

INVESTIGATIVE REPORT

Increased Density of Cutaneous Nerve Fibres in the Affected Dermatomes After Herpes Zoster Therapy

Charalampos ZOGRAFAKIS¹, Dina G. TINIAKOS², Marina PALAIOLOGOU², Mirsini KOULOUKOUSSA², Christos KITTAS² and Nikos STAVRIANEAS¹

¹Second Department of Dermatology and Venereology, University of Athens, Attikon General University Hospital, and ²Laboratory of Histology & Embryology, Medical School, University of Athens, Athens, Greece

Herpes zoster neural injury was assessed by determining cutaneous nerve density in skin biopsies from the affected dermatomes of 35 adult patients with herpes zoster in the acute phase and 3 months post-treatment, using protein gene product 9.5 immunohistochemistry. In contrast to the significant increase in subepidermal nerve fibre density ($11.77 \pm 4.88/\text{mm}$ vs. $13.29 \pm 5.74/\text{mm}$, $p=0.045$) after 3 months, no differences were found in epidermal free nerve endings ($2.43 \pm 2.35/\text{mm}$ and $2.8 \pm 2.86/\text{mm}$, $p=0.168$). Patients with post-herpetic neuralgia had significantly lower subepidermal nerve fibre densities ($9.7 \pm 2.05/\text{mm}$ vs. $14.72 \pm 6.13/\text{mm}$, $p=0.011$) compared to with non-post-herpetic neuralgia patients. No differences in cutaneous nerve density were found in relation to antiviral therapy. In conclusion, 3 months after acute infection, no sign of epidermal innervation recovery is observed, while the increased subepidermal nerve fibre density in the affected dermatomes probably reflects nerve regeneration that is not affected by antiviral agent type. Subepidermal nerve fibre density is decreased in patients with post-herpetic neuralgia 3-months post-acute herpes zoster infection. *Key words: cutaneous nerves density; herpes zoster; immunohistochemistry; PGP 9.5.*

Accepted Apr 10, 2013; Epub ahead of print Aug 27, 2013

Acta Derm Venereol 2014; 94: 168–172.

Charalampos Zografakis, Second Department of Dermatology and Venereology, University of Athens, Attikon General University Hospital, Rimini 1, 12462, Haidari, Athens 12462, Greece. E-mail: chzgr@yahoo.com

Herpes zoster (HZ) is caused by the reactivation of varicella-zoster virus (VZV) from a latent infection of dorsal sensory or cranial nerve ganglia (1). The main risk factor for the development of HZ is increasing age, leading to a decline in VZV-specific cell-mediated immunity. Human immunodeficiency virus infection, chemotherapy, malignancy and chronic corticosteroid use may also increase the risk of developing HZ. With reactivation, the virus spreads transaxonally to the skin, causing a rash with a dermatomal distribution, and severe radicular pain, which, in most patients, disappears completely within a period of 1–2 months (1).

Post-herpetic neuralgia (PHN) is defined as pain that persists for 1–3 months following the HZ rash. PHN affects approximately 50% of patients over 60 years of age and 15% of all HZ patients (2). PHN results when necrosis and scarring of neurones in the dorsal root ganglion (DRG) lead to degeneration and destruction of the emerging motor and sensory projections (3).

Previous histological studies of patients with acute HZ and PHN revealed that cutaneous innervation density is decreased in the affected region (4–6). Rowbotham et al. (7) were the first to quantitate the density of dermal and epidermal innervation in allodynic PHN-affected skin using immunofluorescence. They found a positive correlation between the reduction in cutaneous innervation density with the loss of thermal sensory function, but an inverse correlation with allodynia severity. In a study on the natural history of sensory function after HZ, the severity of initial injury was shown to predict PHN, and especially impaired cold sensation (8).

Oaklander et al. (9) compared neurite densities in PHN-affected skin with densities from previously shingles-affected skin without PHN, using immunohistochemistry for pan-neural marker PGP 9.5. They observed that the presence of PHN strongly correlated with loss of cutaneous sensory nerve terminals. Petersen et al. (10), in a natural history study of cutaneous innervation, following HZ with serial skin biopsies and PGP 9.5 immunofluorescence, have shown that reinnervation of the affected skin in HZ is not evident at 6 months after the rash onset, and resolution of pain and allodynia does not require cutaneous reinnervation.

The aim of the current study was to assess cutaneous nerve fibre density in the affected dermatome during acute HZ infection, and compare it with that of the same dermatome 3 months later. We correlated our findings with the type of antiviral drug prescribed and the development of PHN. To our knowledge this is the first study to evaluate the possible effect of antiviral therapy on cutaneous nerve fibre density in relation to the development of PHN.

MATERIALS AND METHODS

Patients

A total of 35 adult patients (male/female ratio 1.7:1, mean age 62.4 ± 12.5 years) with acute HZ not affecting the cranial nerves

Table I. Patient demographics and affected dermatomes (n = 35)

Variables	
Patients, men, n (%)	22 (62.8)
Age, years, mean \pm standard deviation	62.4 \pm 12.5
Dermatome affected, n (%)	
Cervical	3 (8.6)
Thoracic	19 (54.3)
Lumbar	10 (28.6)
Sacral	3 (8.6)

were included in the study. Patient demographics and the dermatomes affected are shown in Table I. After obtaining written informed consent from all patients, a 4-mm punch biopsy was obtained from clinically not affected skin, between the grouped vesicles of zoster, in the same affected dermatome, during the first 48 h of rash development. Antiviral therapy with 1 of 3 antiviral agents (valacyclovir, famciclovir or brivudine) was randomized as the patients were enrolled in the study, according to known guidelines (11). In detail, 13 patients were treated with brivudine 125 mg \times 1 for 7 days, 11 with valacyclovir 1 g \times 3 for 7 days, and 11 with famciclovir 500 mg \times 3 for 7 days. Repeat biopsies from the same area were performed 3 months later on all patients who completed the study (n = 35). To evaluate normal linear densities of epidermal nerve endings and subepidermal nerve fibres we used 4-mm normal skin biopsies (controls) from the trunk region of 10 age- and gender-matched subjects at autopsy without skin diseases, after obtaining consent according to local guidelines. The study conformed to the standards of the institution's ethics committee and with the Declaration of Helsinki (1975), as amended in 1983.

Table II shows patients' symptoms on admission to the study and 3 months after antiviral treatment. Despite receiving adequate therapy on time, 10 out of 35 patients (28.5%) developed PHN in the affected dermatomes.

Immunohistochemistry

All skin biopsies were fixed in 10% neutral formalin solution and were routinely embedded in paraffin. Serial tissue sections, 5 μ m thick, from pre-treatment and post-treatment HZ skin biopsies were used for every patient; one for routine haematoxylin-eosin staining and 4 for immunohistochemistry using the pan-neural marker protein gene product 9.5 (PGP 9.5) mouse monoclonal antibody (Novocastra Laboratories, Newcastle upon Tyne, UK), incubated at 1:100 dilution for 20 h at 4°C. Anti-PGP 9.5 targets ubiquitin carboxyl-terminal esterase L1, an enzyme found exclusively and ubiquitously in neurones (12). We used the Novolink polymer detection system (Novocastra Laboratories, UK). The final immunostaining reaction was developed using 3'3'diaminobenzidine (DAB) (Sigma, Saint Louis, MO, USA) as chromogen. We used negative controls, in which the staining of the primary antibody was omitted, thus the specific staining patterns of PGP 9.5 were shown.

Immunostained nerve fibres/endings were identified and counted in 4 serial sections for each biopsy by 2 observers (ChZ, DT) at a double-headed microscope. Nerve fibres/endings were evaluated in the epidermis and the upper portion of the papillary dermis close to the basement membrane, in a 50- μ m wide zone parallel to the dermal-epidermal junction, at 40 \times magnification, the minimum

Table II. Herpes zoster (HZ) patient symptoms in the acute phase and after 3 months

Sensory	Acute phase, HZ rash n (%)	After 3 months n (%)
Pain	25 (71.4)	10 (28.5)
Itch	3 (8.6)	0
Pain and itch	4 (11.4)	0
None	3 (8.6)	25 (71.5)

length counted as positive was 3 μ m. Nerves in the reticular dermis were not assessed. The length of epidermis along the upper margin of the stratum corneum was measured using a microscope intraocular lens ruler. The linear nerve fibre/endings density was calculated as the number of fibres/endings per millimetre of epidermal length, as described previously by McArthur et al. (13).

Statistical analysis

Statistical analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The non-parametric Wilcoxon signed-rank test and Mann-Whitney test (for samples with unequal sizes) and the *t*-test (for non-normally and normally distributed variables) were applied, respectively. The normality of the variables' distribution was checked using the Kolmogorov-Smirnov test. Analysis of variance (ANOVA) was used to analyse differences between the 3 groups of patients receiving different antiviral agents, and in the subgroups of PHN and non-PHN patients. The level of significance was set at $p < 0.05$.

RESULTS

Variation in nerve fibre/endings density between serial sections of the same biopsy proved non-significant (data not shown). Table III shows the linear density of free nerve endings in the epidermis and of nerve fibres in the upper papillary (subepidermal) dermis of controls and of our study population during the acute phase and after 3 months, measured as exemplified in (Fig. 1). There was no statistical difference in the epidermal nerve ending density in the acute phase and after 3 months in all patients (Wilcoxon signed-rank test, $p = 0.168$). In contrast, a statistically significant increase was observed in subepidermal nerve fibre density at 3 months compared with that at the acute phase (*t*-test, $p = 0.045$) (Table III).

Significant decrease was observed in the epidermal nerve ending densities between the counted normal values and acute phase HZ ($p < 0.001$) and after 3 months ($p < 0.001$). In addition, statistically significant decrease in subepidermal nerve fibre densities was found between control and acute phase HZ ($p = 0.026$), but no difference was observed 3 months later ($p = 0.58$).

Table III. Linear density (mean number \pm SD/mm epidermis) of free nerve endings and nerve fibres in control skin and in patient skin during the acute phase and after 3 months

Topography of PGP 9.5 immunostaining	Control skin	Acute phase HZ rash	After 3 months	<i>p</i> -value
Free nerve endings in the epidermis	14.4 \pm 2.7	2.43 \pm 2.35**	2.8 \pm 2.86**	NS**
Subepidermal nerves	13.7 \pm 2.3	11.7 \pm 4.88***	13.29 \pm 5.74***	0.045***

Significance is compared between acute phase and after 3 months. NS: non-significant; HZ: herpes zoster.

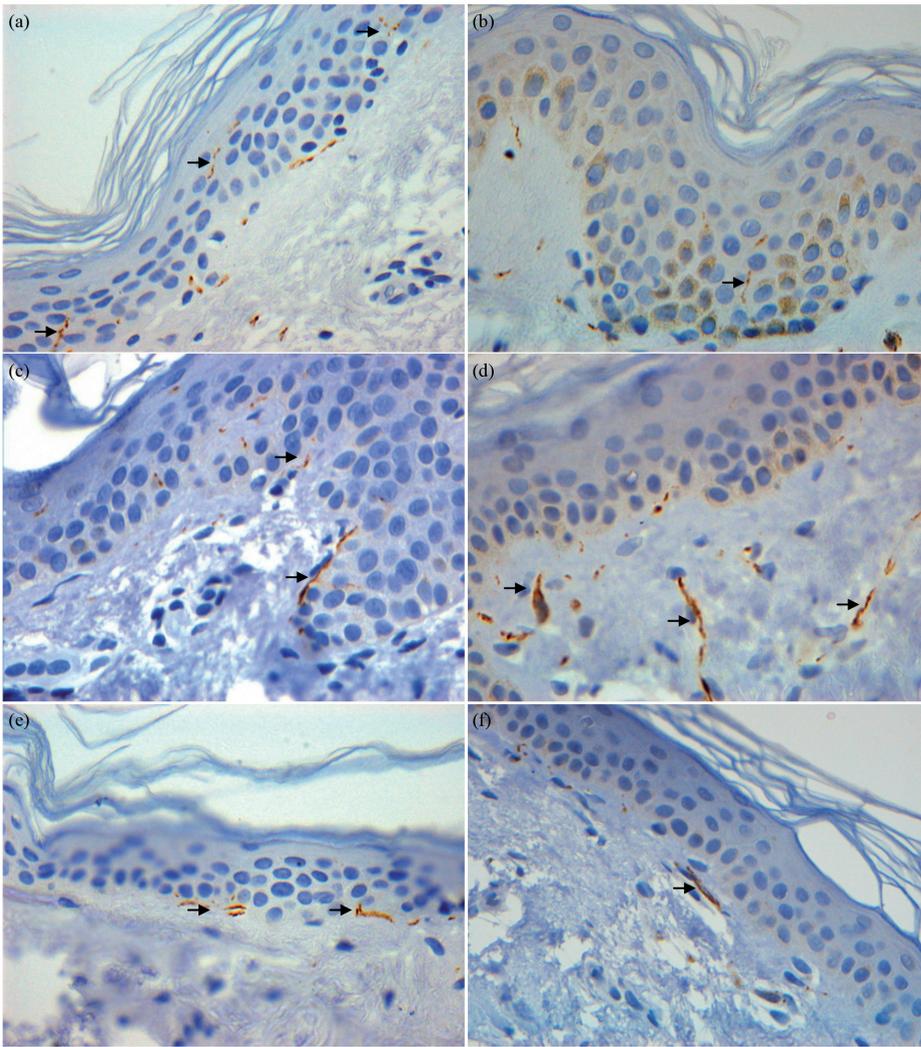


Fig. 1. Protein gene product 9.5 (PGP 9.5)-positive nerve endings and/or fibres (arrows) in the epidermis and papillary dermis of herpes zoster skin biopsies. (a) Epidermis in acute phase, PGP9.5-positive nerve endings ($\times 100$). (b) Epidermis after 3 months, PGP9.5-positive nerve endings, ($\times 100$). (c) Papillary dermis in acute phase, PGP 9.5-positive nerve fibres ($\times 100$). (d) Papillary dermis after 3 months: higher PGP 9.5-positive nerve fibre density compared with C, non-post-herpetic neuralgia (PHN) ($\times 100$). (e) Papillary dermis in acute phase, PGP 9.5-positive nerve fibres ($\times 100$). (f) Papillary dermis in acute phase, PGP 9.5-positive nerve fibres, PHN ($\times 100$).

In the subgroups of patients who developed PHN and of those who did not (non-PHN), no significant differences were observed in the subepidermal nerve fibre density in the acute phase (Mann–Whitney test, $p=0.67$), but a statistically significant decrease was evident after 3 months ($p=0.011$) (Fig. 2).

We compared the linear nerve density in the papillary dermis in the acute phase and 3 months post-treatment in the groups receiving different antiviral agent. From the patients who developed PHN ($n=10$), 4 (30%) received brivudine, 3 (27%) valacyclovir and 3 (27%) famciclovir. No statistically significant differences were observed between the groups of patients receiving different antiviral agents when all patients were tested ($p=0.305$) or in the subgroups of PHN and non-PHN patients ($p=0.811$ and $p=0.254$, respectively).

DISCUSSION

The lower counts of epidermal nerve and subepidermal nerve fibre density in acute HZ in comparison with the normative reference range (13, 14) and normal

skin assessed in our study may signify that the nerve damage occurs early in the course of HZ, probably before the appearance of the characteristic rash, thus resulting in a low density of epidermal nerves at the time of biopsy. Previous studies in painful neuropathic

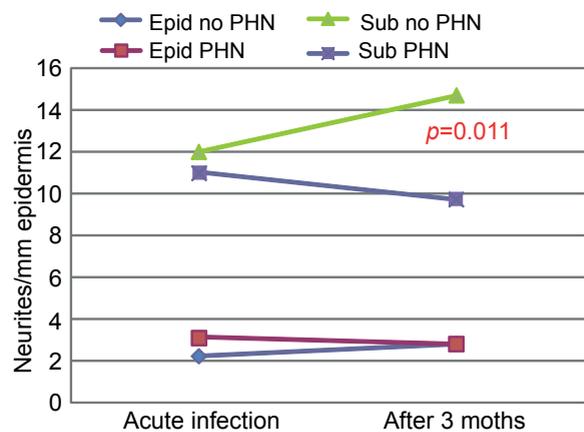


Fig. 2. Changes in the density of epidermal (Epid) and subepidermal (Sub) innervation in patient groups with and without post-herpetic neuralgia (PHN).

syndromes using skin biopsies report a loss of epidermal nerve fibres, and some of them show an inverse correlation between the severity of painful neuropathy and epidermal nerve density (7, 15, 16). In accordance, in our study the patients who developed PHN had a lower subepidermal nerve fibre density 3 months after the acute infection compared with those who did not develop PHN.

The epidermal nerve ending densities' values we observed in our HZ patients are lower compared with those of previous studies. This difference could be partially attributed to the use of neutral formalin as fixative for the skin biopsies, which tends to underestimate by approximately 20% compared with PLP (paraformaldehyde, lysine, and periodate) fixation (17). Most recent studies use PLP or Zamboni (2% paraformaldehyde and picric acid) fixative. According to the European Federation of Neurological Societies/Peripheral Nerve Society (EFNS/PNS) Guidelines on the use of skin biopsy in the diagnosis of small fibre neuropathy, formalin fixation could cause a more fragmented appearance of nerve fibres, but does not affect the measurement of the innervation density (18). Furthermore, the thickness of the evaluated tissue sections may have affected the results, since in previous studies nerve density was assessed in sections 15–50 µm thick (10, 12), while we used thinner (5 µm thick) tissue sections routinely used for immunohistochemistry.

We did not find any significant difference in linear nerve ending density in the epidermis between biopsies taken at diagnosis and those taken after 3 months, indicating that epidermal nerve regeneration has not started at this time. Our findings are in agreement with Petersen et al. (10), who at 6 months still could not observe any evidence of nerve regeneration in the epidermis.

The statistically significant increase in the nerve fibre density in the upper papillary dermis observed 3 months after HZ infection denotes subepidermal nerve regeneration following viral nerve destruction. The reasons for the absence of axonal growth in the epidermis are unknown, it has been suggested that, in the dermal nerves, Schwann cell bands support rapid growth, and the absence of Schwann cells in the epidermis results in a defect in axon support and guidance (19). This is the first study to assess the evolution of nerve injury in HZ evaluating data from the acute stage by examining immunostained nerve fibres fragments in corresponding positions. In this aspect our findings are different from the study by Petersen et al. (10), who did not find any trace of nerve regeneration at 6 months. However, their study population was older than ours (only patients over 50 years old were included) and older subjects reportedly have lower densities of intraepidermal nerves (13). Furthermore, they required a pain score >20 (on the 0–100-mm pain visual analogue scale) for inclusion to their study, and pain is reportedly positively associa-

ted with increased nerve damage (20). In our study the development of PHN was associated with greater nerve damage, as there were differences in the subepidermal nerves densities between PHN and non-PHN patient subgroups at 3-months post-acute phase, indicating that cutaneous re-innervation is probably required for the resolution of PHN pain. Neuropathic pain has been correlated with epidermal nerve loss in small fibre sensory neuropathy, post-herpes neuropathy, HIV sensory neuropathy and diabetic neuropathy. Explanations for this apparent paradox include possible changes in both peripheral and central nervous systems. PHN pain state could be attributed to: (i) increased primary afferent nociceptor firing (as a result of abnormal collections of sodium channels in damaged peripheral nerve fibres, causing ectopic discharge); (ii) decreased inhibition of neuronal activity in central structures (due to loss of inhibitory neurons); (iii) altered central processing (central sensitization) so that sensory input is amplified and sustained; and (iv) alterations in the cutaneous environment could provide an ongoing stimulus; it is possible that cytokines or other inflammatory mediators persist in the painful region without histological evidence of inflammation.

The development of PHN was equally distributed in the 3 groups of patients receiving different antiviral agents. There is insufficient evidence from randomized controlled trials to determine whether antiviral treatments prevent PHN (21, 22). In our study, no antiviral drug proved superior to others in preventing PHN. While patient age, severity of the eruption and severity of acute pain in HZ correlates well with increased risk of PHN development (23), only rash severity (determined based on percentage of dermatome affected by rash) was positively correlated with PHN. One of the objectives of our study was to assess the possibility that epidermal or subepidermal nerve densities during acute infection could be used as a prognostic factor for PHN development. However, no statistically significant differences in nerve density were seen in the acute phase between PHN and non-PHN patients.

The type of antiviral therapy that was promptly prescribed in a randomized fashion did not produce any statistically significant difference in skin innervation 3 months after, or any significant difference in PHN appearance.

This study has some limitations, including the small number of participants and the number of biopsies taken from each patient. However, these limitations are inevitable, since HZ is usually a benign and self-limited disease and, therefore, gaining patient consent to perform at least 2 skin biopsies that are not only painful, but which will also heal leaving scars, is extremely difficult. Our quantitative method of evaluation of PGP 9.5-specific immunostained structures may be less objective than a digital image-analysis; however

this limitation was minimized by the use of 2 observers using a double-headed microscope who agreed on the measurements performed.

In conclusion, 3 months after acute HZ infection, increased subepidermal nerve fibre density is observed in the affected dermatome, probably reflecting nerve regeneration. This result is not affected by antiviral agent type and decreases with development of PHN. In contrast, no sign of innervation recovery is observed in the epidermis. Future studies including larger numbers of patients and skin biopsies taken at the time of diagnosis and at least one year post-infection may shed more light on the time-course of skin reinnervation in patients with HZ, and assess whether completion of nerve regeneration has occurred.

REFERENCES

- Johnson RW. Herpes zoster and postherpetic neuralgia. *Expert Rev Vaccines* 2010; 9: 21–26.
- Niv D, Maltzman-Tseikhin A. Postherpetic neuralgia: the never-ending challenge. *Pain Pract* 2005; 5: 327–340.
- Smith FP. Pathological studies of spinal nerve ganglia in relation to intractable intercostals pain. *Surg Neurol* 1978; 10: 50–53.
- Ebert MH. Histologic changes in sensory nerves of the skin in herpes zoster. *Arch Dermatol Syphil* 1949; 60: 641–648.
- Zacks SI, Langfitt TW, Elliott FA. Herpetic neuritis. A light and electron microscopic study. *Neurology* 1964; 14: 744–750.
- Muller S, Winkelmann R. Cutaneous nerve changes in zoster. *J Invest Dermatol* 1969; 52: 71–77.
- Rowbotham MC, Yosipovitch G, Connolly MK, Finlay D, Forde G, Fields HL. Cutaneous innervation density in the allodynic form of postherpetic neuralgia. *Neurobiol Dis* 1996; 3: 205–214.
- Petersen KL, Rowbotham MC. Natural history of sensory function after herpes zoster. *Pain* 2010; 150: 83–92.
- Oaklander AL, Romans K, Horasek S, Stocks A, Hauer P, Meyer RA. Unilateral postherpetic neuralgia is associated with bilateral sensory neuron damage. *Ann Neurol* 1998; 44: 789–795.
- Petersen KL, Rice FL, Farhadi M, Reda H, Rowbotham MC. Natural history of cutaneous innervation following herpes zoster. *Pain* 2010; 150: 75–82.
- Dworkin RH, Johnson RW, Breuer J, Gnann JW, Levin MJ, Backonja M, et al. Recommendations for the management of herpes zoster. *Clin Infect Dis* 2007; 44 (S 1): S1–26.
- Wilkinson KD, Lee KM, Deshpande S, Duerksen-Hughes P, Boss JM, Pohl J. The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. *Science* 1989; 246: 670–673.
- McArthur JC, Stocks EA, Hauer P, Cornblath DR, Griffin JW. Epidermal nerve fiber density: normative reference range and diagnostic efficiency. *Arch Neurol* 1998; 55: 1513–1520.
- Umapathi T, Tan WL, Tan NC, Chan YH. Determinants of epidermal nerve fiber density in normal individuals. *Muscle Nerve* 2006; 33: 742–746.
- McCarthy BG, Hsieh ST, Stocks A, Hauer P, Macko C, Cornblath DR, et al. Cutaneous innervation in sensory neuropathies: evaluation by skin biopsy. *Neurology* 1995; 45: 1848–1855.
- Oaklander AL. The density of remaining nerve endings in human skin with and without post herpetic neuralgia after shingles. *Pain* 2001; 92: 139–145.
- Kennedy WR, Wendelschafer-Crabb G, Polydefkis M, McArthur JC. Pathology and quantitation of cutaneous innervation. In: Dyck PJ, Thomas PK, editors. *Peripheral neuropathy*. Philadelphia: Saunders, 2005: 869–896.
- Lauria G, Hsieh ST, Johansson O, Kennedy WR, Leger JM, Mellgren SI, et al. 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. *Eur J Neurol* 2010; 17: 903–912.
- Navarro X, Verdu E, Wendelschafer-Crabb G, Kennedy WR. Immunohistochemical study of skin reinnervation by regenerative axons. *J Comp Neurol* 1997; 380: 164–174.
- Decroix J, Partsch H, Gonzalez R, Mobacken H, Goh CL, Walsh L, et al. Factors influencing pain outcome in herpes zoster: an observational study with valaciclovir. *J Eur Acad Dermatol Venereol* 2000; 14: 23–33.
- Li Q, Chen N, Yang J, Zhou M, Zhou D, Zhang Q, et al. Antiviral treatment for preventing postherpetic neuralgia. *Cochrane Database Syst Rev* 2009; 15: CD006866.
- Bruxelle J, Pinchinat S. Effectiveness of antiviral treatment on acute phase of herpes zoster and development of post herpetic neuralgia: review of international publications. *Med Mal Infect* 2012; 42: 53–58.
- Opstelten W, Zuithoff NP, van Essen GA, van Loon AM, van Wijck AJ, Kalkman CJ, et al. Predicting postherpetic neuralgia in elderly primary care patients with herpes zoster: prospective prognostic study. *Pain* 2007; 132: 52–59.