REVIEW

Skin biopsy in
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In the last decade, skin biopsy has enhanced the diagnostic armamentarium in peripheral neuropathies with the opportunity to easily investigate small diameter sensory nerves, in particular somatic unmyelinated fibres. There is no neurophysiological test for the routine examination of this class of nerve fibres. Moreover, although quantitative sensory testing – psychophysical thresholds for warm, cold, and heat pain that are functions of unmyelinated C and small myelinated A□ fibres – may be useful in population studies, they are of no help diagnosing neuropathy in individual patients (Shy *et al.* 2003).

The presence of axons within the human epidermis was denied for more than a century by most investigators, despite Paul Langerhans's first description in 1868 and further studies confirming his original work. This was due to the relative insensitivity of the staining methods, and to the lack of techniques to prevent derangement of skin nerve fibres which are so easily damaged during histological procedures (Lauria 1999a). But nowadays, skin biopsy has become part of the diagnostic work-up in peripheral neuropathies after the extensive innervation of human epidermis was confirmed by immunostaining with the neuronal marker protein gene product 9.5 (PGP 9.5) (Wang *et al.* 1990). Previously, immunohistochemical studies with antibodies against neuropeptides, in particular substance P, calcitonin gene-related product and vasoactive intestinal peptide, had suggested that skin biopsy had a role, but only in the functional study of cutaneous nerve fibres rather than for neuropathological evaluation (Gibbins *et al.* 1985).

Skin biopsy has a number of advantages over conventional nerve biopsy for the study of small fibre sensory neuropathies. It is minimally invasive, painless, easy to perform in every site of the body, repeatable at the same sites, and cheap. This review will illustrate the different methods of doing a skin biopsy, its diagnostic efficiency, and the potential application as an outcome measure for clinical trials in peripheral neuropathies.

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METHODS TO PERFORM SKIN BIOPSY AND TO QUANTIFY NERVE FIBRES

Skin biopsy is most commonly performed using a punch with a diameter of 3 mm and a length of 8 mm. A 'cylinder' of tissue composed of epidermis and superficial (subpapillary and reticular) dermis is obtained. This is carried out using a sterile technique after local anaesthesia (Fig. 1). No suture is needed. Healing is usually complete within a week, and any scar is barely visible after 3 months. Specimens are immediately fixed in 2% paraformaldehyde-lysine-periodate (PLP) for 24 h at 4 °C, then cryoprotected and serially cut in 50-µm thick sections for immunostaining (McCarthy *et al.* 1995). Fixation with PLP causes less fragmentation of the nerve fibres than the formalin used in earlier studies (Lauria *et al.* 1999b). For diagnostic purposes, at least three sections are immunostained with PGP 9.5 and analysed under the light microscope. In each section, the numbers of individual nerve fibres crossing the dermal-epidermal junction are counted, but not secondary branching of the same fibres. The length of the epidermis is measured with computerized software so the linear epidermal innervation density can be calculated, and reported as the number of intraepidermal nerve fibres per millimetre (IENF/mm) (Fig. 2). In addition, qualitative and semi-quantitative examination of dermal

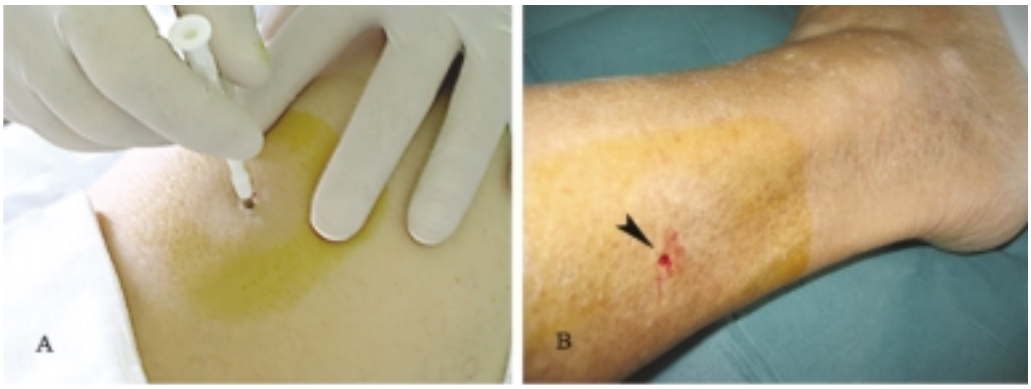

Figure 1 Skin biopsy with a 3-mm punch at (A) the thigh and (B) the distal leg (arrowhead).

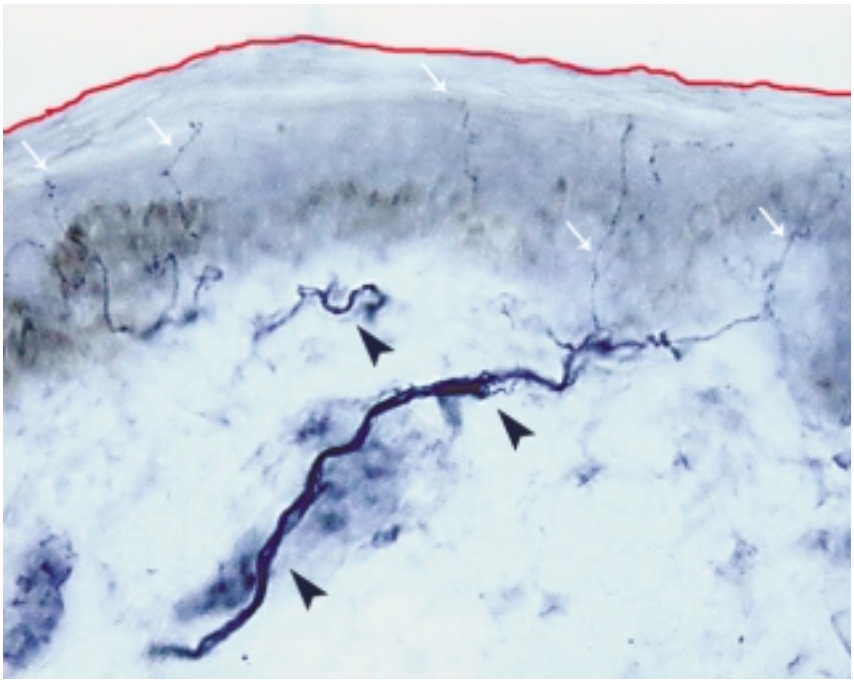

Figure 2 Quantification of intraepidermal nerve fibre density with optical microscopy. Arrows indicate intraepidermal nerve fibres and arrowheads dermal nerve bundles. All nerve fibres are strongly immunostained by anti-PGP 9.5 antibodies. The red line marks the surface of the section, which is measured, and linear intraepidermal nerve fibre density (INEF/mm) is obtained by dividing the number of fibres by the length of the section.

innervation, including of the sweat glands, is carried out.

Another approach is based on indirect immunofluorescence with confocal laser microscopy. This allows three-dimensional reconstruction of 2 □m image stacks of PGP 9.5 immunostained sections cut at 80–100 □m thickness. Linear intraepidermal nerve fibre density is then quantified, using software for image analysis, by tracing nerve fibres in three dimensions (Kennedy & Wendelscafer-Crabb 1996). Double staining with basal membrane markers, such as antibodies against collagen IV, labels the dermal-epidermal junction (Fig. 3). Confocal microscopy is particularly useful in the study of receptors, glands and vessels (Vega *et al.* 1996; Nolano *et al.* 2003).

A less invasive method to examine epidermal innervation is based on the 'blister technique'. A suction capsula, secured to the skin surface with an elastic bandage, creates negative pressure that leads to the formation of a blister in approximately 20–40 min. In effect, the device separates the epidermis from the dermis without bleeding or any need for local anaesthesia (Kennedy *et al.* 1999). This allows quantification of intraepidermal nerve fibres per area in a wider surface that in vertical sections, but does not give any information about dermal nerve fibres.

Skin biopsy can be performed anywhere on the body. This gives the opportunity to specifically examine regions involved in a neuropathic process, such as in post-herpetic neuralgia (Oaklander 2001), truncal neuropathy (Lauria *et al.* 1998), psoriasis (Johansson *et al.* 1991) and leprosy (Karanth *et al.* 1989). Biopsies are usually taken from the proximal thigh (20 cm below the anterior iliac spine) and distal leg (10 cm above the lateral malleolus), to identify the length-dependent denervation typical of dying-back axonopathy, such as in diabetic neuropathy (Lauria *et al.* 2001). Follow-up biopsies can be performed at the same sites, adjacent to the scar and within the same peripheral nerve distribution, to assess any progression of the neuropathy.

CHARACTERIZATION AND FUNCTION OF CUTANEOUS NERVE FIBRES

Early experimental studies showed that intraepidermal nerve fibres were unmyelinated axons with an exclusively somatic function, as demonstrated by their disappearance from the skin after axotomy or ganglionectomy, but not

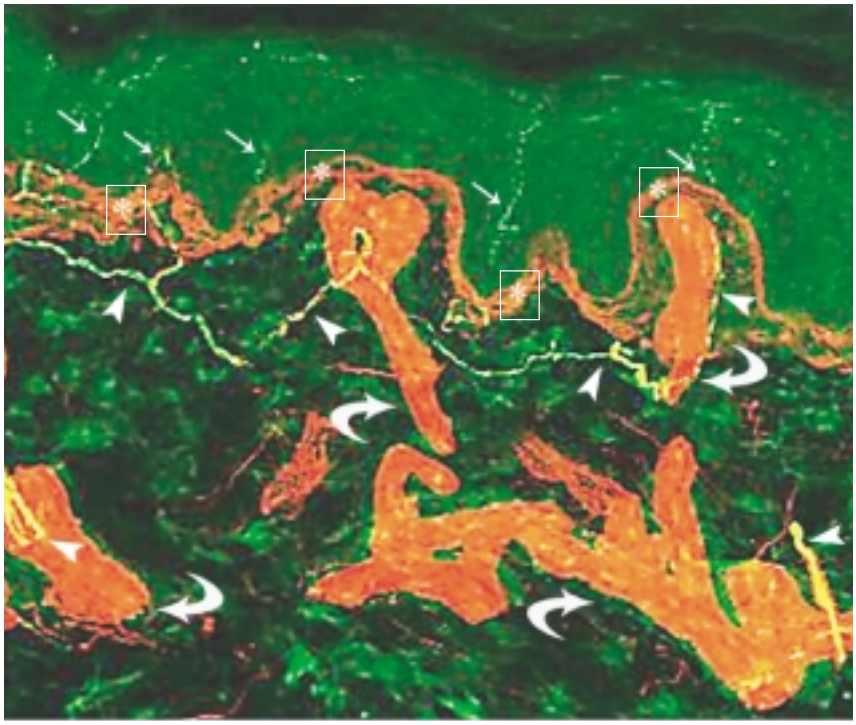

Figure 3 Double-staining confocal microscope study in a healthy subject. Nerve fibres are immunostained by anti-PGP 9.5 antibodies while the dermal–epidermal junction and vessels are immunostained by anti-collagen IV antibodies. Arrows indicate intraepidermal nerve fibres, arrowheads dermal nerve bundles, curved arrows indicate vessels, and asterisks the dermal–epidermal junction.

after dorsal rhizotomy or sympathectomy (Li *et al.* 1997). Autonomic nerve fibres innervating sweat glands, erector pilorum muscles, hair follicles, and vessels can be examined by qualitative or semi-quantitative assessment. Therefore, skin biopsy allows separate investigation of somatic and autonomic axons, giving an advantage over sural nerve biopsy in which small-diameter fibres can be quantified but not differentiated by function.

Intraepidermal nerve fibres innervate all the vital layers of the epidermis and are much better shown by PGP 9.5 than by other markers, such as neuron-specific enolase or neuropeptide immunostaining (Dalsgaard *et al.* 1989). PGP 9.5 is a predominantly neuronal form of ubiquitin carboxyl-terminal hydrolase, a cytosolic enzyme that removes ubiquitin and is transported within the slow component (Sc_b) of axonal transport (Wilkinson *et al.* 1989). Recently, antibodies against specific cytoskeletal components (Lauria *et al.* 2004) and axonal membrane (Polydefkis *et al.* 2004) have been used to immunostain intraepidermal nerve fibres, demonstrating that targeted markers can be used to investigate terminal sensory endings in peripheral neuropathies.

Only a few intraepidermal nerve fibres are immunoreactive to substance P and calcitonin gene-related product (CGRP), which mostly label the autonomic nerves of sweat glands and vessels. However, two groups of intraepidermal nerve fibres showing CGRP immunoreactivity have been described, one larger in which somatostatin is also expressed and another immunoreactive to substance P. Similarly, most CGRP positive fibres innervating cutaneous vessels co-express somastatin, whereas those innervating sweat glands co-express mainly substance P. These groups of CGRP positive fibres project to different layers of the dorsal horns in the spinal cord and are likely to have synergistic functions. Somastatin inhibits the release of substance P, suggesting that stimulation of CGRP-somastatin positive axons might reduce the effects of CGRP-substance P positive activation in inflammatory processes (Gibbins *et al.* 1987).

Intraepidermal nerve fibres arise from subpapillary dermal nerve bundles and run towards the skin surface, without entering the stratum corneum, in a linear course, with few branchings, and slight varicosities due to the uneven distribution of their cytoplasmic components. Double staining shows that Schwann cell ensheathment of subpapillary dermal nerves stops at the dermal–epidermal junction, before the fibres enter the epidermis (Lauria *et al.* 2004). This definitely demonstrates that intraepidermal nerve fibres are naked axons, rather like large diameter nerve fibres without a myelin sheath at the transducing inner core of cutaneous sensory corpuscles (Nolano *et al.* 2003).

Skin biopsy allows separate investigation of somatic and autonomic axons, giving an advantage over sural nerve biopsy in which small-diameter fibres can be quantified but not differentiated by function.

Intraepidermal nerve fibres widely express the capsaicin receptor TRPV1 (Stander *et al.* 2004), confirming they are nociceptors. TRPV1, the first cloned member of the transient receptor potential (TRP) family of vanilloid proteins, is essential for thermal hyperalgesia induced by tissue inflammation and, possibly, has a role in neuropathic pain (Davis *et al.* 2000). Intriguingly, keratinocytes express warm-sensitive receptors of the TRP family that are able to activate cutaneous nociceptors (Peier *et al.* 2002; Southall *et al.* 2003). Overall, these findings suggest that the whole epidermis, not only the axons innervating it, may represent a polymodal nociceptor and that interactions between intraepidermal nerve fibres and resident cells could be involved in the pathophysiology of neuropathic pain.

Skin biopsy also allows investigation of myelinated nerve fibres. In the dermis of hairy skin, a small number of tiny myelinated nerve fibres can be found (Lauria *et al.* 2004). Glabrous skin from the fingertips is rich in myelinated nerves, which include A□ fibres innervating mechanoceptors. Myelinated nerve fibres show intense immunoreactivity to PGP 9.5 as well as to cytoskeletal and myelin markers. This offers the opportunity to correlate the pathology of cutaneous myelinated nerve fibres with neurophysiological examination, and to use skin biopsy in the study of demyelinating neuropathies (Nolano *et al.* 2003).

NORMAL INNERVATION OF HUMAN EPIDERMIS

In healthy subjects, the density of intraepidermal nerve fibres decreases from the proximal to the distal parts of the body, the back being significantly more innervated that the extremities. There is the same paradoxical distribution, considering the higher sensory discriminative property of the distal regions, in both upper and lower limbs. Why this should be we do not know. At the proximal thigh, the normal innervation is 21 ± 11/mm (SD) (McArthur *et al.* 1998) whereas at the distal leg it ranges between 14 ± 7/mm and 12 ± 5/mm (McArthur *et al.*

1998; Gøransson *et al.* 2004). Values from other case series are not very different (Hirai *et al.* 2000; Polydefkis *et al.* 2002; Lauria *et al.* 2003). There is high inter- and intraobserver agreement for intraepidermal nerve fibre counting.

Smaller studies have provided data on intraepidermal nerve fibre density using immunofluorescence and laser scanning confocal microscopy (Kennedy *et al.* 1996; Periquet *et al.* 1999; Pittenger *et al.* 2004). Normal density at the distal leg is significantly higher than with light microscopy, from 33 ± 8/mm in subjects aged 20–59 years to 20 ± 5/mm in subjects over 60 years. Two studies (Periquet *et al.* 1999; Gøransson *et al.* 2004) found that the density decreased with age, and was lower in men than women. Conversely, no age or gender effect was found in other studies (McArthur *et al.* 1998; Lauria *et al.* 1999b).

SKIN BIOPSY IN PERIPHERAL NEUROPATHIES

Positive sensory symptoms are a frequent complaint in peripheral neuropathies. They are caused by dysfunction of sensory nerve fibres, whose assessment is therefore essential for diagnosis and management. Painful neuropathies may involve either the selective impairment of unmyelinated C and small-myelinated A□ nerve fibres, referred to as 'small fibre sensory neuropathies', in which spontaneous and evoked pain, burning, and hyperalgesia dominate the clinical picture, or the simultaneous involvement of large diameter nerve fibres, responsible for paraesthesiae and allodynia. This distinction is not trivial, as different diagnostic and therapeutic strategies may be needed. Small fibre sensory neuropathies are a frequent diagnostic challenge because the patients usually have a normal sensory examination and deep tendon reflexes. Moreover, electrodiagnostic tests may not demonstrate abnormalities in sensory nerve conduction because this investigation provides information only on large myelinated nerve fibres. Further investigations are therefore needed to make the diagnosis. Quantitative sensory testing of thermal (cold and warm) and heat-pain thresholds have limited usefulness in the diagnostic work-up of individual patients (Shy *et al.* 2003). Other methods of examination of small calibre nerve fibres, such as laser evoked potentials, require expensive technology available in only a few centres (Agostino *et al.* 2000). But the neuropathological examination of cutaneous nerve fibres with quantification of epidermal innervation density has proved to be an excellent test to confirm the diagnosis of small fibre sensory neuropathies.

Several studies have shown that in patients with painful neuropathy of different aetiologies, including diabetes, impaired glucose tolerance, HIV infection and sarcoidosis, intraepidermal nerve fibre density is significantly reduced (Omdal *et al.* 2002; Kennedy *et al.* 1996; Holland *et al.* 1998; Herrmann *et al.* 1999; Hoitsma *et al.* 2002; Polydefkis *et al.* 2002; Sumner *et al.* 2003) (Fig. 4). Skin biopsy is more sensitive than the quantitative sudomotor axon test, quantitative sensory tests, and sural nerve biopsy in the diagnosis of small fibre sensory neuropathy. In a study on 36 patients with peripheral neuropathy, quantification of intraepidermal nerve fibre density and sural nerve morphometry correlated in 73% of patients, whereas reduced epidermal innervation density was the only indicator of a small fibre neuropathy in 23% (Periquet *et al.* 1999; Herrmann *et al.* 1999).

The diagnostic efficiency (percentage of correctly classified) of skin biopsy performed at the distal leg is 88%, with a specificity (percentage of true negative) of 97% and a sensitivity (percentage of true posi-

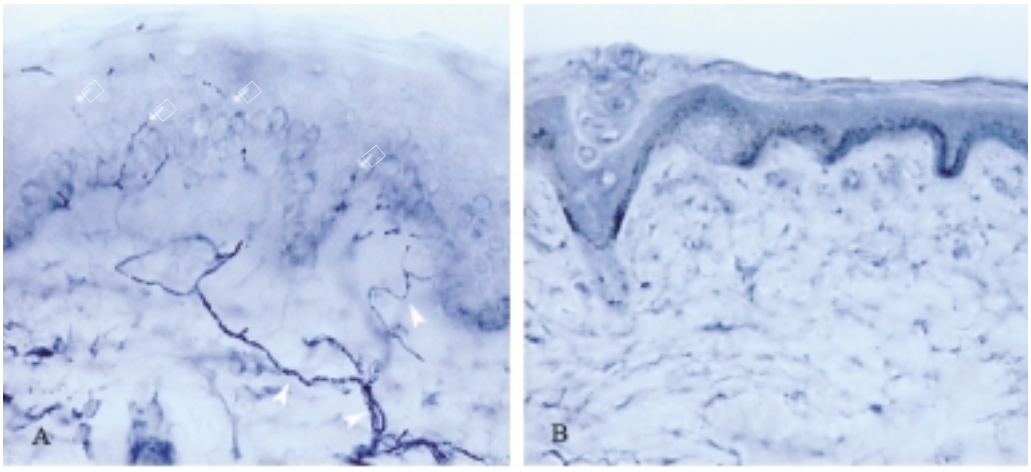

Figure 4 Skin innervation at (A) proximal thigh and (B) distal leg in a patient with diabetic neuropathy. Arrows indicate intraepidermal nerve fibres and arrowheads indicate dermal nerve bundles. At the proximal thigh, intraepidermal nerve fibre (arrows) density is reduced and dermal nerve bundles (arrowheads) show diffuse morphological changes reflecting axonal degeneration. At the distal leg, the skin appears completely denervated.

tive) of 45%. The high specificity means that quantification of intraepidermal nerve fibre density is an ideal test to verify the presence of a neuropathy for which there is little clinical or neurophysiological evidence, or when it is mandatory to confirm the diagnosis. This is the case for small fibre sensory neuropathy, in which clinical and electrophysiological examinations are frequently normal. The relatively low sensitivity implies that normal intraepidermal nerve fibre density does not rule out the presence of sensory neuropathy. Sensitivity and specificity reflect the probabilities of skin biopsy confirming the presence or absence of a neuropathy diagnosed on the basis of clinical symptoms and signs. Most importantly for clinicians, skin biopsy has a positive predictive value of 92% and a negative predictive value of 90%. This means that the probability of detecting or ruling out a small fibre sensory neuropathy, given a positive or negative test result, is very high (McArthur *et al.* 1998).

Some patients, despite persisting sensory symptoms in the feet, have normal intraepidermal nerve fibre density at the distal leg (Holland *et al.* 1998; Herrmann *et al.* 1999; Periquet *et al.* 1999). However, we have recently shown that quantification of intraepidermal nerve fibre swellings (Fig. 5), which are axonal changes diffusely present in these patients, can support an earlier diagnosis of small fibre sensory neuropathy and predict its progression (Lauria *et al.* 2003). Moreover, in patients with neuropathy, dermal nerve fibres usually show diffuse morphological changes of the dermal nerve bundles, characterized by weaker and fragmented PGP 9.5 immunoreactivity, and reduced innervation of sweat glands. This reflects degeneration of somatic and cholinergic sympathetic axons, respectively (Fig. 6).

Recently, skin biopsy has been used to demonstrate abnormalities in unmyelinated axons in neuropathies previously supposed to affect exclusively large nerve fibres, such as the Guillain–Barré syndrome and chronic inflammatory demyelinating polyradiculoneuropathy (Chiang *et al.* 2002; Pan *et al.* 2003). Moreover, in neuropathies associated with antibodies against myelin-associated glycoprotein, skin biopsy can be used to detect specific deposits of IgM and complement in dermal small myelinated nerves (Lombardi *et al.* 2005). These findings suggest that skin biopsy is potentially useful in the study of immune-mediated neuropathies.

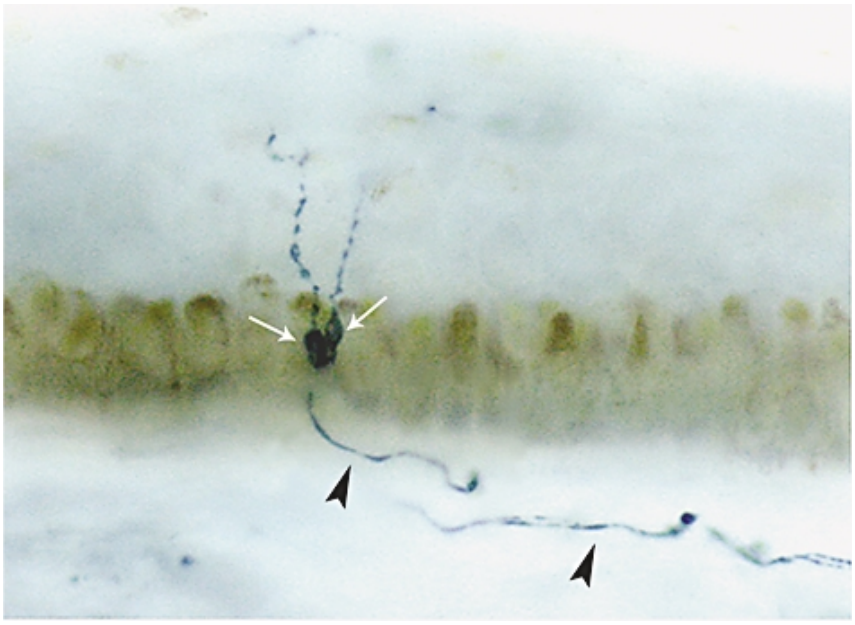

Figure 5 PGP 9.5 positive skin nerve fibres in a patient with painful neuropathy. Arrows indicate large swellings in a branched epidermal axon. Arrowheads indicate the dermal nerve bundles from which the epidermal axon arises. Note the weak and fragmented immunoreactivity reflecting axonal degeneration.

REGENERATION OF SKIN NERVE FIBRES

Cutaneous nerve fibres can spontaneously regenerate after remission of neuropathies, and after chemical skin denervation with topical capsaicin (Lauria *et al.* 1998; Nodera *et al.* 2003). Parallel to the disappearance of intraepidermal nerve fibres and dermal nerves, capsaicin induces loss of heat pain and pinprick sensation that recovers after skin reinnervation

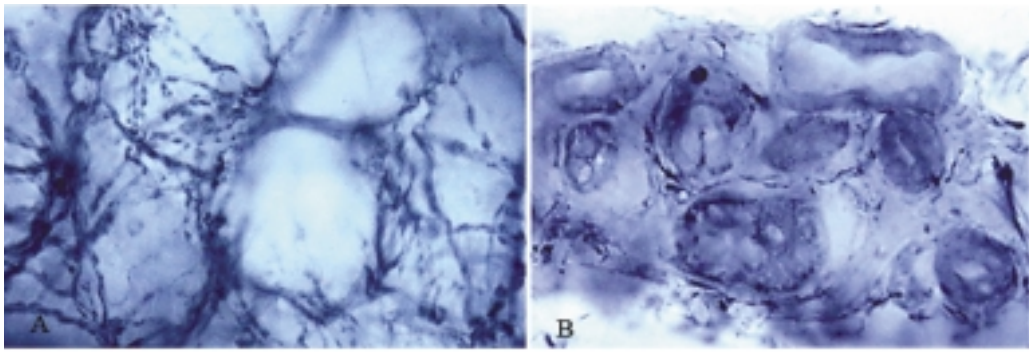

Figure 6 Sweat gland innervation in (A) a healthy subject and (B) a patient with peripheral neuropathy. Nerve fibres densely surround the secretory tubules of normal sweat glands. Note the nearly complete loss of nerve fibres in the patient with peripheral neuropathy.

(Nolano *et al.* 1999). This suggests a primary role of intraepidermal nerve fibres as polymodal receptors for nociceptive stimuli. However, their functions are likely to be broader and have not yet been characterized. They could be 'nocieffectors' and interact with keratynocites expressing warm-sensitive receptors, and with Langerhans cells, which are immune-competent cells (Hosoi *et al.* 1993; Gaudillere *et al.* 1996).

Recently, Polydefkis *et al.* (2004) used the capsaicin model to investigate the regeneration rate of intraepidermal nerve fibres in healthy and diabetic subjects. Interestingly, they found that the rate was lower in diabetes irrespective of the presence of a neuropathy. This suggests that diabetes *per se* causes functional impairment of peripheral axons that can eventually evolve to an overt neuropathy. These results strengthen the potential role of skin biopsy with quantification of intraepidermal nerve fibre density as an outcome measure in clinical trials, as already shown in experimental models of neuropathy (Bianchi *et al.* 2004).

WHEN IS SKIN BIOPSY INDICATED?

The ability of skin biopsy to investigate somatic unmyelinated axons in the epidermis makes this technique a useful tool for studying patients with painful neuropathy. These patients complain of persisting positive sensory symptoms such as burning, shooting pain, hyperaesthesia, allodynia, and paraesthesia, which severely affect their quality of life. Frequently, they do not have any signs of neuropathy on clinical examination and they have normal sensory and motor nerve conduction in the lower limbs. In clinical practice, skin biopsy is primarily indicated to confirm the diagnosis of small fibre sensory neuropathy in patients with sensory symptoms in the distal lower limbs and no or little evidence of neuropathy on clinical and electrophysiological grounds. The possibility of examining dermal small myelinated nerve fibres, and even large myelinated nerve fibres if glabrous skin from the fingertips is taken, could widen the diagnostic role of skin biopsy to demyelinating neuropathies. Finally, the study of sweat gland innervation can provide evidence of autonomic nervous system involvement in peripheral neuropathies.

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CONCLUSIONS

- Painful neuropathies are characterized predominantly by the degeneration of small diameter sensory nerve fibres, which is difficult if not impossible to detect with routine neurophysiological examination.
- Skin biopsy has become part of the diagnostic work-up in sensory neuropathies because the density of the small diameter nerve fibres that innervate epidermis and superficial dermis can be quantified.
- Skin biopsy is usually performed with a 3-mm punch. No suture is needed and healing is complete within a week. Alternatively, the 'blister technique' can be used to separate the epidermis from the dermis, though this does not give any information on dermal innervation.
- Skin biopsies are usually taken from the proximal thigh and the distal leg to identify the length-dependent denervation typical of dying-back axonopathies, such diabetic neuropathy. Follow-up biopsies can be performed at the same sites, adjacent to the scar and within the same peripheral nerve distribution, to assess any progression of the neuropathy.
- After fixation, sections are immunostained with the panaxonal marker anti-PGP 9.5. All intraepidermal axons are counted, the length of each section is measured, and the linear epidermal innervation density (IENF/mm) is calculated. Qualitative and semi-quantitative examination of dermal nerve fibres and sweat glands is also performed.
- In healthy subjects, the intraepidermal nerve fibre density is significantly higher at proximal than distal sites of the body, about 21 fibres/mm at the proximal thigh and 13 fibres/mm at the distal leg.
- Skin biopsy is more sensitive than the quantitative sudomotor axon test, quantitative sensory tests, and sural nerve biopsy in making the diagnosis of small fibre sensory neuropathy. The probability of detecting or ruling out a small fibre sensory neuropathy using skin biopsy is very high.
- Skin biopsy is primarily indicated to confirm the diagnosis of small fibre sensory neuropathy in patients with persisting painful symptoms and little or no clinical and electrophysiological evidence of a neuropathy. Skin biopsy can also be used to investigate cutaneous myelinated nerve fibres and has additional potential in the study of immune-mediated neuropathies.
- Skin biopsy can be used to investigate the regeneration rate of nerve fibres, of potential as an outcome measure for clinical trials in peripheral neuropathies.

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