



Universiteit
Leiden
The Netherlands

Pain-related changes in cutaneous innervation of patients suffering from bortezomib-induced, diabetic or chronic idiopathic axonal polyneuropathy

Bechakra, M.; Nieuwenhoff, M.D.; Rosmalen, J. van; Groeneveld, G.J.; Huygen, F.J.P.M.; Zeeuw, C.I. de; ... ; Jongen, J.L.M.

Citation

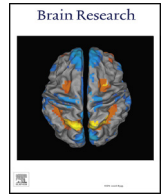
Bechakra, M., Nieuwenhoff, M. D., Rosmalen, J. van, Groeneveld, G. J., Huygen, F. J. P. M., Zeeuw, C. I. de, ... Jongen, J. L. M. (2020). Pain-related changes in cutaneous innervation of patients suffering from bortezomib-induced, diabetic or chronic idiopathic axonal polyneuropathy. *Brain Research*, 1730. doi:10.1016/j.brainres.2019.146621

Version: Publisher's Version

License: [Creative Commons CC BY 4.0 license](https://creativecommons.org/licenses/by/4.0/)

Downloaded from: <https://hdl.handle.net/1887/3182793>

Note: To cite this publication please use the final published version (if applicable).



Research report

Pain-related changes in cutaneous innervation of patients suffering from bortezomib-induced, diabetic or chronic idiopathic axonal polyneuropathy

Malik Bechakra^{a,b,1}, Mariska D. Nieuwenhoff^{c,1}, Joost van Rosmalen^d, Geert Jan Groeneveld^e, Frank J.P.M. Huygen^c, Chris I. de Zeeuw^{b,f}, Pieter A. van Doorn^a, Joost L.M. Jongen^{a,*}

^a Dept. of Neurology, Erasmus MC, Dr. Molewaterplein 40, 3015GD Rotterdam, the Netherlands

^b Dept. of Neuroscience, Erasmus MC, Dr. Molewaterplein 50, 3015GE Rotterdam, the Netherlands

^c Dept. of Anesthesiology, Erasmus MC, Dr. Molewaterplein 40, 3015GD Rotterdam, the Netherlands

^d Dept. of Biostatistics, Erasmus MC, Wytemaweg 80, 3015 CN Rotterdam, the Netherlands

^e Centre for Human Drug Research, Zernikedreef 8, 2333CL Leiden, the Netherlands

^f Netherlands Institute for Neuroscience, Royal Academy of Arts and Sciences, Meibergdreef 47, 1105 BA Amsterdam, the Netherlands

HIGHLIGHTS

- Distinctive cutaneous innervation changes in acute versus chronic neuropathic pain.
- Specific clinical-pathological associations in purely neuropathic, not mixed pain.
- A distinct role for non-peptidergic nociceptors in BiPN and CIAP patients.

ARTICLE INFO

Keywords:

Neuropathic pain
Cutaneous innervation
Peptidergic nerve fibers
Non-peptidergic nerve fibers
McGill Pain Questionnaire

ABSTRACT

Consistent associations between the severity of neuropathic pain and cutaneous innervation have not been described. We collected demographic and clinical data, McGill Pain Questionnaires (MPQ) and skin biopsies processed for PGP9.5 and CGRP immunohistochemistry from patients with bortezomib-induced peripheral neuropathy (BiPN; $n = 22$), painful diabetic neuropathy (PDN; $n = 16$), chronic idiopathic axonal polyneuropathy (CIAP; $n = 16$) and 17 age-matched healthy volunteers. Duration of neuropathic symptoms was significantly shorter in patients with BiPN in comparison with PDN and CIAP patients. BiPN was characterized by a significant increase in epidermal axonal swellings and upper dermis nerve fiber densities (UDNFD) and a decrease in subepidermal nerve fiber densities (SENFDF) of PGP9.5-positive fibers and of PGP9.5 containing structures that did not show CGRP labeling, presumably non-peptidergic fibers. In PDN and CIAP patients, intraepidermal nerve fiber densities (IENFD) and SENFD of PGP9.5-positive and of non-peptidergic fibers were decreased in comparison with healthy volunteers. Significant unadjusted associations between IENFD and SENFD of CGRP-positive, i.e. peptidergic, fibers and the MPQ sensory-discriminative, as well as between UDNFD of PGP9.5-positive fibers and the MPQ evaluative/affective component of neuropathic pain, were found in BiPN and CIAP patients. No significant associations were found in PDN patients. Cutaneous innervation changes in BiPN confirm characteristic features of early, whereas those in CIAP and PDN are in line with late forms of neuropathic pathology. Our results allude to a distinct role for non-peptidergic nociceptors in BiPN and CIAP patients. The lack of significant associations in PDN may be caused by mixed ischemic and purely neuropathic pain pathology.

1. Introduction

Neuropathic pain is a frequent complication of peripheral neuropathies, such as bortezomib-induced peripheral neuropathy (BiPN;

occurring in 25–80% of patients (Jongen et al., 2015; Rampen et al., 2013), painful diabetic neuropathy (PDN; in 16–40% of patients (Javed et al., 2015; Jongen et al., 2018) and chronic idiopathic axonal polyneuropathy (CIAP; in 42% of patients (Erdmann et al., 2010;

* Corresponding author at: Dept. of Neuro-oncology, Erasmus MC Cancer Institute, Room Nt-540, Dr. Molewaterplein 40, 3015GD Rotterdam, the Netherlands.
E-mail address: j.jongen@erasmusmc.nl (J.L.M. Jongen).

¹ These authors contributed equally to the manuscript

Hanewinkel et al., 2016; Warendorf et al., 2017).

PDN and CIAP are both examples of chronic peripheral neuropathies, while an acute or subacute neuropathy often presents with BiPN, in contrast to other chemotherapy-induced peripheral neuropathies (Jongen et al., 2015; Rampen et al., 2013; Richardson et al., 2012). Specific alterations have been observed in (sub)acute as opposed to chronic neuropathies. Axonal swellings, containing accumulations of mitochondria, usually occur early in the course of distal symmetric peripheral neuropathies, while (epi)dermal nerve fiber loss and degenerative Schwann cell changes occur as late consequences (Bennett et al., 2014; Ebenezzer et al., 2007; Lauria et al., 2003).

Apart from a recent study that showed a correlation between GAP43 intraepidermal nerve fiber density and the severity of burning pain in PDN patients (Galosi et al., 2018), no consistent associations between cutaneous innervation and the severity of neuropathic pain have been described (Kalliomaki et al., 2011; Lindenlaub and Sommer, 2002; Schley et al., 2012; Vlckova-Moravcova et al., 2008). This may be explained by mixed pathology, for example in painful diabetic neuropathy, or by the fact that selective degeneration of a subset of nociceptors, which may not be detected using the pan axonal marker PGP9.5, may drive hyperalgesia and eventually neuropathic pain. We have recently published two papers, one in a rat-model of nerve-injury induced pain (Bechakra et al., 2017) and one in patients with BiPN (Bechakra et al., 2018), suggesting that selective degeneration of non-peptidergic nerve fibers may directly or indirectly (via parasympathetic sprouting) contribute to the affective and evaluative component of neuropathic pain. Non-peptidergic nerve fibers have already previously been considered to be more characteristically involved in neuropathic pain (Willcockson and Valtchanoff, 2008), since sensory qualities that are distinct in neuropathic pain, like paresthesias, burning pain and tactile allodynia, are typically experienced in skin, which is predominantly innervated by non-peptidergic nerve fibers (Guedon et al., 2016). Peptidergic nerve fiber loss on the other hand may contribute to the sensory-discriminative component of neuropathic pain in BiPN patients (Bechakra et al., 2018). The McGill Pain questionnaire (Melzack and Torgerson, 1971; Melzack, 2005), a reliable and extensively validated test in many languages, was specifically designed to discern the sensory-discriminative, affective and evaluative components of neuropathic pain. More recently it has been suggested that separate anatomical pathways exist for these respective components (Braz et al., 2005; Craig, 2003).

The aim of the current study is to further explore the hypothesis that selective degeneration of nociceptors in neuropathic pain syndromes can be associated with distinctive pain qualities, by comparing the pathology and pain perception among BiPN, PDN and CIAP patients.

2. Results

In Table 1, demographic data and clinical characteristics of 17 healthy volunteers, 22 patients with BiPN (previously described in (Bechakra et al., 2018)), 16 patients with PDN (previously described in (Emanuel et al., 2017)) and 16 patients with CIAP are listed. Median ages and percentages of males were not significantly different among the four groups ($p = 0.453$ and $p = 0.139$, using Kruskal-Wallis and chi-square test respectively). Median and range of duration of neuropathy symptoms until the moment of study entry was significantly shorter in BiPN patients (2 [0.5–23] months) than in PDN (36 [8–60] months) and in CIAP patients (60 [12–132]), while the difference between PDN and CIAP patients was not significantly different ($p < 0.001$, $p < 0.001$ and $p = 0.831$ respectively; Kruskal-Wallis test with post-hoc comparisons using Dunn's test). Additionally, 16 out of 22 BiPN patients were considered to have (sub)acute neuropathies (i.e. duration of neuropathy symptoms ≤ 3 months), whilst none of the PDN or CIAP patients had. Median time between a diagnosis of diabetes and inclusion in the study of PDN patients was 144 [12–408] months.

In Fig. 1 representative PGP9.5 and CGRP immunohistochemical

Table 1

Demographic data and clinical characteristics of healthy volunteers (HV), bortezomib-induced peripheral neuropathy (BiPN), painful diabetic neuropathy (PDN) and chronic idiopathic axonal polyneuropathy (CIAP) patients. PRI = Pain Rating Index, NWC = Number of Words Count. Adjuvant medication included anti-epileptics and anti-depressants. *** $p < 0.001$, Kruskal-Wallis test with post-hoc comparisons using Dunn's test.

Patients	Median (range) or n (%)			
	HV	BiPN	PDN	CIAP
	n = 17	n = 22	n = 16	n = 16
Demographic data				
Age (years)	63 (27–75)	63 (39–79)	66 (30–76)	67 (49–76)
Sex (male)	10 (59%)	19 (86%)	9 (56%)	12 (75%)
Duration of neuropathy (months)		2 (0.5–23)	144 (12–408)	60 (12–132)
Neuropathic pain				
McGill pain questionnaire				
PRI-Sensory (0–36 points)		11 (4–22)	10 (4–22)	11 (3–23)
PRI-Affective (0–15 points)		3 (0–8)	2 (0–11)	4 (0–11)
PRI-Evaluative (0–12 points)		6 (2–9)	5 (3–7)	5 (3–9)
PRI-Total (0–63 points)		20 (10–37)	18 (9–38)	22 (6–54)
NWC-Sensory (0–12 words)		7 (3–12)	7 (4–12)	7 (3–12)
NWC-Affective (0–5 words)		7 (0–5)	2 (0–4)	3 (0–5)
NWC-Evaluative (0–8 words)		3 (2–3)	3 (3–3)	3 (3–3)
NWC-Total (0–25 points)		13 (7–20)	13 (7–20)	14 (6–20)
Pain Medication				
Adjuvant medication		12 (55%)	6 (38%)	4 (25%)

staining patterns in the epidermis, subepidermal layer and upper dermis are presented, from healthy volunteers (Fig. 1A, B, I and J), patients with BiPN (Fig. 1C, D, K and L), patients with PDN (Fig. 1E, F, M and N) as well as patients with CIAP (Fig. 1G, H, O and P). Characteristic staining patterns of these fibers, including orientation, morphology and branching of PGP9.5 and CGRP positive fibers as well as immunohistochemical control experiments have been previously described by our group (Bechakra et al., 2018). The density of PGP9.5 positive intraepidermal nerve fibers appeared lower in PDN and in CIAP patients, while the density of upper dermal fibers appeared higher in BiPN patients. Looking in close detail (see insets in Fig. 1), PGP9.5-positive intraepidermal nerve fibers also showed axonal swellings, both small ($2\text{--}3 \times$ the nerve diameter) and large ($> 5 \times$ the nerve diameter). These nerve swellings appeared more abundant in BiPN patients compared to the other groups.

In Fig. 2 the results of IENFD (Fig. 2A), SENFD (Fig. 2C) and UDNFD (Fig. 2D) of PGP9.5, CGRP and (PGP9.5-CGRP) are summarized. Swelling ratios of intraepidermal PGP9.5 fibers are presented in Fig. 2B. In CIAP patients, IENFD of PGP9.5 and of (PGP9.5-CGRP), i.e. presumed non-peptidergic fibers, were significantly decreased in comparison with healthy volunteers ($p = 0.007$ and $p = 0.015$ respectively), while in PDN patients IENFD of (PGP9.5-CGRP) was significantly decreased ($p = 0.030$) and the decrease in IENFD of PGP9.5 almost reached statistical significance ($p = 0.054$; Kruskal-Wallis test with post-hoc comparisons using Dunn's test). Similarly, significant decreases were found for SENFD of PGP9.5 ($p = 0.006$) and of (PGP9.5-CGRP) ($p = 0.006$) in CIAP and in PDN patients ($p = 0.006$ and $p = 0.006$ respectively). BiPN patients were characterized by a significant increase in epidermal axonal swellings ($p < 0.001$) and upper

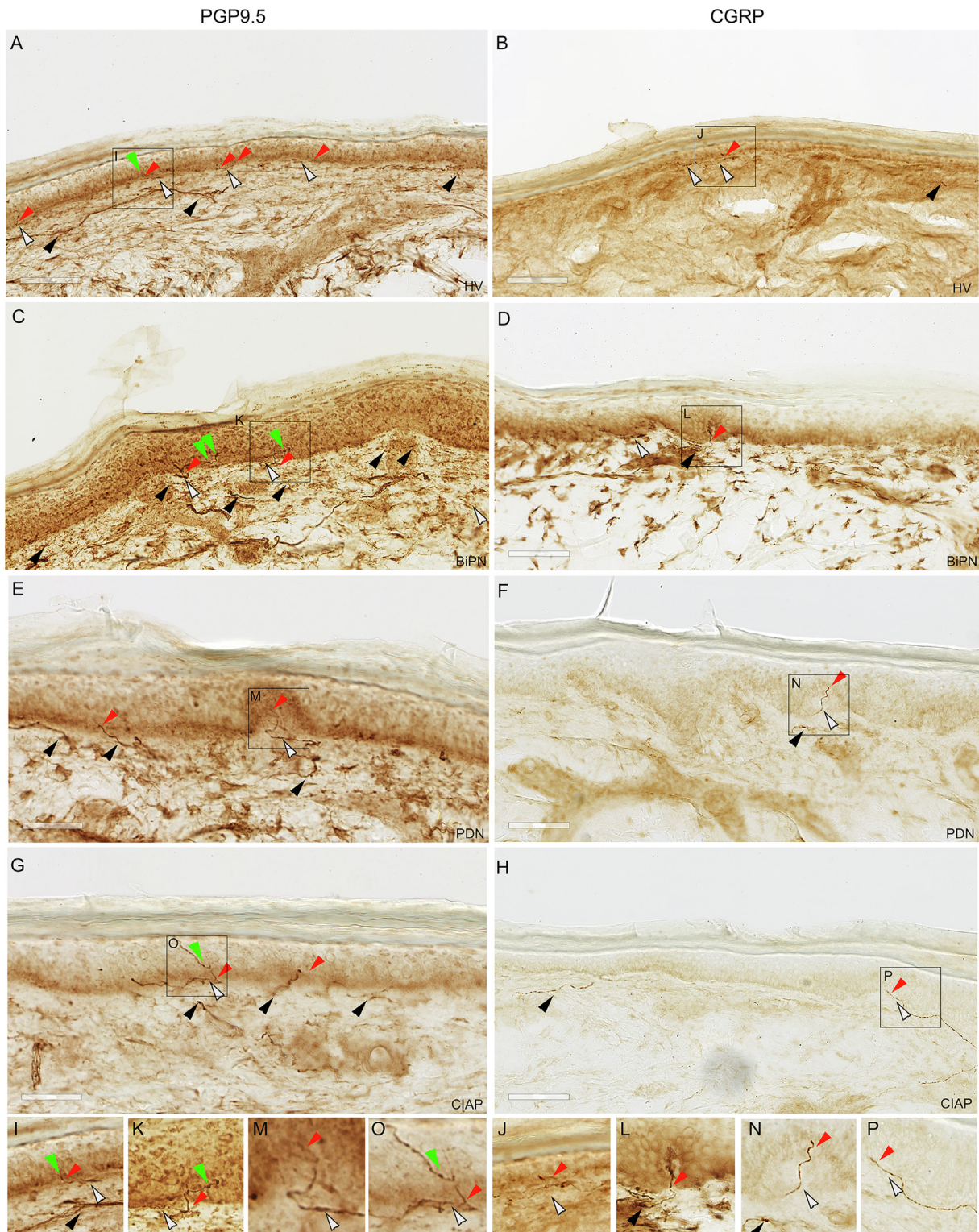


Fig. 1. Immunohistochemical staining patterns of PGP9.5 (A, C, E, G) and CGRP (B, D, F, H), in healthy volunteers (A, B), in BiPN patients (C, D), in PDN patients (E, F) and in CIAP patients (E, F). I, K, M, O, J, L, N and P represent high-power insets, which enable to visualize the length of the intra-epidermal fibers, branching pattern and intra-epidermal axonal swellings. Red arrowheads represent intra-epidermal nerve fibers, green arrowheads axonal swellings, white arrowheads sub-epidermal nerve fibers and black arrowheads upper-dermal nerve fibers. The white bars measure 50 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dermis nerve fiber densities (UDNFD) of PGP9.5 ($p = 0.015$) and of (PGP9.5-CGRP) ($p = 0.015$), whilst a significant decrease was found in SENFD of PGP9.5 ($p < 0.001$) and of presumed non-peptidergic fibers ($p < 0.001$; Kruskal-Wallis test with post-hoc comparisons using

Dunn's test), as previously described (Bechakra et al., 2018). IENFD, SENFD and UDNFD of CGRP fibers in BiPN, PDN and CIAP patients were not significantly different from healthy volunteers.

In Table 2 correlations between the nerve fiber densities for each

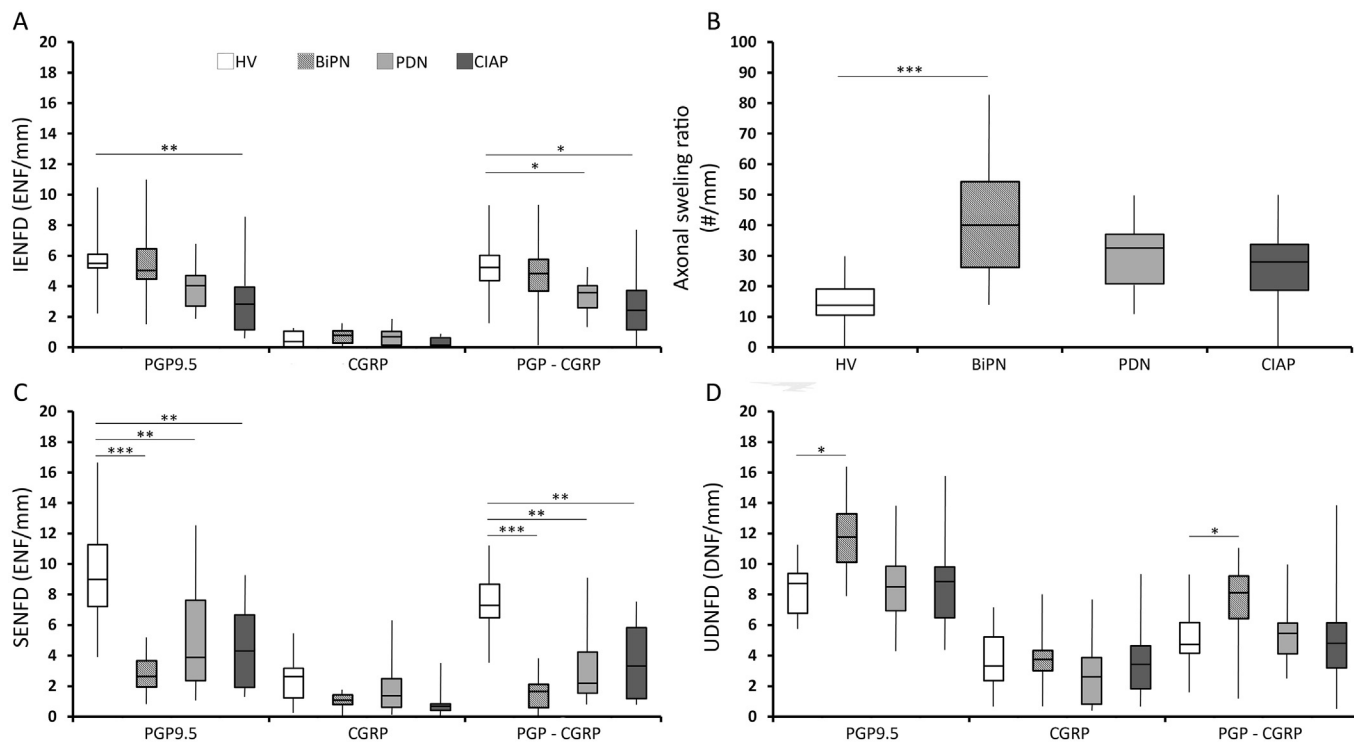


Fig. 2. Skin innervation measurements in healthy volunteers ($n = 17$), BiPN patients ($n = 22$), PDN patients ($n = 16$) and CIAP patients ($n = 16$). Box-plots showing the median, interquartile range and total range of the number of intra-epidermal (IENFD; A), sub-epidermal (SENF; C), upper-dermal (UDNFD; D) nerve fiber densities and the axonal swelling ratios (B), using PGP9.5, CGRP and (PGP9.5-CGRP) as markers to measure the total number of fibers and peptidergic and non-peptidergic subclasses, $*p \leq 0.05$, $**p \leq 0.01$ $***p \leq 0.001$; Kruskal-Wallis test with post-hoc comparisons using Dunn's test.

immunohistochemical marker and the sensory-discriminative, affective and evaluative PRIs and NWCs with corresponding p -values and Spearman's rank correlation coefficients are presented. In BiPN patients, the correlations between UDNFD of PGP9.5 and the evaluative MPQ PRI and NWC were $\rho = 0.447$; $p = 0.037$ and $\rho = 0.427$; $p = 0.047$ respectively (not significant following Bonferroni correction with an adjusted significance level of 0.017) and there was an unadjusted significant negative correlation between SENFD of CGRP and the sensory-discriminative MPQ NWC with $\rho = -0.423$; $p = 0.050$, as previously described (Bechakra et al., 2018). In CIAP patients, the correlation between UDNFD of PGP9.5 and the affective MPQ PRI was $\rho = 0.542$ ($p = 0.030$; not significant following Bonferroni correction with an adjusted significance of 0.017), and there were unadjusted significant correlations between IENFD of CGRP and the sensory-discriminative MPQ PRI was $\rho = 0.574$ ($p = 0.020$) and NWC ($\rho = 0.517$; $p = 0.040$). The evaluative MPQ NWC was 3 in all CIAP patients and therefore no correlation coefficients could be calculated. Finally, correlation coefficients in PDN patients were not statistically significant.

3. Discussion

This study describes changes in (epi)dermal innervation and associations with pain qualities in cohorts of BiPN, PDN and CIAP patients with neuropathic pain. Cutaneous innervation changes in BiPN patients, which mostly presented as (sub)acute neuropathies, were characterized by a decrease in SENFD, as opposed to an increase in UDNFD of PGP9.5 and of presumed non-peptidergic nerve fibers as well as by an increase in epidermal axonal swellings. PDN and CIAP on the other hand, which invariably presented as chronic neuropathies, were characterized by a decrease in IENFD and SENFD of PGP9.5 and of presumed non-peptidergic fibers. Significant unadjusted associations between IENFD and SENFD of peptidergic fibers and the sensory-discriminative component, and between UDNFD of PGP9.5 and the evaluative/affective component of neuropathic pain, were found in

BiPN and CIAP patients. No significant associations were found in PDN patients.

Concerning the immunohistochemical quantification of cutaneous innervation, one should be aware that PGP9.5 may be expressed not only in nerve terminals, but also in Langerhans cells in denervated skin (Hsieh et al., 1996) and under certain conditions in fibroblasts (Olerud et al., 1998). Thus, it could have been of additional value to incorporate additional specific markers of cutaneous innervation, especially to label the non-peptidergic nerve fiber population and possibly also functional markers of excitability such as sodium channel subtypes (Kalliomaki et al., 2011; Schley et al., 2012). However, as we are aware thus far there have been no reports of reproducible immunohistochemical staining patterns allowing for quantification of non-peptidergic fibers and of sodium channels in humans. Furthermore, we do believe that based upon morphology and predefined quantification criteria nerve fiber (terminals) can be selectively separated from non-nerve cells.

Since no consistent associations between cutaneous innervation and neuropathic pain intensities have been described so far, mainly in patient cohorts containing different types of nerve-injury induced pain (Kalliomaki et al., 2011; Schley et al., 2012), we analyzed three cohorts representing distinctive types of painful peripheral neuropathy separately, i.e. (sub)acute (BiPN), chronic (CIAP) and chronic mixed pathology (PDN) neuropathic pain. The specific epidermal innervation changes that we found in BiPN (increased axonal swellings) as opposed to the changes in PDN and CIAP (decreased IENFD of PGP9.5 and of (PGP9.5-CGRP) fibers) are consistent with previously described differential neuropathic changes in (sub)acute versus chronic neuropathies (Bennett et al., 2014; Ebenezer et al., 2007; Lauria et al., 2003). This match with prior results enhances the notion that any further results should be valid. The decrease in IENFD of PGP9.5 in PDN patients as compared to healthy volunteers just failed to reach statistical significance ($p = 0.054$), but this may be due to the sample size. The increased density of upper dermis PGP9.5 fibers that we observed in BiPN patients has been described in an animal model of subacute

Table 2

Correlations between immunohistochemical markers and McGill Pain Questionnaire (MPQ) Pain Rating Index (PRI) and Number of Words Count (NWC), in bortezomib-induced peripheral neuropathy (BiPN) ($n = 22$), painful diabetic neuropathy (PDN) ($n = 16$) and chronic idiopathic axonal polyneuropathy (CIAP) patients ($n = 16$). Numerals in the upper left part of the cells refer to p values, numerals in the lower right part of all the cells refer to Spearman's correlation coefficients. Correlation coefficients with an uncorrected $p \leq 0.05$ are printed in bold with an asterisk. IENFD = IntraEpidermal Nerve Fiber Density, SENFD = SubEpidermal Nerve Fiber Density, UDNFD = Upper Dermis Nerve Fiber Density.

Patients	MPQ Pain Glossary	IENFD			SENFD			UDNFD		
		PGP9.5	CGRP	PGP-CGRP	PGP9.5	CGRP	PGP-CGRP	PGP9.5	CGRP	PGP-CGRP
BiPN	PRI-Sensory	0,637	0,079	0,694	0,648	0,139	0,240	0,553	0,050	0,596
		0,106	-0,382	0,089	-0,103	-0,326	0,261	-0,134	-0,422	0,120
		0,056	0,825	0,075	0,466	0,903	0,206	0,576	0,831	0,533
	PRI-Affective	0,413	-0,050	0,388	0,164	-0,028	0,280	0,126	-0,048	0,140
		0,271	0,276	0,388	0,192	0,618	0,943	0,037	0,427	0,231
		0,245	0,243	0,193	0,289	0,113	0,016	0,447*	0,179	0,266
	PRI-Evaluative	0,998	0,064	0,939	0,405	0,050	0,312	0,189	0,071	0,883
		-0,001	-0,401	0,017	-0,187	-0,423*	0,226	-0,291	-0,392	-0,033
		0,457	0,421	0,453	0,760	0,216	0,926	0,260	0,407	0,674
	NWC-Sensory	0,167	-0,181	0,169	0,069	-0,022	0,275	-0,007	-0,186	0,095
		0,621	0,088	0,805	0,935	0,279	0,279	0,047	0,870	0,107
		0,111	0,373	0,056	-0,019	0,241	-0,241	0,427*	-0,037	0,353
PDN	PRI-Sensory	0,127	0,307	0,311	0,598	0,635	0,806	0,780	0,356	0,427
		0,398	0,273	0,270	0,143	0,129	0,067	0,076	0,247	-0,214
		0,329	0,510	0,371	0,240	0,119	0,350	0,877	0,351	0,217
	PRI-Affective	0,261	0,178	0,240	0,612	0,240	0,250	-0,042	0,250	-0,327
		0,851	0,349	0,609	0,400	0,320	0,859	0,467	0,329	0,400
		0,051	0,251	-0,139	0,226	0,266	0,048	-0,196	0,261	-0,226
	NWC-Sensory	0,142	0,299	0,331	0,531	0,643	0,610	0,743	0,359	0,411
		0,384	0,277	0,260	0,169	0,126	0,138	0,089	0,246	-0,321
		0,288	0,450	0,342	0,158	0,078	0,252	0,864	0,352	0,204
	NWC-Affective	0,283	0,203	0,254	0,370	0,453	0,304	-0,047	0,249	-0,336
		0,918	0,754	0,918	0,346	0,757	0,166	0,346	0,105	0,105
		-0,028	-0,085	-0,028	0,252	0,084	0,364	-0,252		-0,420
CIAP	PRI-Sensory	0,970	0,020	0,892	0,542	0,446	0,710	0,595	0,404	0,718
		0,010	0,574*	-0,037	-0,165	0,205	-0,101	0,144	0,224	0,098
		0,465	0,249	0,386	0,468	0,052	0,497	0,030	0,278	0,327
	PRI-Affective	0,197	0,306	0,233	0,196	0,494	0,183	0,542*	0,289	0,262
		0,830	0,784	0,736	0,687	0,793	0,996	0,139	0,741	0,195
		0,058	0,075	0,091	-0,109	0,071	0,001	0,387	-0,090	0,342
	NWC-Sensory	0,375	0,040	0,432	0,904	0,247	0,926	0,637	0,456	0,669
		0,238	0,517*	0,211	0,033	0,307	0,025	0,128	0,201	0,116
		0,649	0,224	0,601	0,881	0,215	0,643	0,191	0,613	0,302
	NWC-Affective	0,123	0,322	0,141		0,328	0,126	0,344	0,137	0,275
					0,041					
		-	-	-	-	-	-	-	-	-

neuropathy, see also below (Grelík et al., 2005; Ramien et al., 2004; Taylor and Ribeiro-da-Silva, 2011; Taylor et al., 2012).

Mixed pathology is common in PDN, especially in patients with long standing diabetes as was the case in our cohort of PDN patients. In long standing diabetes, pain in the feet may be explained by other factors than nociceptor degeneration, like myelinated nerve fiber degeneration (Vickova-Moravcova et al., 2008), autonomic nerve dysfunction (Vickova-Moravcova et al., 2008), ischemia and inflammation (Schmidt and Holmes, 2018). This may explain why no significant associations of cutaneous innervation parameters (mainly representing nociceptors) and neuropathic pain descriptors were found in our cohort of PDN patients, which is in line with previous findings (Shun et al., 2004).

In a previous publication (Bechakra et al., 2017) we have demonstrated changes in cutaneous innervation following nerve injury in rats, of peptidergic nerve fibers that were labeled by CGRP-ir and of non-peptidergic nerve fibers that were labeled by P2X₃-ir. It is generally known that these two classes of nociceptors target specific neurons in the spinal dorsal horn (Jongen et al., 2005), are modality-specific (Zhang et al., 2013) and supposedly may each convey specific information about pain along labeled lines to the spinal cord and brain (Bechakra et al., 2017, 2018; Braz et al., 2005; Craig, 2003). Peptidergic nerve fibers can be labeled by CGRP-ir, substance P-ir, but also contain the TrkA receptor for Nerve Growth Factor and the TRPV1

receptor for capsaicin. Non-peptidergic nerve fibers can be labeled with P2X₃-ir, Isolectin B4, Mrgprd-ir and contain the RET receptor for glial cell line-derived neurotrophic factor (GDNF) (Jongen et al., 2007). While these two classes of neurons are for the greatest part mutually exclusive, there is some overlap depending on the markers used to label them (Bechakra et al., 2017; Price and Flores, 2007). Thus, peptidergic and non-peptidergic nerve fibers may be considered complementary, because they serve different functions and are more or less mutually exclusive. Since we and others were unable to immunohistochemically label cutaneous non-peptidergic nerve fibers for quantification in the human skin, we decided to use IENFD, SENFD and UDNFD of the difference between PGP9.5 and CGRP labeled fibers as surrogate markers for the number of nonpeptidergic fibers in order to get a complete picture of cutaneous innervation in our cohorts of BiPN, PDN and CIAP patients. Our findings in BiPN and CIAP patients on associations of peptidergic nerve fiber innervation with the sensory-discriminative component of neuropathic pain on the one hand and that of upper-dermis nerve fiber sprouting resulting from non-peptidergic nerve fiber degeneration (see below) with the affective/evaluative component on the other hand are both in line with the labeled lines hypothesis mentioned above (see also Grelík et al., 2005; Ramien et al., 2004; Taylor and Ribeiro-da-Silva, 2011; Taylor et al., 2012).

As far as the upper dermis is concerned, a rapid decrease followed

by a slow return (at 10 weeks after ligation) to normal values of UDNFD of NF-200-labeled myelinated nerves has been described in rats with partial nerve ligation (Duraku et al., 2013). However, although myelinated nerves are affected in BiPN as well as in CIAP patients given EMG abnormalities (Bechakra et al., 2018; Hanewinkel et al., 2016), (neuropathic) pain is a cardinal symptom alluding to significant small nerve-fiber involvement. It has been shown repeatedly in experimental animals (Grelík et al., 2005; Ramien et al., 2004; Taylor et al., 2012) that peptidergic nerve-fiber degeneration causes sympathetic nerve fibers to sprout in the upper dermis, while non-peptidergic nerve fiber degeneration, which was demonstrated in our BiPN patients in the sub-epidermal layer and in CIAP patients in the epidermis as well as in the sub-epidermal layer, induces parasympathetic fibers to sprout. Thus, the increased UDNFD of PGP9.5 in BiPN patients likely represents parasympathetic sprouting as a consequence of non-peptidergic nerve fiber degeneration. This upregulation is temporary (Grelík et al., 2005) and may therefore explain why an absolute increase in UDNFD of PGP9.5 was not observed in chronic neuropathies like PDN and CIAP. Although the correlations between UDNFD of PGP9.5 and the evaluative/affective pain components in BiPN and CIAP patients just failed to reach statistical significance after correction for multiple testing ($p \leq 0.05$, but $p > 0.017$), we still conclude that our results allude to a distinct role for non-peptidergic nociceptors in BiPN and CIAP patients, in light of consistent findings across the BiPN and CIAP groups, our previous data in rats, clinical observations and the literature regarding labeled lines.

The inverse association of subepidermal peptidergic nerve fibers with the sensory-discriminative component of neuropathic pain in BiPN patients may imply that in (sub)acute neuropathies this pain component is driven by increased degeneration or impaired regeneration of CGRP fibers in the subepidermal layer, while the positive associations in the epidermis of CIAP patients may imply that in chronic neuropathies this component is driven by decreased degeneration or increased regeneration of CGRP fibers in the epidermis. However, the significant associations between IENFD of CGRP and the sensory-discriminative pain component in CIAP patients should be interpreted with caution due to the scarcity of intraepidermal CGRP fibers.

Finally, although the evaluative component is classified as a separate entity within the MPQ, we analyzed it here in conjunction with the affective pain component, because many of its descriptors have an emotional-affective connotation (Melzack and Torgerson, 1971; van der Kloot et al., 1995).

4. Conclusion

Changes in cutaneous innervation in BiPN represent early, whereas those in PDN and CIAP represent late neuropathic pathology. Furthermore, our results allude to a distinct role for non-peptidergic nociceptors in BiPN and CIAP patients. The significant associations between IENFD of CGRP and the sensory-discriminative pain component in CIAP patients should be interpreted with caution due to the scarcity of intraepidermal CGRP fibers. The lack of significant associations in PDN may be caused by mixed ischemic and purely neuropathic pain pathology. Although the MPQ may be impractical for use in routine clinical practice, we suggest to rate pain intensity as well as pain unpleasantness separately in neuropathic pain patients using a numerical rating scale, to consider both sensory-discriminative and affective components.

5. Methods and materials

5.1. Patients, clinical data and skin biopsies

The study was approved by the medical ethical committees of Leiden University Medical Centre, Leiden (NL46921.058.13) and of Erasmus MC, Rotterdam (NL24284.078.08) in the Netherlands and was

performed in accordance with the Declaration of Helsinki of 2013 (World Medical, 2013). All participants had given written informed consent. Parts of the study results have been published previously (Bechakra et al., 2018; Emanuel et al., 2017), which is indicated in the results section.

A total of 71 subjects were included: 17 healthy volunteers (HV), 22 patients with BiPN, 16 patients with PDN, and 16 patients with CIAP. The diagnosis of BiPN was established on clinical grounds by a neurologist as a new-onset peripheral neuropathy or a (sub)acute clear deterioration of previously minimally symptomatic peripheral neuropathy following start of bortezomib, fulfilling the ACTTION-APS Pain Taxonomy (AAPT) diagnostic criteria for a diagnosis of CiPN (Paice et al., 2017). Patients were treated with either intravenous bortezomib monotherapy or intravenous bortezomib in combination with non-neurotoxic chemo/immunotherapy, that is, hydroxydaunorubicin ($n = 8$) (Sonneveld et al., 2012), lenalidomide ($n = 2$) (Broijl et al., 2016), or rituximab ($n = 2$). The diagnosis of PDN was established by a neurologist based on the medical history, signs and symptoms upon clinical examination in patients with diabetes mellitus type 2 (Emanuel et al., 2017). The diagnosis of CIAP was established by a neurologist who interpreted the combination of clinical manifestation, nerve conduction parameters as well as relevant laboratory tests as an axonal peripheral neuropathy in the absence of identifiable underlying etiology (Hanewinkel et al., 2016).

The study consisted of the collection of demographic data and clinical data, including pain intensity on a numerical rating scale (NRS) and McGill Pain Questionnaires (Dutch ($n = 70$) or English ($n = 1$) language versions) (Melzack and Torgerson, 1971; van der Kloot et al., 1995). For the McGill Pain Questionnaire, the sum of the sensory-discriminative, affective and evaluative Pain Rating Indices (PRI) and the overall sum of PRIs were calculated. In addition, the Number of Words Chosen (NWC) for these items were used.

5.2. Obtaining, processing and analysis of skin biopsies

Three-mm skin biopsies were taken 10 cm proximal to the lateral ankle under local anesthesia and stored, according to international guidelines (Lauria et al., 2010). From these biopsies, 50 μ m sections were cut on a freezing microtome and processed for free-floating immunohistochemistry using rabbit anti-PGP9.5 (Catalog# ADI-905-520; Enzo Life Sciences, Farmingdale, NY), representing all cutaneous nerve fibers, and guinea-pig anti-CGRP (Catalog # 16013; Progen Biotechnik, Heidelberg, DE), representing peptidergic nerve fibers, as previously described (Bechakra et al., 2018). After the sections had been mounted to glass slides they were scanned, digitized using a Hamamatsu Nano-Zoomer 2.0-HT slide scanner (Hamamatsu Photonics, Hamamatsu City, JP), analyzed and quantified using Leica Aperio ImageScope freeware, as previously described (Bechakra et al., 2018) (see also Supplemental Methods file).

Cutaneous innervation was expressed as intra-epidermal nerve fiber density (IENFD), subepidermal nerve fiber density (SENFD), upper dermis nerve fiber density (UDNFD) of PGP9.5- and CGRP-fibers and as the axonal swelling ratio of PGP9.5-fibers. Definitions of IENFD, SENFD, UDNFD and axonal swelling ratio were previously published (Lauria et al., 2010; Schley et al., 2012) and extensively described and validated in our recent publications (Bechakra et al., 2017, 2018). As a surrogate for non-peptidergic innervation, we also calculated IENFD, SENFD and UDNFD of the difference between the number of PGP9.5 fibers (i.e. the total number of nerve fibers) and the number of CGRP fibers (i.e. peptidergic nerve fibers) and called this (PGP9.5-CGRP). As we are aware, thus far there are no reports of reproducible immunohistochemical staining patterns allowing for quantification of these fibers in humans (Bechakra et al., 2018). Besides, the population of peptidergic and non-peptidergic nerve fibers are mostly complementary (Bechakra et al., 2017).

5.3. Statistical analysis

Given that most variables had a non-normal distribution, as assessed with Kolmogorov-Smirnov test, data were summarized using medians and ranges. The Kruskal-Wallis test with post-hoc comparisons using Dunn's test and the chi-square test were used to compare age, duration of neuropathy symptoms and sex of healthy volunteers, BiPN, PDN and CIAP patients. The Kruskal-Wallis test with post-hoc comparisons using Dunn's test were used to compare IENFD, SENFD and UDNFD of PGP9.5, CGRP and (PGP9.5-CGRP) and to compare axonal swelling ratios of healthy volunteers with those of BiPN, PDN and CIAP patients. The Dunn's tests were performed for each comparison between healthy volunteers and a patient group, with Bonferroni-adjusted *p*-values to correct for multiple testing due to these three comparisons. Spearman's rank correlation coefficients between immunohistochemical markers and neuropathic pain descriptors were determined. Correction for multiple testing was not applied to the correlation analysis, apart from Bonferroni correction with an adjusted significance level of 0.017 for comparisons of UDNFD of PGP9.5 with sensory-discriminative, affective and evaluative components of the MPQ, since this part of the analysis was hypothesis driven. All remaining statistical tests were two-sided with a significance level of 0.05. The statistical analysis was performed using IBM SPSS Statistics v.24.0.0.0 software (IBM Corp., Armonk, NY).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was funded by a NeuroSipe/STW grant 2009 (#10730) and an Erasmus MC Grant 2011. CidZ was funded by ERC and ERC-PoC of the EU as well as by ZonMW and NWO-ALW. The funding sources had no role in study design, data collection, data analysis, manuscript preparation and/or publication decisions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.brainres.2019.146621>.

References

Bechakra, M., Schuttenhelm, B.N., Pederzani, T., van Doorn, P.A., de Zeeuw, C.I., Jongen, J.L.M., 2017. The reduction of intraepidermal P2X3 nerve fiber density correlates with behavioral hyperalgesia in a rat model of nerve injury-induced pain. *J. Comp. Neurol.* 525, 3757–3768.

Bechakra, M., M.D. Nieuwenhoff J. van Rosmalen G.J. Groeneveld, Scheltens-de Boer, M., Sonneveld, P., van Doorn, P.A., de Zeeuw, C.I., Jongen, J.L., 2018. Clinical, electrophysiological, and cutaneous innervation changes in patients with bortezomib-induced peripheral neuropathy reveal insight into mechanisms of neuropathic pain. *Mol. Pain* 14 1744806918797042.

Bennett, G.J., Doyle, T., Salvemini, D., 2014. Mitotoxicity in distal symmetrical sensory peripheral neuropathies. *Nat. Rev. Neurol.* 10, 326–336.

Braz, J.M., Nassar, M.A., Wood, J.N., Basbaum, A.I., 2005. Parallel “pain” pathways arise from subpopulations of primary afferent nociceptor. *Neuron* 47, 787–793.

Broijl, A., Kersten, M.J., Alemayehu, W.G., Levin, M.D., de Weerd, O., Vellenga, E., Meijer, E., Wittebol, S., Tanis, B.C., Cornelisse, P.B., Stevens-Kroef, M., Bos, G.M., Wijermans, P.W., Lokhorst, H., Sonneveld, P., 2016. Phase I/II trial of weekly bortezomib with lenalidomide and dexamethasone in first relapse or primary refractory myeloma. *Haematologica* 101, e149–e152.

Craig, A.D., 2003. Pain mechanisms: labeled lines versus convergence in central processing. *Annu. Rev. Neurosci.* 26, 1–30.

Duraku, L.S., Hossaini, M., Schuttenhelm, B.N., Holstege, J.C., Baas, M., Ruigrok, T.J., Walbeehm, E.T., 2013. Re-innervation patterns by peptidergic Substance-P, non-peptidergic P2X3, and myelinated NF-200 nerve fibers in epidermis and dermis of rats with neuropathic pain. *Exp. Neurol.* 241, 13–24.

Ebenezer, G.J., McArthur, J.C., Thomas, D., Murinson, B., Hauer, P., Polydefkis, M.,

Griffin, J.W., 2007. Denervation of skin in neuropathies: the sequence of axonal and Schwann cell changes in skin biopsies. *Brain* 130, 2703–2714.

Emanuel, A.L., Nieuwenhoff, M.D., Klaassen, E.S., Verma, A., Kramer, M.H., Strijers, R., Vrancken, A.F., Eringa, E., Groeneveld, G.J., Serne, E.H., 2017. Relationships between type 2 diabetes, neuropathy, and microvascular dysfunction: evidence from patients with cryptogenic axonal polyneuropathy. *Diabetes Care* 40, 583–590.

Erdmann, P.G., van Genderen, F.R., Teunissen, L.L., Notermans, N.C., Lindeman, E., van Wijck, A.J., van Meeteren, N.L., 2010. Pain in patients with chronic idiopathic axonal polyneuropathy. *Eur. Neurol.* 64, 58–64.

Galosi, E., La Cesa, S., Di Stefano, G., Karlsson, P., Fasolino, A., Leone, C., Biasiotta, A., Cruccu, G., Truini, A., 2018. A pain in the skin: regenerating nerve sprouts are distinctly associated with ongoing burning pain in patients with diabetes. *Eur. J. Pain* 22, 1727–1734.

Grelik, C., Bennett, G.J., Ribeiro-da-Silva, A., 2005. Autonomic fibre sprouting and changes in nociceptive sensory innervation in the rat lower lip skin following chronic constriction injury. *Eur. J. Neurosci.* 21, 2475–2487.

Guedon, J.M., Longo, G., Majuta, L.A., Thomson, M.L., Fealk, M.N., Mantyh, P.W., 2016. Dissociation between the relief of skeletal pain behaviors and skin hypersensitivity in a model of bone cancer pain. *Pain* 157, 1239–1247.

Hanewinkel, R., Drenthen, J., van Oijen, M., Hofman, A., van Doorn, P.A., Ikram, M.A., 2016. Prevalence of polyneuropathy in the general middle-aged and elderly population. *Neurology* 87, 1892–1898.

Hsieh, S.T., Choi, S., Lin, W.M., Chang, Y.C., McArthur, J.C., Griffin, J.W., 1996. Epidermal denervation and its effects on keratinocytes and Langerhans cells. *J. Neurocytol.* 25, 513–524.

Javed, S., Petropoulos, I.N., Alam, U., Malik, R.A., 2015. Treatment of painful diabetic neuropathy. *Ther. Adv. Chronic. Dis.* 6, 15–28.

Jongen, J.L., Haasdijk, E.D., Sabel-Goedknegt, H., van der Burg, J., Vecht Ch, J., Holstege, J.C., 2005. Intrathecal injection of GDNF and BDNF induces immediate early gene expression in rat spinal dorsal horn. *Exp. Neurol.* 194, 255–266.

Jongen, J.L., Jaarsma, D., Hossaini, M., Natarajan, D., Haasdijk, E.D., Holstege, J.C., 2007. Distribution of RET immunoreactivity in the rodent spinal cord and changes after nerve injury. *J. Comp. Neurol.* 500, 1136–1153.

Jongen, J.L., Broijl, A., Sonneveld, P., 2015. Chemotherapy-induced peripheral neuropathies in hematological malignancies. *J. Neurooncol.* 121, 229–237.

Jongen, J.L.M., Verhamme, C., van Dam, P.S., Schaper, N.C., Versteegen, G., van Wijck, A.J.M., 2018. Dutch national guideline painful diabetic neuropathy. *FMS Richtlijnen Database* 1–325.

Kalliomaki, M., Kieseritzky, J.V., Schmidt, R., Hagglof, B., Karlsten, R., Sjogren, N., Albrecht, P., Gee, L., Rice, F., Wiig, M., Schmelz, M., Gordh, T., 2011. Structural and functional differences between neuropathy with and without pain? *Exp. Neurol.* 231, 199–206.

Lauria, G., Morbin, M., Lombardi, R., Borgna, M., Mazzoleni, G., Sghirlanzoni, A., Pareyson, D., 2003. Axonal swellings predict the degeneration of epidermal nerve fibers in painful neuropathies. *Neurology* 61, 631–636.

Lauria, G., Hsieh, S.T., Johansson, O., Kennedy, W.R., Leger, J.M., Mellgren, S.I., Nolano, M., Merkiys, L.S., Polydefkis, M., Smith, A.G., Sommer, C., Valls-Sole, J., 2010. European federation of neurological societies/peripheral nerve society guideline on the use of skin biopsy in the diagnosis of small fiber neuropathy. *Eur. J. Neurol.* 17 (903–12), e44–e49.

Lindenlaub, T., Sommer, C., 2002. Epidermal innervation density after partial sciatic nerve lesion and pain-related behavior in the rat. *Acta. Neuropathol.* 104, 137–143.

Melzack, R., Torgerson, W.S., 1971. On the language of pain. *Anesthesiology* 34, 50–59.

Melzack, R., 2005. The McGill pain questionnaire: from description to measurement. *Anesthesiology* 103, 199–202.

Olerud, J.E., Chiu, D.S., Usui, M.L., Gibran, N.S., Ansel, J.C., 1998. Protein gene product 9.5 is expressed by fibroblasts in human cutaneous wounds. *J. Invest. Dermatol.* 111, 565–572.

Paice, J.A., Mulvey, M., Bennett, M., Dougherty, P.M., Farrar, J.T., Mantyh, P.W., Miaskowski, C., Schmidt, B., Smith, T.J., 2017. AAPT diagnostic criteria for chronic cancer pain conditions. *J. Pain* 18, 233–246.

Price, T.J., Flores, C.M., 2007. Critical evaluation of the colocalization between calcitonin gene-related peptide, substance P, transient receptor potential vanilloid subfamily type 1 immunoreactivities, and isolectin B4 binding in primary afferent neurons of the rat and mouse. *J. Pain* 8, 263–272.

Ramien, M., Ruocco, I., Cuello, A.C., St-Louis, M., Ribeiro-Da-Silva, A., 2004. Parasympathetic nerve fibers invade the upper dermis following sensory denervation of the rat lower lip skin. *J. Comp. Neurol.* 469, 83–95.

Rampen, A.J., Jongen, J.L., van Heuvel, I., Scheltens-de Boer, M., Sonneveld, P., van den Bent, M.J., 2013. Bortezomib-induced polyneuropathy. *Neth. J. Med.* 71, 128–133.

Richardson, P.G., Delforge, M., Beksac, M., Wen, P., Jongen, J.L., Sezer, O., Terpos, E., Munshi, N., Palumbo, A., Rajkumar, S.V., Harousseau, J.L., Moreau, P., Avet-Loiseau, H., Lee, J.H., Cavo, M., Merlini, G., Voorhees, P., Chng, W.J., Mazumder, A., Usmani, S., Einsele, H., Comenzo, R., Orlovski, R., Vesole, D., Lahuerta, J.J., Nievesvizky, R., Siegel, D., Mateos, M.V., Dimopoulos, M., Lonial, S., Jagannath, S., Blade, J., Miguel, J.S., Morgan, G., Anderson, K.C., Durie, B.G., Sonneveld, P., 2012. Management of treatment-emergent peripheral neuropathy in multiple myeloma. *Leukemia* 26, 595–608.

Schley, M., Bayram, A., Rukwied, R., Dusch, M., Konrad, C., Benrath, J., Geber, C., Birklein, F., Hagglof, B., Sjogren, N., Gee, L., Albrecht, P.J., Rice, F.L., Schmelz, M., 2012. Skin innervation at different depths correlates with small fiber function but not with pain in neuropathic pain patients. *Eur. J. Pain* 16, 1414–1425.

Schmidt, B.M., Holmes, C.M., 2018. Updates on diabetic foot and charcot osteopathic arthropathy. *Curr. Diab. Rep.* 18, 74.

Shun, C.T., Chang, Y.C., Wu, H.P., Hsieh, S.C., Lin, W.M., Lin, Y.H., Tai, T.Y., Hsieh, S.T., 2004. Skin denervation in type 2 diabetes: correlations with diabetic duration and

- functional impairments. *Brain* 127, 1593–1605.
- Sonneveld, P., Schmidt-Wolf, I.G., van der Holt, B., El Jarari, L., Bertsch, U., Salwender, H., Zweegman, S., Vellenga, E., Broyl, A., Blau, I.W., Weisel, K.C., Wittebol, S., Bos, G.M., Stevens-Kroef, M., Scheid, C., Pfreundschuh, M., Hose, D., Jauch, A., van der Velde, H., Raymakers, R., Schaafsma, M.R., Kersten, M.J., van Marwijk-Kooy, M., Duehrsen, U., Lindemann, W., Wijermans, P.W., Lokhorst, H.M., Goldschmidt, H.M., 2012. Bortezomib induction and maintenance treatment in patients with newly diagnosed multiple myeloma: results of the randomized phase III HOVON-65/ GMMG-HD4 trial. *J. Clin. Oncol.* 30, 2946–2955.
- Taylor, A.M., Ribeiro-da-Silva, A., 2011. GDNF levels in the lower lip skin in a rat model of trigeminal neuropathic pain: implications for nonpeptidergic fiber reinnervation and parasympathetic sprouting. *Pain* 152, 1502–1510.
- Taylor, A.M., Osikowicz, M., Ribeiro-da-Silva, A., 2012. Consequences of the ablation of nonpeptidergic afferents in an animal model of trigeminal neuropathic pain. *Pain* 153, 1311–1319.
- van der Kloot, W.A., Oostendorp, R.A., van der Meij, J., van den Heuvel, J., 1995. The Dutch version of the McGill pain questionnaire: a reliable pain questionnaire. *Ned. Tijdschr. Geneesk.* 139, 669–673.
- Vlckova-Moravcova, E., Bednarik, J., Belobradkova, J., Sommer, C., 2008. Small-fibre involvement in diabetic patients with neuropathic foot pain. *Diabet. Med.* 25, 692–699.
- Warendorf, J., Vrancken, A.F., van Schaik, I.N., Hughes, R.A., Notermans, N.C., 2017. Drug therapy for chronic idiopathic axonal polyneuropathy. *Cochrane Database Syst. Rev.* 6, CD003456.
- Willcockson, H., Valtchanoff, J., 2008. AMPA and NMDA glutamate receptors are found in both peptidergic and non-peptidergic primary afferent neurons in the rat. *Cell Tissue Res.* 334, 17–23.
- World Medical, A., 2013. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. *JAMA* 310, 2191–2194.
- Zhang, J., Cavanaugh, D.J., Nemenov, M.I., Basbaum, A.I., 2013. The modality-specific contribution of peptidergic and non-peptidergic nociceptors is manifest at the level of dorsal horn nociceptive neurons. *J. Physiol.* 591, 1097–1110.