in these studies varied from 32 to 60 μm. With confocal microscopy, the most important variable that may account for the different results in IENF density is the number of optical sections used to create the image on which quantification is performed. Analysis of sixteen 2 μm optical sections taken by confocal microscopy from fresh fixed 60 μm frozen sections correspond to analysis of 50 μm sections after correction for shrinkage and compression.

Nolano *et al.* (2003) estimated the density of 11.3 ± 2.9 IENF/mm (mean ± SD) in the glabrous skin of fingertip in 14 healthy subjects (age 22–53). The authors also estimated a density of 59.0 ± 29.3 (mean ± SD) myelinated endings per square millimetre, with a mean diameter of 3.3 ± 0.5 μm (SD) and an internodal length of 79.1 ± 13.8 μm (SD). The mean density of Meissner corpuscles in the fingertip of digit III was 33.02 ± 13.2 (SD) per square millimetre. No age effect was found, but a higher number of neural structures was observed in females related to a smaller fingertip surface, suggesting that spatial distribution of nerve endings might also depend on body growth.

Diagnostic yield. Data on IENF density in neuropathies come from a more limited number of studies, including 198 patients from five studies (Kennedy *et al.*, 1996; Periquet *et al.*, 1999; Novak *et al.*, 2001; Hoitsma *et al.*, 2002; Pittenger *et al.*, 2004). In all studies, IENF density was significantly lower in neuropathy patients than in controls. In 89 patients with SFSN and no electrophysiological abnormalities reported in two studies from the same laboratory (Periquet *et al.*, 1999; Novak *et al.*, 2001), density ranged between 9.2 ± 6.2 and 14.9 ± 11.0 (mean ± SD). Median density was 5.4/mm in seven patients with sarcoidosis-associated SFSN and no electrophysiological abnormalities (Hoitsma *et al.*, 2002). In 48 diabetic and non-diabetic neuropathy patients, IENF density was 17.5 ± 3.3 (Pittenger *et al.*, 2004). IENF density was not altered in patients with diabetes of < 5 years duration (37.4 ± 7.1/mm; mean ± SD), whereas it was significantly decreased in patients with > 5 years duration (7.8 ± 7.1/mm; mean ± SD). These data are in contrast to previous studies showing reduced IENF density in patients with neuropathy and impaired glucose tolerance (Smith *et al.*, 2001; Sumner *et al.*, 2003).

Overall, cut-off values and mean densities quantified using confocal microscopy were higher than in light microscopy studies. Nevertheless, sensitivity and specificity of skin biopsy in the diagnosis of SFSN, separately examined in a meta-analysis (N. Rosenberg personal communication), were not influenced by different microscopy techniques.

The median density of dermal nerve fibres in healthy subjects quantified by stereological methods in the hand, upper arm, shoulder, back and thigh was 23.7/mm, with no significant differences between sites (Liang *et al.*, 1996). Dermal nerve fibres were never quantified in patients with peripheral neuropathy.

Description of morphological changes reflecting axonal derangement was reported in several case series. Lombardi *et al.* (2005) examined 14 patients with neuropathy associated with anti-myelin-associated glycoprotein antibodies. All patients showed specific IgM deposits on dermal myelinated fibres, with a higher prevalence at the distal site of the extremities. Conversely, no patient with chronic inflammatory demyelinating polyradiculoneuropathy or IgM paraproteinemic neuropathy had deposits of IgM. These results suggest that skin biopsies can be a potential tool for investigating immune-mediated demyelinating neuropathies.

Recently, ultrastructure and myelin gene protein expression of dermal nerve fibres from finger and forearm of healthy subjects and patients with Charcot Marie Tooth disease and hereditary neuropathy with liability to pressure palsies were investigated. This study demonstrated that dermal myelinated nerves not only show abnormalities previously detected in sural nerve biopsies, but also detect abnormal features not previously reported. Results suggest that biopsy of glabrous skin may be a potential tool to investigate the morphological markers of disease progression and the genotype–phenotype correlations in patients with

demyelinating or dysmyelinating neuropathies (Li *et al*., 2005).

Recommendations

Diagnostic efficiency and predictive values of skin biopsy with linear quantification of IENF in the diagnosis of peripheral neuropathy were very high (level A recommendation). Immunohistochemical technique does not seem to influence the ability of skin biopsy to demonstrate SFSN. For diagnostic purposes or as outcome measure in clinical trials we recommend rigorous quantitative assessment with appropriate quality controls (level B recommendation). Cut-off values for epidermal densities in studies based on immunofluorescence microscopy appeared to be higher than in bright-field microscopy studies. Thus far, only the bright-field microscopy method was used to establish normative reference range and diagnostic performances. For quantitative purposes in evaluating peripheral neuropathies, we recommend determination of IENF density using either immunohistochemistry with bright-field microscopy or immunofluorescence (level A recommendation). Appropriate normative data from healthy subjects matched for age, gender, ethnicity and anatomical site should be used. Quality control should include all the steps of the procedure, in particular, the aspect of intra- and inter-observer ratings.

Studies comparing the diagnostic yield of bright-field microscopy and immunofluorescence with and without confocal microscopy in homogeneous groups of neuropathy patients are warranted. We emphasize that the confocal microscopy technique may be useful to investigate cutaneous nerve fibres in demyelinating neuropathies. Furthermore, the diagnostic yield of dermal nerve fibre quantification needs to be addressed. Confocal microscopy technique applied to glabrous skin allows investigation of dermal receptors and their myelinated endings and might provide morphological information that potentially enlarges the usefulness of skin biopsy in sensory neuropathies.

Assessment of morphological changes

Besides the estimation of epidermal innervation density, several papers included morphological changes of both IENF (i.e. axonal swellings and branching) and dermal nerve bundles (i.e. weaker and fragmented immunoreactivity to PGP 9.5) amongst pathological features in patients with peripheral neuropathy. Two studies (Lauria *et al*., 2003; Herrmann *et al*., 2004b) investigated the diagnostic yield of IENF swellings in sensory neuropathies in 72 patients. Swellings were defined as enlargements either above 1.5 μm or twice the diameter of the parent IENF. Both studies found a significantly higher prevalence of swellings at the distal leg in neuropathies, including patients with normal IENF density and persisting painful symptoms in the feet, than in controls. Increased swellings at the distal leg correlated with impaired heat-pain threshold, development of symptomatic neuropathy and progression of neuropathy.

Increased branching of IENF was also considered as a common feature in peripheral neuropathies (Kennedy *et al*., 1996; Herrmann *et al*., 1999; Scott *et al*., 1999; Smith *et al*., 2001). One study (Lauria *et al*., 1999) reported significantly higher branching ratio (number of branch points/density) and normal density of IENF at the proximal thigh in patients with sensory neuropathy. Increased branching complexity in unaffected sites suggested that predegenerative changes might precede the loss of fibres. These data need to be confirmed by further studies.

Morphological abnormalities of dermal nerve bundles, such as fragmented immunoreactivity to PGP 9.5, were described in most patients with peripheral neuropathy. Nevertheless, no study designed to quantify the changes of either unmyelinated or small-myelinated nerve fibres was performed.

Recommendations

Quantification of IENF swellings at the lower limb could have a predictive value to the progression of neuropathy, especially if large (level B recommendation). Further studies are warranted to establish whether increased IENF swellings could support the diagnosis of sensory neuropathy and whether this morphological change occurs prior to decreasing IENF density. Further studies are also needed to verify whether increased branching is an early diagnostic finding in peripheral neuropathy.

Quantification of sweat gland innervation

Several studies (Karanth *et al*., 1989; McCarthy *et al*., 1995; Kennedy *et al*., 1996; Facer *et al*., 1998; Nolano *et al*., 2000, 2001; Pan *et al*., 2003; Perretti *et al*., 2003) described reduced innervation of sweat glands in patients with peripheral neuropathies using both PGP 9.5 and neuropeptide (substance P, calcitonin gene-related peptide and vasointestinal peptide) immunostaining. Two studies (Hirai *et al*., 2000; Sommer *et al*., 2002) quantified the density of sweat gland nerve fibres using different methods. Hirai *et al*. (2000) found decreased nerve fibre length around sweat glands in 32 patients with diabetic neuropathy. Sommer *et al*. (2002) reported significant correlation between anhidrosis and reduced sweat gland innervation per area in four patients with Ross syndrome.

Hilz *et al.* (2004) used a semiquantitative approach based on a 5-degree rating scale [0 = normal, 1 = reduction < 50% of normal density (mild), 2 = reduction > 50% of normal density (moderate), 3 = sparse innervation, 4 = no nerve fibres] to classify sweat gland innervation in 10 patients with familial dysautonomia. Facer *et al.* (1998) focused on leprosy neuropathy and found a correlation between reduced nicotine-induced axon-reflex sweating and decreased innervation of sweat glands. Pan *et al.* (2003) examined cutaneous innervation in Guillain-Barré syndrome (GBS). Although about 60% of patients had clinical manifestations of autonomic dysfunction, no correlation between sweat gland innervation and RR interval variability or sympathetic skin response was observed.

Recommendation

Data on sweat gland innervation density in healthy subjects and in patients with peripheral neuropathy as well as data on correlation between sweat gland nerve fibre density and autonomic assessment are limited (class III evidence). Although part of the neuropathological examination of skin biopsy, assessment of sweat gland innervation still lacks extensive validation.

Correlation between IENF density and clinical, neurophysiological, psychophysical, autonomic, and sural nerve biopsy examinations

Correlation with clinical measures of neuropathy

Only a few studies correlated epidermal innervation density with validated clinical scales. Decrease in IENF density correlated with progression of neuropathy and duration of diabetes (Holland *et al.*, 1997; Lauria *et al.*, 2003; Shun *et al.*, 2004). In HIV-associated sensory neuropathy, IENF density inversely correlated with the severity of neuropathic pain measured by patient and doctor evaluation score, but not by the Gracely Pain Scale (Polydefkis *et al.*, 2002). Herrmann *et al.* (2004b) showed that assessment of IENF density could not differentiate between patients with symptomatic or asymptomatic HIV neuropathy. However, IENF densities at the distal leg showed a non-significant trend towards an inverse correlation with overall pain intensity amongst patients with symptomatic neuropathy.

In patients with diabetic neuropathy, a negative correlation between IENF density and duration of diabetes, neurological impairment score, and the results of sensory evaluation was reported (Pittenger *et al.*, 2004; Shun *et al.*, 2004). However, no correlation between IENF density and the presence of neuropathic pain was found (Pittenger *et al.*, 2004).

In patients with GBS, reduced IENF values were significantly associated with higher disability grade, need of ventilatory support and dysautonomia (Pan *et al.*, 2003).

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 fibre versus small fibre). Overall, concordance between sural SNAP amplitude and IENF density was found in patients with clinical impairment of large nerve fibres, whereas skin biopsy appeared more sensitive than sensory nerve conduction study (NCS) in diagnosing SFSN (Holland *et al.*, 1997; Herrmann *et al.*, 1999; Periquet *et al.*, 1999; Smith *et al.*, 2001; Lauria *et al.*, 2003; Shun *et al.*, 2004). Herrmann *et al.* (2004a) described a linear correlation between medial plantar SNAP amplitude and IENF density in patients with normal sural NC values. Hirai *et al.* (2000) reported significant correlation between sural nerve conduction velocity and length of dermal nerve fibres in patients with diabetic neuropathy.

Correlation with non-conventional neurophysiological examinations

No study was specifically designed to correlate skin innervation with non-conventional methods for assessing small fibre nerve conduction, such as laser-evoked potentials, microneurography and nociceptive reflex recording. Available data rely on single case studies. In a patient with congenital insensitivity to pain, microneurography revealed the loss of sensory and skin sympathetic C fibre activity that correlated with the loss of IENF and sweat gland nerves (Nolano *et al.*, 2000). In two patients with generalized anhidrosis, microneurography and skin biopsy allowed differentiation between specific postganglionic autonomic nerve fibre impairment and eccrine gland dysfunction (Donadio *et al.*, 2005). In two patients with Ross syndrome, abnormal laser-evoked potentials correlated with decreased IENF density and increased thermal thresholds (Perretti *et al.*, 2003).

Correlation with quantitative sensory testing and autonomic nervous system testing

Psychophysical assessment of thermal, heat-pain and vibratory thresholds provides information on Aδ, C and Aβ fibres respectively. IENF density inversely correlated more closely with warm and heat-pain threshold (Pan *et al.*, 2001, 2003; Chiang *et al.*, 2002; Pittenger *et al.*, 2004; Shun *et al.*, 2004) than with cooling threshold (Holland *et al.*, 1997; Periquet *et al.*, 1999; Novak *et al.*, 2001). The size of the QST probe is likely to affect the analysis (Khalili *et al.*, 2001).

Correlation with impaired vibratory threshold is more likely when patients have clinical and electrophysiological evidence of large fibre neuropathy (Lauria *et al*., 2003).

A significant correlation was found between the decrease in IENF density and abnormal autonomic function assessed by quantitative sudomotor axonal reflex test in patients with painful neuropathy (Novak *et al*., 2001). However, no correlation with other measures of autonomic dysfunction, such as RR interval variability and sympathetic skin response, was found in GBS patients.

Correlation with sural nerve biopsy

Herrmann *et al*. (1999) compared IENF density at the distal leg and sural nerve morphometry in 26 patients with peripheral neuropathy. IENF density correlated with total myelinated, small myelinated and large myelinated fibres, whereas there was a trend towards correlation with unmyelinated fibres. IENF and sural nerve small myelinated fibre density were concordant in 73% of patients. Decreased IENF density was the only indicator of SFSN in 23% patients. Similar findings were reported in smaller case series (Holland *et al*., 1998; Scott *et al*., 1999).

Recommendations

Correlation between IENF density and the severity of neuropathic pain needs extensive validation. Decrease in IENF density might represent a further index to predict poorer outcome in patients with GBS.

Quantification of IENF density can better assess the diagnosis of SFSN (level A recommendation) than sural NCS and sural nerve biopsy. 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.

The inverse correlation between IENF density and warm threshold assessed by QST in patients with SFSN demonstrates that both methods can reliably assess the impairment of unmyelinated nerve fibres in peripheral neuropathies (level A recommendation). Correlation with heat-pain and cooling thresholds as well as measures of autonomic dysfunction needs more extensive validation (level C recommendation).

Studies of skin reinnervation

Two distinct patterns of skin reinnervation have been described. After transecting the subepidermal plexus (incision or intracutaneous axotomy model), Wallerian degeneration is followed by fast collateral sprouting from the epidermal axons outside the incision line, leading to complete reinnervation of the epidermis by 30–75 days. Conversely, removal of the incised cylinder of skin (excision model) leaves a denervated area in which Schwann cells are absent and causes a slower reinnervation rate, which is not achieved after 23 months (Rajan *et al*., 2003). These findings suggest that skin biopsy might be used to study the effect of growth factors on small fibre reinnervation in peripheral neuropathies.

Previous studies showed that cutaneous nerve fibres could spontaneously regenerate after nerve injury (Lauria *et al*., 1998; Nodera *et al*., 2003) or following chemical denervation with topical capsaicin. Parallel to the disappearance of IENF and dermal nerves, capsaicin induced loss of heat-pain and pinprick sensation that recovered after skin reinnervation (Simone *et al*., 1998; Nolano *et al*., 1999).

Polydefkis *et al*. (2004) investigated the regeneration rate of IENF after capsaicin treatment in 31 healthy and 20 diabetic subjects. The authors found that the regeneration rate of IENF was lower in diabetic patients, irrespective of the presence or absence of neuropathy, suggesting that diabetes *per se* causes a functional impairment of peripheral axonal regrowth. The relationship of this finding to the eventual development of peripheral neuropathy is uncertain.

Recommendation

Skin biopsy with quantification of IENF density can be used to assess the regeneration rate of sensory axons in peripheral neuropathies and could represent a potential outcome measure in clinical trials (level B recommendation).

EU standards

Skin biopsy is a reliable technique to assess loss and regeneration of sensory nerve fibres in peripheral neuropathies. For diagnostic purposes, we endorse 3 mm punch skin biopsy at the distal leg, and quantification of linear epidermal innervation density in at least three 50 μm thick sections per biopsy, fixed in 2% PLP or Zamboni's solution, by immunohistochemistry using anti-PGP 9.5 antibodies and bright-field microscopy or immunofluorescence with or without confocal microscopy.

We strongly recommend training in an established cutaneous nerve laboratory before performing and processing skin biopsies in the diagnosis of peripheral neuropathies. Appropriate normative data from healthy subjects matched for age, gender, ethnicity and anatomical site should be always used. 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

Collaborative studies should be designed to compare the diagnostic predictive values of IENF quantification by light and confocal microscopy technique in homogeneous groups of patients with peripheral neuropathy of different pathogenesis (i.e. axonal versus demyelinating). Standardization of methods for quantification of dermal and sweat gland nerve fibres with both the techniques should be addressed.

Correlation studies of IENF density to clinical measures, QST, nerve conductions and non-conventional neurophysiological tests should be designed in order to assess the relative diagnostic values to the progression of neuropathy.

Longitudinal studies of IENF density and regeneration rate should be performed both in healthy subjects and in patients with early neuropathy, in order to confirm the potential usefulness of skin biopsy as an outcome measure in peripheral neuropathy trials.

Statement of the likely time when the guidelines will need to be updated

We estimate that the guideline will need to be updated in 3 years.

Conflicts of interest

No member of the Task Force has conflict of interest in this report.

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