Experimental Dermatology **Letter to the Editor**

Title: There is no functional small-fiber neuropathy in prurigo nodularis despite neuroanatomical alterations.

Authors:
Pereira MP[1], Pogatzki-Zahn E[2], Snels C[3], Vu T[1], Üçeyler N[4], Loser K[1], Sommer C[4], Evers A[3], van Laarhoven, AIM [3], Agelopoulos K[1], Ständer S[1]

Institutions.
[1]Department of Dermatology and Center for Chronic Pruritus, University Hospital Münster, Germany
[2]Department of Anaesthesiology, Intensive Care and Pain Medicine, University Hospital Münster, Germany.
[3]Institute of Psychology, Health, Medical and Neuropsychology Unit, University of Leiden, the Netherlands
[4]Department of Neurology, University of Würzburg, Germany

Corresponding author:
Sonja Ständer M.D.
Department of Dermatology and Center for Chronic Pruritus, University Hospital Münster, Von-Esmarch-Straße 58, D-48149, Münster, Germany
Phone: +49-251-83 58950; E-mail: sonja.staender@uni-muenster.de

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Abstract
Prurigo nodularis (PN) is a pruritic condition with altered epidermal neuroanatomy as demonstrated previously. Here we elucidated neuroimmunological mechanisms by combining functional, morphological, and gene expression experiments in twelve subjects with PN and eight healthy controls. Subjects with PN showed a reduced intraepidermal nerve fiber density (IENFD) in lesional skin. Quantitative sensory testing indicated maintenance of somatosensory function compared to controls. None of the tested molecular markers including the neuron-distracting SEMA3A and neuron-attracting NGF were altered in lesional vs. non-lesional skin in PN subjects. Accordingly, we speculate that scratching may contribute to reduced IENFD rather than an authentic endogenous neuropathy.

Background

Chronic pruritus (CP) may arise from many different diseases, and its peripheral and central neuroimmunological mechanisms still remain unclear[1]. Previous data suggest an important role of both histamine-dependent mechano-insensitive and histamine-independent mechano- and heat-sensitive epidermal C-fibers in the pathophysiology of CP[2-42-4]. However, molecular markers for CP are still ambiguous and only scarce data exist on the role of the anatomy and function of the peripheral nervous system for the development and maintenance of pruritus. In subjects with prurigo nodularis (PN), a condition characterized by the development of hyperkeratotic itching nodules as a result of CP and prolonged scratching, we previously demonstrated a reduced intraepidermal nerve fiber density (IENFD) in lesions, which reconstitutes after healing[5]. A reduced IENFD may hint towards a small-fiber neuropathy (SFN), which can be induced by diseases such as diabetes and results in pathologic functionality of cutaneous peripheral nerves[6].

Questions addressed

We speculate that the reduced IENFD in PN is not related to a SFN causing pain in neuropathic diseases. Rather, external injury such as long standing scratching as observed in subjects with PN may contribute to nerve destruction without showing functional signs of a SFN (e.g. small-fiber function related abnormalities). As inflammation may contribute to peripheral neuronal sensitization[7], we aimed to characterize potential molecular markers for PN in lesional and non-lesional skin.

Experimental design

Participants

Twelve subjects with PN (m:f=5:7; age=50±14 years) and eight healthy controls (m:f=4:4; age=49±10 years) were included. Inclusion/exclusion criteria are detailed in supplementary table 1. There was no difference in age between groups (p=0.91). Asked for their scratching behavior, subjects with PN confirmed scratching of itchy nodules.

Histology and IENFD

Histology and the distribution of inflammatory cells were assessed by H&E staining of lesional (only subjects with PN, lower leg) biopsies. The IENFD was determined in biopsies from lesional skin by immunostaining with antibodies against protein gene product 9.5 (PGP 9.5; rabbit polyclonal, 1:200, Zytomed, Berlin, Germany) as previously described[8]. Since a normal IENFD was already shown in non-lesional skin of subjects with PN, we did not determine non-lesional IENFD[5].

Expression analysis

Gene expression was analyzed using biopsies from lesional and non-lesional skin from the lower leg in eight subjects with PN. Total RNA was isolated using the innuPrep RNA Mini-Kit (Analytik Jena, Jena, Germany) and reverse transcription was performed with oligo-dT primers and the RevertAid® First Strand cDNA Synthesis Kit (both by Thermo Scientific, Kalamazoo, USA) according to the instructions of the manufacturers. Gene expression was analyzed by means of quantitative real-time PCR using the ABI 7300-Real Time-PCR System (Applied Biosystems, Darmstadt, Germany). Gene expression of known or presumed molecular markers for PN and/or inflammation like interleukin (IL)-6, IL-8, tyrosine kinase A (TRKA), nerve growth factor (NGF), semaphorin-3A (SEMA3A), and IL-31 were analyzed in lesional and non-lesional skin of subjects with PN (supplementary section, supplementary table 2).

Quantitative sensory testing (QST)

To assess possible dysfunctions of peripheral large and small myelinated and unmyelinated nerve fibers, subjects with PN and controls underwent standardized QST performed at the left lower leg in lesional skin adjacent to the itchy nodules. The prurigo nodules were avoided in the examination. Briefly, the subjective response to graded stimuli of various modalities was assessed according to a standardized procedure[9] (see supplementary section).

Participants signed an informed consent and the study was approved by the local ethics committee (2007-135-f-S) and conducted according to the declaration of Helsinki.

Statistics

SPSS 22.0 (IBM, Armonk, NY, USA) was used for group comparisons using the non-parametric Mann-Whitney test or Wilcoxon test, as appropriate. Two-tailed statistical tests with a level of significance of $p<0.05$ were used.

Results

Histology and IENFD

All lesional skin biopsies of subjects with PN showed typical signs of PN with pseudoepitheliomatous hyperplasia of the epidermis, fibrosis of the dermal collagen fibers, a weak to moderately dense perivascular and interstitial inflammatory infiltrate, and a reduced IENFD in lesional skin (range: 0.2-5.2 fibers/mm; median=1.3; normal values: >11 fibers/mm (female), >10 fibers/mm (male); data not shown).

Gene expression analyses

We found no differential gene expression of SEMA3A and IL-31 ($p=0.07$ and $p=0.09$) in lesional compared to non-lesional skin of subjects with PN (figure 1, supplementary figure 1).

QST

We did not find differences in QST parameters between subjects with PN and healthy controls ($p>0.05$, supplementary table 3). Although significance was not reached, our data suggests that mechanical pain sensitivity (MPS) may be reduced in subjects with PN compared to controls ($p=0.08$, figure 2) suggesting an Aδ-fiber malfunction.

Conclusions

In these subjects with PN, IENFD in lesional skin was reduced, while QST profiles representative for C-fiber function were normal in lesional skin. In QST, a malfunction of cutaneous sensory C and Aδ-fibers would be reflected by pathological non-noxious and noxious temperature thresholds, which we did not observe here. We demonstrated previously in PN, that healed lesions without itch and scratching showed a full reconstitution of IENFD. Accordingly, we can speculate that the disturbed epidermal neuroanatomy most likely does not result from a functional neuropathy but rather from a mechanical damage such as scratching. Future studies should correlate scratching activity with the IENFD. In agreement with this hypothesis, SEMA3A as nerve distracting factor and NGF as nerve attracting factor[10] were not up or down-regulated. These factors have been reported to be regulated in inflammatory diseases such as atopic dermatitis and alter the neuroanatomical structure[5]. Future studies should analyze the expression at protein level. Impaired descending inhibition is an alternative explanation for the absence of QST abnormalities is spite of neuroanatomical changes and should be addressed in future studies.

In the experimental testing, we found indications for decreased MPS to pinprick stimulation, which may represent a reduced function of Aδ-fibers. However, MPS was not reduced and other parameters mediated by Aδ-fiber function were unchanged as well (e.g. cold detection and pain thresholds). Thus, the role of Aδ-fibers for pruritus needs further investigations.

A limitation of this pilot study is that it might have been underpowered to show possible differences. Larger studies are needed to confirm our findings. Thus, these shall be seen as hypothesis-generating.

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The authors declare no conflicts of interest.

Author contributions

MPP performed the statistical analysis, wrote the manuscript and approved the final version of the manuscript; EPZ designed the study, performed and supervised QST/DNIC experiments, wrote the manuscript and approved the final version of the manuscript; CS collected the data and approved the final version of the manuscript; TV collected the data and approved the final version of the manuscript; NU: performed PCR and approved the final version of the manuscript; KL: performed PCR and approved the final version of the manuscript; CS: performed PCR and approved the final version of the manuscript; AE designed the study and approved the final version of the manuscript; AIMvL designed the study and approved the final version of the manuscript; KA performed the statistical analysis, wrote the manuscript and approved the final version of the manuscript; SS designed the study, performed the histological investigations and determination of the intraepidermal nerve fiber density, wrote the manuscript and approved the final version of the manuscript

Supporting Information

Additional supporting data may be found in the supplementary information of this article.

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Figures

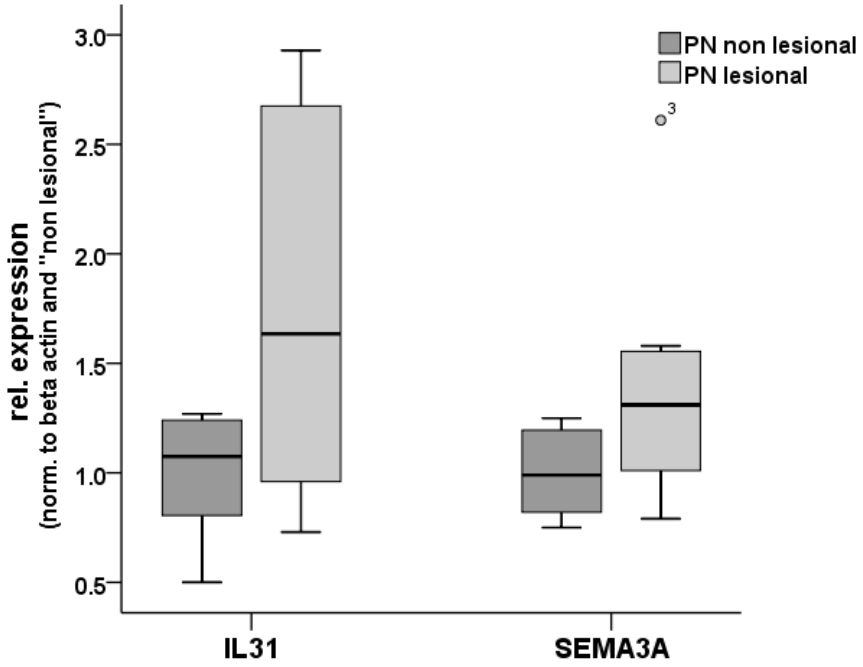


Figure 1. Gene expression: IL-31 and SEMA3A. Relative gene expression was measured by means of quantitative real time PCR. Our data suggests that the expression of IL-31 (p=0.09) and SEMA3A (p=0.07) may be higher in lesional compared to non-lesional skin in subjects with prurigo nodularis (PN), however significance was not reached. Statistics was done with SPSS 22.0. P-values were calculated using Related-Samples Wilcoxon Signed Rank Test. Dots indicate outlier data points lying between 1.5 and 3x of the individual interquartile range.

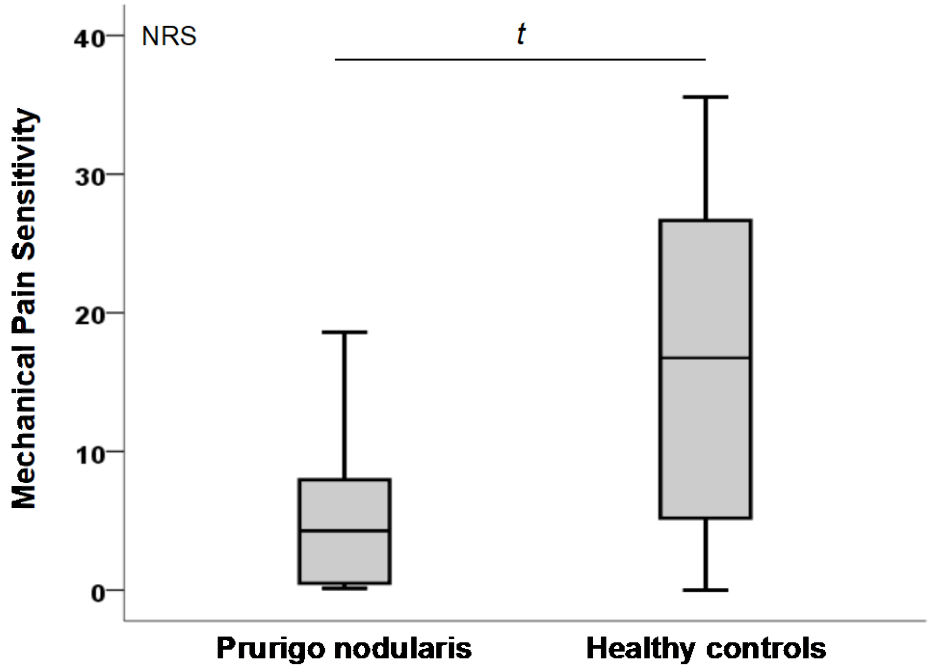


Figure 2. Quantitative sensory testing: mechanical pain sensitivity. Boxplots showing distribution of mechanical pain sensitivity to pinprick stimulation (8-512 mN) in subjects with PN and matched healthy controls. Although significance was not reached, our data suggests that mechanical pain sensitivity (MPS) may be reduced in subjects with PN compared to controls (p=0.08), NRS: numerical rating scale (0-100). PN: prurigo nodularis. The p-value was calculated using the Mann-Whitney U test.

Supplementary methods

Inclusion and exclusion criteria

Inclusion and exclusion criteria are presented in supplementary table 1.

Supplementary table 1. Inclusion and exclusion criteria.

Inclusion Criteria	Exclusion Criteria
• 18≤age≤70	• Neurological, psychosomatic or severe psychiatric disorders
• Generalized prurigo nodularis (subjects with PN)	• Diabetes mellitus
	• Previous myocardial infarction
	• Localized itch syndromes
	• Pain syndrome
	• Polyneuropathy
	• Diseases that prevented study participation
	• Pregnant and lactating women

Gene expression analysis

Gene expression was analyzed by quantitative real time PCR using the Kappa SYBR Fast Universal Kit (Peqlab, Erlangen, Germany). PCR reactions were carried out in duplicates using the gene specific primer pairs listed below. Amplification and detection was done using an ABI 7300-Real Time-PCR System (Applied Biosystems, Darmstadt, Germany). Threshold cycles were analyzed with the 7500 Fast System Software and relative gene expression was calculated by the 2^-delta delta CT method with beta actin as housekeeping gene.

Supplementary table 2. Genes analyzed for expression by means of qPCR

Gene ID	encoded gene / protein	primer sequence
ACTB	actin beta	AAGGAGAAGCTGTGCTACGTC AACCGCTCATTGCCAATGGTG
CASP1	caspase 1	CAAACTTTTTCAGAGGGGATCG GCATACTGTTTCAGCATGGCAC
CXCL2	C-X-C motif chemokine ligand 2	CGCCCCTGGCCACTGAACTGC CTTAACCATGGGCGATGCGG
GAP43	growth associated protein 43	CCATGCTGTGCTGTATGAGAA TGTTATGTGTCCACGGAAGC
NFKBIA	NFKB inhibitor alpha	GTCAAGGAGCTGCAGGAGAT CCATGGTCAGTGCCTTTTCT
NFKB1	nuclear factor kappa B subunit 1	ATGTATGTGAAGGCCCATCC ATAACCTTTGCTGGTCCCAC
NGF	nerve growth factor	ACACTGAGGTGCATAGCGTAA CAGTAATGTTGCGGGTCTGC
NGFR	nerve growth factor receptor	CTGTTGCTGCTTCTGGGG GCTCACACACGGTCTGGTTGGC

IL1A	interleukin 1 alpha	TGTGACTGCCCAAGATGAAG AAGTTTGGATGGGCAACTGA
IL1B	interleukin 1 beta	AAATACCTGTGGCCTTGGGC TTTGGGATCTACACTCTCCAGCT
IL6	interleukin 6	ACAGCCACTCACCTCTTCAG AGTGATGATTTTCACCAGGCA
IL8	interleukin 8	GCCTTCCTGATTTCTGCAGC CAGTTTTCCTTGGGGTCCAGAC
IL31	interleukin 31	CATCCGGGCATATCTCAAGAC GATGAAGCGTTTACATTCATGGG
RASA1	RAS p21 protein activator 1	GGACACCCTCTGACCCTCG GCTTGCTAAACTTCCTCGCTC
RELA	RELA proto-oncogene, NF-kB subunit	CACCGACAAGTGGCCATTGTG TTCTCCTCAATCCGGTGACG
RELB	RELB proto-oncogene, NF-kB subunit	ATCCTTGGGGAGAGCAGC GAGGCCAGTCCTTCCACAC
SEMA3A	semaphorin 3A	AATGGGAAGAACAATGTGCC ACAGCCTACTCCGTTCCTCA
TAC1	tachykinin precursor 1 / substance P	TTAATGGGCAAACGGGATGC TGCCCATTGACACAAATGAAGC
TACR1	tachykinin receptor 1	AATGACAGGTTCCGTCTGGG GAGCAGTTGGAGGTCAGGTC
TNF	tumor necrosis factor	CTCCAGGCGGTGCTTGTT CATGGGCTACAGGCTTGTCA

Quantitative sensory testing (QST)

To assess a possible dysfunction of sensory fibers and signs of central sensitization we performed a test battery of somatosensory testing by QST in fixed order according to the protocol developed by the German Research Network for Neuropathic Pain[9]. A non-lesioned area on the left lower leg was chosen for the assessments.

Thermal thresholds were determined using a 3x3 cm contact thermode of Peltier elements (TSA II NeuroSensory Analyzer, Medoc Ltd., Israel) from a baseline temperature of 32°C with a ramp rate of 1.0°C/s (cut-off: 0ºC and 50ºC). To assess detection (CDT: cold detection threshold; WDT: warmth detection threshold) and pain thresholds (CPT: cold pain threshold; HPT: heat pain threshold), participants were instructed to press a button as soon as the sensation induced by the contact thermode changed from a neutral temperature to a cold or warmth sensation or to a painful sensation, respectively. The thermal sensory limen (TSL), i.e. the difference limen for alternating warm and cold stimuli, was assessed by asking the subjects to press a button as soon as the sensation induced by the thermode changed from a neutral temperature to a cold or warmth sensation. Additionally, the number of paradoxical heat sensations (PHS), i.e. reports of hot or burning sensations to innocuous cold stimuli was recorded. To assess mechanical detection (MDT) and mechanical pain thresholds (MPT), subjects were asked to report when they perceived the stimulation by a set of von Frey filaments (Optihair2-Set, Marstock Nervtest, Germany [forces between 0.25 and 512 mN; diameter: 0.5 mm]) or whether stimulation with a series of weighted pins PinPrick, MRC Systems, Heidelberg, Germany [forces between

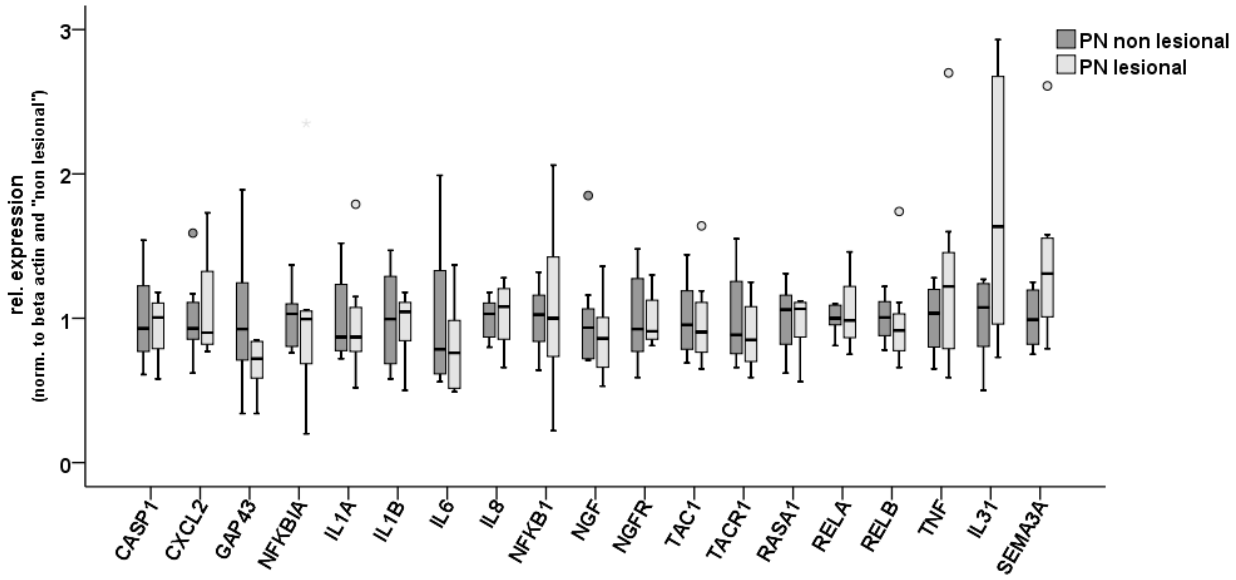
8 and 512 mN; diameter: 0.25 mm]) were painful. Mechanical pain sensitivity (MPS) was assessed as the stimulus-response function for pinprick stimulation using the pinprick set described above, while dynamic mechanical allodynia (DMA) was measured as the pain to stimulation with a cotton wisp (3 mN), a cotton wool tip (100 mN) and a brush (200–400 mN). The difference in pain intensity evoked by a single pinprick stimulation (256 mN) and by the application of a series of 10 pinprick stimuli (256 mN, 1 Hz) determined the wind-up ratio. Finally vibration detection thresholds (VDT) were assessed using a tuning fork (AESCULAP, B. Braun Company, Germany; 64 Hz, 8/8 scale) and pressure pain thresholds (PPT) with a pressure algometer (FDN200, Wagner Instruments, USA; 1-cm^2 probe).

Supplementary results

Supplementary table 3. Comparison of quantitative sensory testing parameters in subjects with PN and healthy controls. There was a trend in decreased MPS in subjects with PN compared to controls (p=0.08). No significances or trends were observed for the remaining parameters (p>0.1). Data are shown as median [interquartile range]. PN: prurigo nodularis, HC: healthy controls. CDT: cold detection threshold; CPT: cold pain threshold; DMA: dynamic mechanic allodynia; HPT: heat pain threshold; MDT: mechanical detection threshold; MPS: mechanical pain sensitivity; MPT: mechanical pain threshold; PHS: paradoxical heat sensation; PPT: pressure pain threshold; TSL: thermal sensory limen; VDT: vibration detection threshold; WDT: warmth detection threshold; WUR: wind-up ratio.

QST parameter	PN	HC	p-value
CDT (ºC)	4.0 [2.1;5.7]	3.2 [2.3;-4.3]	0.68
WDT (ºC)	7.8 [4.2;12.0]	5.9 [4.0;9.1]	0.68
TSL (ºC)	9.7 [7.5;12.3]	7.8 [7.0;11.0]	0.38
PHS (/3)	0.5 [0;1]	0 [0;0]	0.16
CPT (ºC)	0.8 [0;3.5]	0 [0;0]	0.18
HPT (ºC)	48.7 [47.4;49.5]	46.7 [45.6;48.9]	0.38
MDT (mN)	15.5 [6.3;20.1]	5.1 [2.6;10.8]	0.12
MPT (mN)	9.5 [6.6;13.7]	8.1 [5.7;10.2]	0.38
MPS (NRS)	4.3 [0.5;7.4]	16.8 [7.1;23.2]	0.08
DMA (NRS)	0 [0;0]	0 [0;5]	0.43
WUR	1.8 [1.6;2.8]	1.6 [1.3;1.9]	0.30
VDT (/8)	6.3 [5.9;6.8]	6.2 [5.6;8]	0.91
PPT (kPa)	271 [203;328]	319 [301;425]	0.12

Supplementary figures



Supplementary figure 1. Relative gene expression was measured by means of quantitative real time PCR in subjects with prurigo nodularis (PN) (n=8). Only two genes showed a trend towards higher expression in lesional compared to non lesional skin, IL31 (p=0.09) and SEMA3A (p=0.07), respectively. Statistic was done with SPSS 22. P-values were calculated using Related-Samples Wilcoxon Signed Rank Test.