Article published online: 2024-06-06

Immunohistochemical and histopathological analyses of cutaneous innervation to improve the diagnostic efficacy in hansen disease skin lesion

Análises imuno-histoquímica e histopatológica da inervação cutânea para aumento da eficácia diagnóstica nas lesões de pele da hanseníase

Eduardo Alves Freire da Costa[1] Thaís Porto Amadeu[2] Ximena Illarramendi[1,3] Bernardo Pascarelli[1] José Augusto da Costa Nery[1] Anna Maria Sales[1] Sérgio Luiz Gomes Antunes[1]

[1] Instituto Oswaldo Cruz, Laboratório de Hanseníase, Rio de Janeiro RJ, Brazil.
[2] Universidade do Estado de Rio de Janeiro, Laboratório de Imunopatologia, Rio de Janeiro RJ, Brazil.
[3] Fundação Oswaldo Cruz, Centro de Desenvolvimento Tecnológico em Saúde, Rio de Janeiro RJ, Brazil.

Address for correspondence Sérgio Luiz Gomes Antunes (email: santunes3@gmail.com)

Arq. Neuro-Psiquiatr. 2024;82(5):s00441787136.

Abstract

Background The diagnosis of Hansen disease (HD) can be difficult when acid-fast bacilli are not detected in the patient's skin sample.

Objective To demonstrate that detailed morphological analysis of nonspecific inflammatory and/or noninflammatory alterations in dermal nerves as well as skin adnexa in leprosy-suspected biopsy samples could improve the efficacy of histopathological diagnosis.

Methods Patients with one to five skin lesions were enrolled in the study and classified into three groups by skin histopathology findings: Hansen disease (HD, $n = 13$), other diseases (OD, $n = 11$), and inconclusive cases (INC, $n = 11$). We quantified dermal nerve damage via the nerve lesion index (NLI) and PGP9.5-immunoreactive axon quantitative index in dermal nerves (AQI). We also measured inflammatory involvement of adnexa in cutaneous samples as indirect evidence of HD.

Results We observed a higher median endoneurial inflammatory infiltrate NLI (HD = 0.5; INC = 0; OD = 0; $p < 0.001$) and more frequent inflammatory involvement of skin adnexa in samples of the HD group compared with those of the INC and OD groups (HD = 7; INC = 1; OD = 0). However, samples from the INC and OD groups also showed inflammatory and noninflammatory damage of dermal nerves, with 2 or more kinds of alterations in nerves in the same sample (respectively: INC = in 1 and 2 samples;

Keywords
- Leprosy
- Diagnosis, Differential
- Immunohistochemistry
- Nerve Fibers

received
October 31, 2023
received in its final form
March 16, 2024
accepted
April 27, 2024

DOI https://doi.org/10.1055/s-0044-1787136.
ISSN 0004-282X.

© 2024. The Author(s).
This is an open access article published by Thieme under the terms of the Creative Commons Attribution 4.0 International License, permitting copying and reproduction so long as the original work is given appropriate credit (https://creativecommons.org/licenses/by/4.0/).

Thieme Revinter Publicações Ltda., Rua do Matoso 170, Rio de Janeiro, RJ, CEP 20270-135, Brazil

OD = in 3 and 5 respectively). The quantification of PGP9.5-immunoreactive axons in dermal nerves revealed no difference between the groups.
Conclusion A detailed morphological analysis of cutaneous nerves in lesions with a suspicion of HD enabled us to select patients with nonspecific inflammatory or non-inflammatory lesions in the dermal nerves in the INC and OD groups, so they may be clinically monitored aiming at a possible future diagnosis of the disease. These INC and OD patients cannot have the HD diagnosis definitely excluded, and HD may coexist with another disease as a comorbidity.

Resumo

Antecedentes A hanseníase pode ter o seu diagnóstico histopatológico dificultado quando bacilos álcool-ácido resistentes não são encontrados nas amostras de pele dos pacientes.
Objetivo Demonstrar que uma análise morfológica detalhada de alterações histopatológicas dos nervos dérmicos pode aumentar a eficácia diagnóstica.
Métodos Foram selecionadas amostras de pele de pacientes com uma a cinco lesões suspeitas de hanseníase. Os casos selecionados foram classificados conforme achados histopatológicos: hanseníase (HD, $n = 13$), casos inconclusivos (INC, $n = 11$), e outras doenças (OD, $n = 11$). Quantificamos as lesões dos nervos cutâneos por meio do índice de lesão de nervos (*nerve lesion index*, NLI, em inglês) e do índice quantitativo de axônios (axon quantitative index, AQI, em inglês) imunorreativos a PGP9.5 nos nervos cutâneos. Também medimos o envolvimento inflamatório dos anexos em amostras de pele como evidência indireta de hanseníase.
Resultados Foram observadas no grupo HD medianas mais altas do NLI com relação a infiltrados inflamatórios endoneurais (HD = 0,5; INC = 0; OD = 0; $p < 0,001$) e mais alta frequência de acometimento inflamatório de anexos cutâneos (HD = 7; INC = 1; OD = 0). Entretanto, as amostras dos grupos INC e OD também mostraram comprometimento inflamatório e não inflamatório dos nervos cutâneos, com 2 ou mais tipos de alterações de nervos na mesma amostra (respectivamente: INC = 1 e 2; OD = 3 e 5). Não houve diferença significativa na quantidade de axônios endoneurais imunorreativos a PGP9.5 entre os grupos.
Conclusão A análise morfológica detalhada dos nervos cutâneos em lesões suspeitas de hanseníase permitiu selecionar pacientes com lesões inespecíficas inflamatórias ou não inflamatórias nos nervos dérmicos nos grupos INC e OD, para que sejam monitorados clinicamente visando um possível diagnóstico futuro da doença. Esses pacientes INC e OD não podem ter o diagnóstico de HD definitivamente excluído, e a hanseníase pode coexistir com outra doença como uma comorbidade.

Palavras-chave
- Hanseníase
- Diagnóstico Diferencial
- Imuno-histoquímica
- Fibras Nervosas

INTRODUCTION

Peripheral neuropathy is the main problem in Hansen disease (HD).[1] It predominantly affects cutaneous autonomic and sensory nerve fibers, causing impairment of neural function in the skin. Inflammatory processes that affect dermal nerves and are induced by the causative agent *Mycobacterium leprae* are a characteristic of HD, which subsequently enables the histopathological diagnosis by skin biopsy. Peripheral nerve trunks can also be compromised, with destruction of motor fibers accompanied by motor deficit, disability, and deformities.[1,2] The disease may evolve to severe forms due to immunological reactive episodes that worsen nerve damage and cause ocular and testicular lesions. Osteopathic resorption expressed in the extremities may accompany the course of the disease.[3]

M. leprae is an intracellular acid-fast bacillus (AFB) harbored predominantly in macrophages and other cells of the connective tissue (fibroblasts and endothelial cells). *M. leprae* can be found in peripheral nerves, particularly in Schwann cells, which are in charge of producing myelin sheath for axons of peripheral nerves.[4] *M. leprae* is known to alter the regulation of genes involved in myelin production and affect the Schwann cell-axon interaction.[5,6]

The presence of *M. leprae* in the skin and nerve environment elicits a local cutaneous inflammatory process. Clinically, HD manifests as patches, plaques, papules, or nodules with single or multiple lesions spread throughout regions of

the body together with peripheral neuropathy of the limbs and face.

Inflammation disrupts both the dermal and epidermal skin layers and is responsible for neuropathy, because Schwann cells become compromised and are unable to protect axons and organize nerve compartments.[7,8] The inflammatory effect on free sensory and autonomic nerve endings or unmyelinated fibers that convey thermal, noxious, and vascular autonomic stimuli, as well as the autonomic function of adnexa (sweat glands and pilosebaceous complex),[4] results in impairment of sensory and autonomic function in the skin. Impairment of thermal sensation mediated by unmyelinated fibers can be one of the first signs of impaired neural function. Tactile sensation, meanwhile, is mediated by small, myelinated fibers (of up to 7 µm) that are also present in the dermal nerves and are frequently affected in HD.[9]

Inflammatory alterations affecting the dermal nerve branches as well as the skin adnexa in a cutaneous biopsy specimen taken from a hypoesthetic skin lesion are strong indications of HD.[10,11] The inflammatory infiltrate may surround the perineurial outline of dermal nerves, but it can also pass through perineurial boundaries and penetrate the endoneurial compartment,[4] where it causes axonal destruction of the endoneurial axons. *M. leprae* itself and its accompanying inflammatory process elicit demyelination, followed by Schwann cell proliferation, nerve fiber destruction, an increase in the endoneurial extracellular matrix (ECM), and replacement of nerve fibers by collagen fibers.[5,7] Inflammatory macrophage differentiation to epithelioid cells (epithelioid granuloma) in a cutaneous lesion of suspected HD is also strong evidence for its diagnosis, even in the absence of *M. leprae* detection.[12]

Other diseases such as nontuberculous mycobacteriosis, sarcoidosis, secondary and tertiary syphilis, borreliosis, leishmaniasis, lupus erythematosus, and Behcet disease can also affect cutaneous nerve branches and adnexa.[10] Therefore, the differential diagnosis of HD can be difficult and relies on the detection of *M. leprae* by histological staining procedures, *M. leprae* antigens in histological sections of the skin and nerve by immunohistochemical labeling, or *M. leprae* DNA in human material by polymerase chain reaction (PCR) techniques. In the absence of these laboratory tools, however, HD diagnosis can be presumed by taking into account multiple factors, including the dermatological manifestation, the histopathological lesion of the dermal nerves, and the patient's epidemiological context.[7]

Therefore, the aim of the present study was to identify and quantify histological alterations in dermal nerves and adnexa suggestive of HD. We explored the nonspecific histopathological changes in dermal nerves to develop criteria to improve the differential diagnosis of HD in patients, thus favoring an early diagnosis of the disease.

The project was approved by the Ethics Review Board of Instituto Oswaldo Cruz/Fundação Oswaldo Cruz (IOC/Fiocruz), under registration number CAAE 242191215.2.0000.5248, which also waived the need for informed consent.

METHODS

Specimens were obtained from routine skin biopsies performed for the diagnosis of patients with suspicion of HD. All patients ($n = 35$) were screened at Ambulatório Souza Araújo (ASA/IOC/Fiocruz), a referral center for HD in Brazil. The sample was composed of 29 female and 6 male patients. Regarding skin color, 14 subjects were white, 11 were brown, and 9 were black. The mean age was of 42.3 (range: 12 to 78) years, and the patients had 1 to 5 skin lesions (►**Table 1**). They came from HD contact groups, or they were walk-ins requiring medical assistance, or patients who had been forwarded from other medical services.

Criteria for HD diagnosis and selection of patients

The bacilloscopy index (BI) was assessed in every patient. The PCR test was not available for the detection of *M. leprae* DNA in the patient samples at the time of the present study.

All of the selected patients except one had a negative BI, and the final diagnosis of HD was supported by the presence of specific histological inflammatory alterations, such as epithelioid granulomas or mononuclear inflammatory infiltration surrounding cutaneous dermal nerves. Criteria for the HD diagnosis based on the histopathology of dermal nerves, employed in the present investigation, were adapted from Antunes et al[7], who originally employed it for the diagnosis of pure neural leprosy in the absence of AFB. In addition, epidemiological evidence, such as being an HD contact, contributed to the final HD diagnosis.

Patient assessment and collection of skin lesion biopsies

We conducted general dermatological and neurological assessments of skin lesions and impairment of sensory, motor, proprioceptive, and autonomic neural function.

Table 1 Number and types of lesions

Types	Patients (n)	HD (single lesion)	HD (2–5 lesions)
Hypoesthetic area	0	0	0
Patches	7	2	5
Plaque	6	5	1
		INC (single lesion)	INC (2–5 lesions)
Hypoesthetic area	1	1	0
Patches	9	4	5
Plaque	1	1	0
		OD (single lesion)	OD (2–5 lesions)
Hypoesthetic area	2	2	0
Patches	3	2	1
Plaque	6	3	3

Abbreviations: HD, Hansen disease group; INC, inconclusive group; OD, other diseases group.

We performed the quantitative sensory testing (QST) using the Medoc TSA-II NeuroSensory Analyzer (Medoc - Advanced Medical Systems, Ramat Yishay, Israel).[13] In brief, it consists of the comparative evaluation of thermal and pain sensation thresholds on the suspected cutaneous lesions and contralateral healthy skin region on the same dermatome in response to different temperatures using the aforementioned thermal sensory analyzer. The equipment container is filled with water and can be heated or cooled. The 5-mm^2 thermode attached to the skin can be cooled or warmed to evaluate thermal sensation, or set to 0°C or 50°C to elicit pain.

We also measured tactile sensation on the lesions as well as on the most commonly HD-affected innervated cutaneous regions by monofilament esthesiometry,[14] and assessed thermal and pain sensations on the same regions by the classic methods, using test tubes containing heated and cooled water and with a metal pin respectively.

The samples were sorted into three groups based on the neurological and clinical evaluations and laboratory test results (BI and histopathology of the cutaneous biopsy): Hansen disease (HD), with 13 patients; inconclusive (INC), with 11 patients; and other diseases (OD), with 11 patients.

Neurological and histopathological alterations in the study groups

HD group

The HD group was composed of patients presenting very strong histopathological evidence of HD. Only one patient exhibited AFB in the biopsy sample, albeit scarcely. Therefore, the inclusion of patients in this group was primarily based on histopathology together with the accompanying clinical characteristics. In the histopathological examination, the presence of inflammatory infiltrate clearly affecting dermal nerves was the main sign that defined this diagnosis. In addition, the presence of epithelioid granulomas increased the probability of HD in the AFB-negative patients. The number and types of lesions in the HD group are shown in ►**Table 1**. The histopathological alterations in biopsy specimens are shown in ►**Table 2**. We classified the patients in the HD group according to the Ridley & Jopling classification as follows: 11 patients as borderline tuberculoid (BT), 1 as indeterminate HD, and 1 as reversal reaction.

Eight and four patients in the HD group showed alterations in tactile and thermal sensations respectively, as evaluated with esthesiometric monofilaments and test tubes filled with heated and cooled water (►**Table 3**).

The results of the QST regarding the lesions in the HD group were as follows: thermal hypoesthesia to cold and heat and pain hypoesthesia to cold in one patient; thermal and pain anesthesia to cold and heat in one patient; and thermal anesthesia to heat and pain anesthesia to cold in one patient.

Furthermore, the assessment of the peripheral nerves revealed tactile hypoesthesia involving the sural and plantar nerves in three patients and involving one or more nerves (median, sural, tibial, and fibular) in five patients. Tactile, thermal, and pain hypoesthesia on the territories innervated by the median, ulnar, and radial nerves, as well as the sural, fibular, saphenous, calcaneal, and plantar nerves (right and left) occurred in one patient. We detected decreased strength in the anterior tibial muscle in one patient.

Table 2 Histopathological nerve alterations

Type of histopathological alteration	HD	INC	OD
Endoneurial infiltrate	9	0	3
Perineurial infiltrate	11	1	5
Perineurial enlargement	6	2	0
Sweat gland infiltrate	4	1	0
Pilosebaceous infiltrate	1	0	0
Epithelioid granuloma	12	0	2*
Mononuclear infiltrate	1	1	5
Increased ECM	1	0	4

Abbreviations: ECM, extracellular matrix; HD, Hansen disease group; INC, inconclusive group; OD, other diseases group.

INC group

The patients in the INC group did not present strong histopathological evidence of HD: only nonspecific perivascular inflammation and inflammatory cells surrounding the perineurium of dermal nerves and periadnexal infiltrate were observed in one patient sample. Nine patients exhibited hypochromic patches, one presented a hypoesthesic area, and one had a plaque (►**Table 1**).

Of the patients in the INC group, eight showed alterations in tactile sensation (monofilament esthesiometry) and eight, in thermal sensation (classic test tube method) on the skin lesion (►**Table 3**).

The assessment of the peripheral nerves revealed one patient complaining of paresthesia in the limbs where the lesion was located. No degree of physical disability was found in this group.

On the QST exam, thermal hypoesthesia to heat stimulus was observed in four patients, and to cold stimulus, in two patients. Pain hypoesthesia to cold was observed in two patients, and to heat, in another two patients.

OD group

Four patients in the OD group exhibited patches, six presented plaques, and one had a hypoesthesic area. The evaluation of the peripheral nerve function showed: tactile and pain hypoesthesia on the sural, fibular, saphenous, and calcaneal nerve territories in one patient; burning pain on the feet in one patient; hypoesthesia on the dorsal region of the thorax in one patient; and slightly decreased strength on the anterior tibial muscle in one patient.

Table 3 Neurological evaluation

A- Frequency of hypoesthesia on lesions as assessed with esthesiometric monofilaments (tactile) and heated or cooled water in test tubes (thermal)				
	Tactile		Thermal	
HD	9		4	
INC	8		8	
OD	11		8	
B- Frequency of quantitative sensory test alterations in the lesions				
	Thermal		Pain	
	Cold	Heat	Cold	Heat
HD	2	3	3	1
INC	2	4	2	2
OD	3	6	3	3
C- Frequency of neurological alterations				
HD	- Three patients: tactile hypoesthesia involving the sural and plantar nerves; - Five patients: paresthesia involving one or more nerves (median, sural, tibial, and fibular); - One patient: tactile, thermal, and pain hypoesthesia on the regions innervated by the median, ulnar, and radial nerves, as well as the sural, fibular, saphenous, calcaneal, and plantar nerves (right and left); - One patient: decreased strength in the anterior tibial muscle.			
INC	- One patient: limb paresthesia.			
OD	- One patient: tactile and pain hypoesthesia on the sural, fibular, saphenous, and calcaneal nerve territories; - One patient: burning pain on the feet; - One patient: hypoesthesia on the dorsal region of the thorax; - One patient: slightly decreased strength in the anterior tibial muscle.			

Abbreviations: HD, Hansen disease group; INC, inconclusive group; OD, other diseases group.

Eleven and eight patients in the OD group showed alterations in tactile (monofilament esthesiometry) and thermal (classical test tube method) sensations, respectively.

The QST regarding the lesions of the patients in the OD group revealed thermal hypoesthesia to cold stimulus in three patients and to heat stimulus in six patients. Pain hypoesthesia to cold was observed in three patients, and to heat, in three patients.

The final histopathological diagnoses of the OD group (diseases other than leprosy) were the following: reaction to an insect bite (1); granuloma annulare (2); eczematid (1); Sweet syndrome (1); mucinosis (1); lichen nitidus (1); erythema dyschromicum perstans (1); Lyme disease (1); psoriasiform dermatitis (1); and mycosis fungoides (1).

The decisive criteria to allocate patients into the HD, INC, and OD groups were the results of the histopathological examination, somewhat supported by the major neurological alterations in the peripheral nerves and the sensation assessment on the lesions and the innervation territories mostly affected by HD (ulnar, median, radial, tibial, fibular, plantar, calcaneal). None of the patients were included in any of the groups based exclusively on the clinical picture presented. We assigned the label HD to patients presenting mild sensory alterations on the cutaneous lesion sampled who exhibited somewhat specific histopathological alterations of HD. ►**Table 3** shows that it was impossible to clearly distinguish among the groups based exclusively on the neurological assessment, probably because of the subjectivity of the sensation examination.

Despite the neurological alterations found in the OD group, both in the QST and the assessment of peripheral nerves, the histopathological description related to the disease other than leprosy prevailed and justified the inclusion of these patients in the OD group.

Two dermatopathologists performed routine histopathological diagnoses and the assignment of the samples to each group. One pathologist, blinded to the group allocations, evaluated the samples stained with hematoxylin-eosin (H&E), Wade fuchsin (for AFB detection), and Gomori trichrome (for the examination of the endoneurial ECM). The pathologist examined all samples under a 400× objective and 10× optical lens in 2 histological sections for each sample and also counted all cutaneous nerve branches in each section. Another pathologist reviewed the findings and both concluded the exam by consensus. They quantified the different types of damage to nerve branches and adnexa as explained in the following section.

Quantification of cutaneous nerve branch alterations

The nerve lesion index (NLI), an arbitrary index, was calculated as the ratio of altered nerves affected by one of the

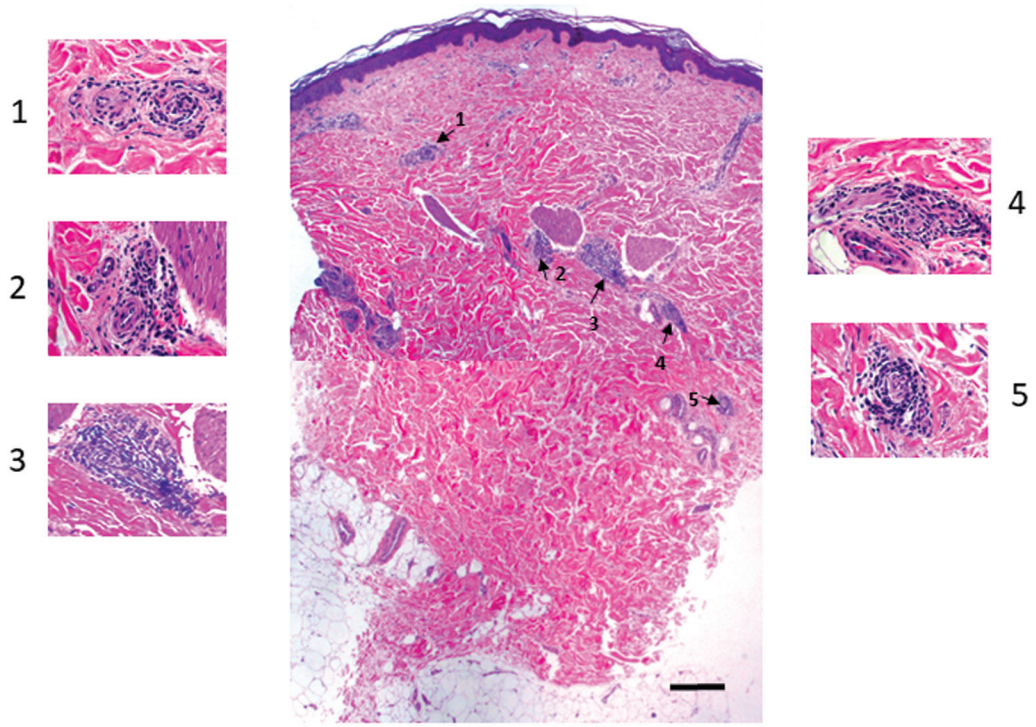

Figure 1 Calculation of the nerve lesion index (NLI). The NLI is the ratio of the number of dermal nerves in a section affected by each histopathological alteration (**A**) to the total number of dermal nerves found in the section (**B**). In the case illustrated, a = 5/b = 5m yielding an NLI of 1. Arrows 1–5 indicate dermal nerves found in the section. Each dermal nerve has a corresponding insert (1 to 5) providing a closer view of the inflammatory nerve damage. Inflammatory infiltrate surrounding and/or invading the endoneurial compartment of the dermal nerves can be seen. The NLI can vary from its minimal value of 0, which represents absence of dermal nerves presenting a histopathological damage in the section, to its maximal value of 1, when all the nerves in the section have a type of histopathological damage. Scale bar: 100 µm.

histopathological variables listed below to the total number of nerves observed in the respective section (►**Figure 1**). The NLI was determined for each sample regarding each of the following histopathological parameters:

- endoneurial inflammatory infiltrate;
- perineurial inflammatory infiltrate;
- perineurial enlargement (reaction to perineurial injury); and
- increase in endoneurial ECM.

Mean and a median NLIs were obtained for each group and then compared. The increase in ECM was evaluated semi-quantitatively in samples stained with Gomori trichrome. The presence of homogeneous light-green stained ECM in 60% or more of the cross-sectional area of the cutaneous nerve branch indicated increased ECM.

Quantification of the frequency of inflammatory involvement of skin adnexa

We investigated the frequency of inflammatory involvement of skin adnexa by assigning each histological section to either the "absent" or "present" categories. This assignment was made according to the detection of inflammatory infiltrate affecting each type of adnexa (sweat gland, pilosebaceous complex, and hair follicle). The intensity of the inflammatory involvement was not quantified in this procedure, only the frequency. We excluded from the analysis sections without visible adnexa structures in the microscopic examination.

Immunohistochemical procedure for the detection of protein gene product (PGP)9.5-immunoreactive axons

We stained sections with the rabbit anti-human PGP9.5 polyclonal antibody diluted 1/200 (Thermo Fisher Scientific, Waltham, MA, United States) and the EnVision FLEX peroxidase kit (Agilent Technologies, Santa Clara, CA, United States), as previously described by Antunes et al.[15]

We examined the histological sections with a Nikon E400 microscope (Nikon, Tokyo, Japan) under a 400× objective and 10× optical lens, and captured images of all cross-sectioned dermal nerves present in 2 histological sections per sample with a digital camera (Teledyne Lumenera, Ottawa, ON, Canada) attached to the microscope.

Quantification of endoneurial PGP9.5-immunoreactive fibers in nerve branches (AQI)

We calculated the axonal quantification index (AQI) for each dermal nerve found in each section as the ratio of the endoneurial PGP9.5-immunoreactive axonal area (EndonPGP9.5-ira) of one cross-sectioned dermal nerve within the cross-sectional endoneurial area (Endonarea) of the same branch examined (►**Figure 2**). Each biopsy sample was represented by the mean AQI of the cross-sectioned nerves found in the two

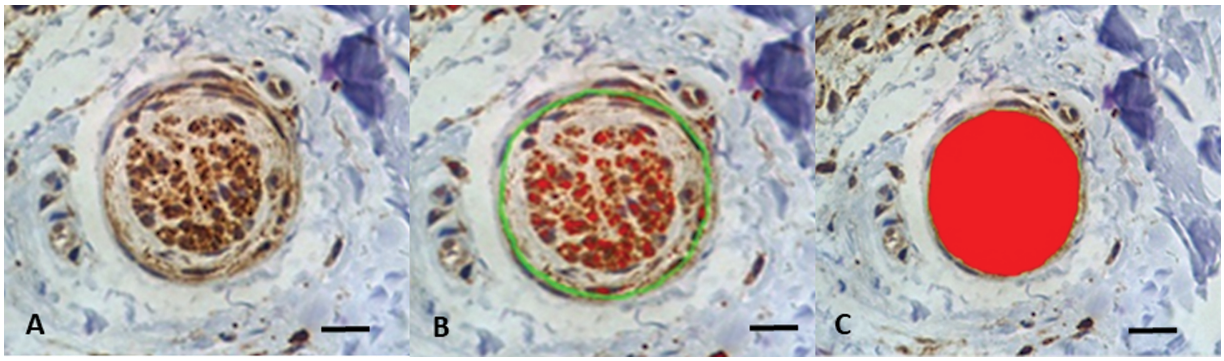

Figure 2 Calculation of the axonal quantification index (AQI). The AQI is calculated as the ratio of the endoneurial PGP9.5-immunoreactive area to the endoneurial area of the nerve examined. (**A**) PGP9.5-immunoreactive axons of a dermal peripheral nerve. (**B**) Image analyzer software definition of the endoneurial PGP9.5-immunoreactive area (EndonPGP9.5-ira), which is marked in red by the software. (**C**) Definition and calculation of the endoneurial area (EndonArea) of the same dermal nerve by the image analyzer. Scale bars: 45 µm.

sections. The EndonPGP9.5-ira and the Endonarea were measured and analyzed with the Image-Pro software (Media Cybernetics, Rockville, MD, United States).

Statistical analysis

We performed descriptive and bivariate analyses using the Epi Inf software (CDC, Atlanta, GA, United States). We used the Kruskal–Wallis test and Fisher exact test, with a 95% confidence interval (95%CI) for the inferential analysis, because of the small sample size and non-normal distribution of the variables.

RESULTS

Comparison of the mean NLI for inflammatory and non-inflammatory involvement of dermal nerves among the study groups

We found inflammatory and non-inflammatory damage to the nerves and adnexa in all three groups (►**Table 4A**; ►**Figures 3A-F**; **4A**). Regarding the inflammatory involvement of nerves, the HD group, as expected, presented higher median NLI values than the INC and OD groups (►**Table 4A**). The inflammatory infiltrate was more evident in samples of the HD group

Table 4 Indexes and frequencies of inflammatory and morphological alterations of dermal nerves and skin adnexa

A- Nerve lesion index (NLI) values of morphological alterations observed in cutaneous nerve branches of lesions: median (minimum–maximum)				
	HD ($n^*=13$)	**INC ($n=11$)**	**OD ($n=11$)**	***p*-value****
Endoneurial inflammatory infiltrate	0.5 (0–1)	0 (0–0)	0 (0–1)	13–0.001
Perineurial inflammatory infiltrate	1 (0–1)	0 (0–0.5)	0 (0–1)	13.6–0.001
Perineurial enlargement	0.2 (0–1)	0 (0–0.5)	0 (–0)	10.2–0.06
Increased ECM	0 (0–0.5)	0 (0–0)	0 (0–0.3)	5.9–0.05
B- Cutaneous nerve branches and skin adnexa alterations: *n* (%)				
B1- Dermal nerve branches		**HD ($n=13$)**	**INC ($n=11$)**	**OD ($n=11$)**
Endoneurial inflammatory infiltrate		9 (69%)	0	3 (27%)
Perineurial inflammatory infiltrate		11 (85%)	1 (9%)	5 (45%)
Perineurial enlargement		6 (46%)	2 (18%)	0
Increased ECM		1 (8%)	0	4 (36%)
B2- Skin adnexa: inflammatory involvement				
Sweat glands		7 (54%)	1 (9%)	0
Hair follicle		4 (40%)	0	0
Sebaceous glands		1 (11%)	0	0
C- Axon quantification index (AQI): mean ± SD				
Ratios	HD ($n=6$)	INC ($n=10$)	OD ($n=10$)	*p*-value
AQI	0.07 ± 0.02	0.06 ± 0.02	0.08 ± 0.03	0.2

Abbreviations: ECM, extracellular matrix; HD, Hansen disease group; INC, inconclusive group; OD, other diseases group; SD, standard deviation.
Notes: *number of patiens in the sample; **p value lower than 0.005 were considered statistically signficant.

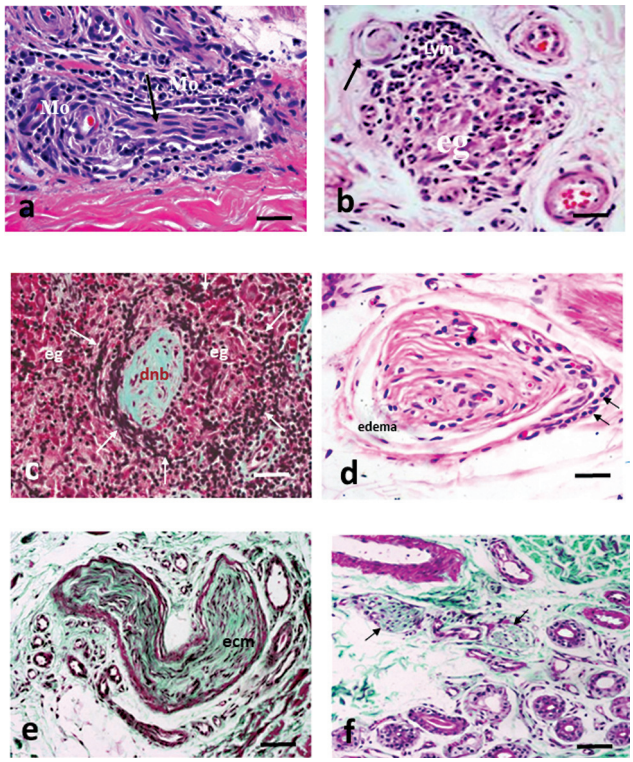

Figure 3 (**A**) Mononuclear inflammatory cells (Mo) consisting of lymphocytes and macrophages surrounding and invading a nerve branch (arrow). HD group. hematoxylin-eosin (H&E). Scale bar: 55 µm. (**B**) Epithelioid granuloma (EGF) in contact with the perineurial layer of a small dermal nerve branch (arrow). Abbreviation: Lym: lymphocytes. HD group. H&E. Scale bar: 55 µm. (**C**) Dermal nerve branch (DNB) showing increased endoneurial extracellular matrix (ECM) stained in green. Few macrophages, lymphocytes, fibroblasts, and other chromotrope 2R-stained structures can be seen in the endoneurial compartment. The referred branch is surrounded by EG and lymphocytic halo (delimited by white arrows). HD group. Gomori trichrome. Scale bar: 40 µm. (**D**) dermal nerve branch showing few lymphocytes surrounding its perineurial layers and enlargement of subperineurial space (edema). INC group. H&E. Scale bar: 40 µm. (**E**) Increased percentage of green-stained ECM in the endoneurial space due to the loss of nerve fibers. INC group. Gomori trichrome. Scale bar: 110 µm. (**F**) Two segments of a dermal nerve branch (arrows), parts of a single nerve, exhibiting normal quantity of endoneurial ECM and endoneurial cells. INC group. Gomori trichrome. Scale bar: 110 µm.

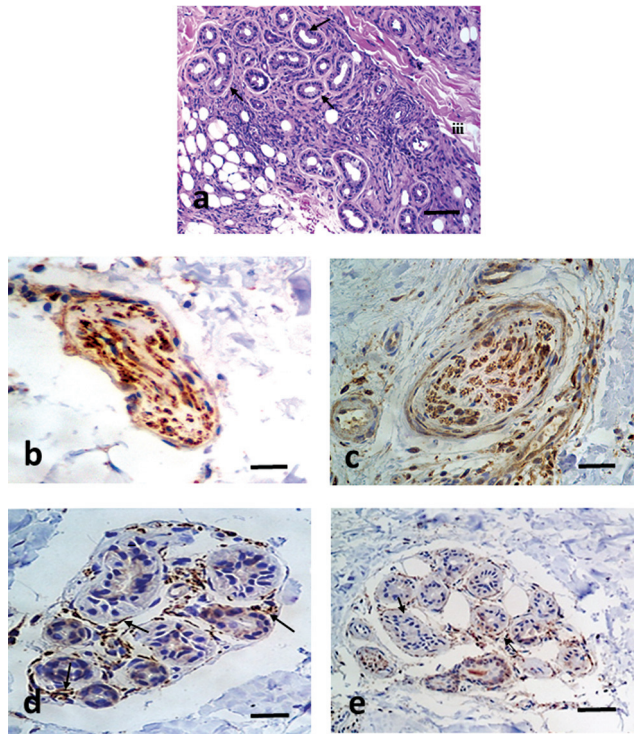

Figure 4 (**A**) Cross-section of coiled sweat gland acini (arrows), dissociated by intervening inflammatory infiltrate (III) among acinar loops. HD group. H&E. Scale bar: 80 µm. (**B**) PGP9.5-immunoreactive axons in the endoneurium of cutaneous nerve branch. INC group. Immunoperoxidase. Scale bar: 40 µm. (**C**) PGP9.5-immunoreactive axons in a cutaneous nerve branch. OD group. Immunoperoxidase. Scale bar: 60 µm. (**D**) PGP9.5-immunoreactive axons surrounding sweat gland acini (arrows). OD group. Immunoperoxidase. Scale bar: 40 µm. (**E**) Scarce PGP9.5-immunoreactive axons surrounding sweat gland acini (arrows). HD group. Immunoperoxidase. Scale bar: 40 µm. Abbreviations: HD, Hansen disease group; INC, inconclusive group; OD, other diseases group.

through the presence of a higher NLI corresponding to endoneurial and perineurial infiltrates (►**Figures 3A–C**). The median perineurial and endoneurial infiltrate NLI values of INC samples, on the other hand, were of zero (►**Table 4A**); however, one INC sample showed scarce perineurial lymphocytic infiltrate. The NLI regarding perineurial enlargement was higher in the HD group than in the INC group, and was null in the OD group (►**Table 4A**).

Frequency of histopathological alterations in dermal nerves and adnexa in the study groups

Regarding the frequency of nerve histomorphological alterations in the INC group, one sample exhibited perineurial inflammatory infiltration, seven presented an increased number of cells in the endoneurium, and two, perineurial enlargement (►**Table 4B1**), while only one sample showed any inflammatory involvement of adnexa, and that was in the sweat glands (►**Table 4B2**). In the OD group, endoneurial inflammatory infiltrate were observed in three samples, perineurial inflammatory infiltrate, in five, and increased endoneurial ECM, in four (►**Table 4B1**, ►**Figure 3E**).

Comparison of inflammatory involvement of adnexa among the study groups

Inflammatory involvement of sweat glands and hair follicles was more frequent in the samples of the HD group, but it was also present in the INC group (►**Table 3** and **4B2**, ►**Figure 4A**).

Comparison of the AQI among the study groups

We did not find significant differences in the AQI among the three groups (►**Table 4C**; ►**Figures 4B, C**). Samples of the HD group exhibited decreased innervation of sweat glands compared with those of the INC and OD groups (►**Figures 4D, E**).

DISCUSSION

The NLI was efficient in discriminating HD from INC and OD skin samples. It was also helpful to detect probable or possible cases of HD among INC and OD samples. We created

the NLI, an arbitrary index, to quantify the intensity of nerve damage in the sample. The NLI is a quantitative method with its own internal control that expresses the fraction of the dermal nerves affected by the characteristic HD inflammatory process within histological sections. The greater the number of affected nerves in a section, the higher the fraction of compromised nerve in the skin and the greater the chance of HD etiology. This is particularly useful when the search for AFB in the material of suspected HD patients does not yield positive results. This method, however, exhibits limitations when the examination of the histological section does not present any visible cross-sectioned dermal nerves. The absence of visible nerves in skin sections cannot be interpreted as damaged nerve according to Ridley.[12]

Studies on HD histopathology with a similar objective as that of the present study are lacking in the recent literature. Histopathological diagnosis is a classic method that, together with clinical dermatological and neurological examinations, guided the studies that led to the classifications of HD, including the Madrid[16] and Ridley–Jopling classifications.[12] Histopathological diagnosis of HD still prevails as an important tool in the study of the disease, providing morphological evidence to support the results of newer technological procedures in the diagnostic field.

A classic reference that highlights the importance of the histopathological examination for HD diagnosis comes from the article on the Ridley–Jopling classification.[12] The author asserts that, in an HD biopsy sample, the finding of a cross-sectioned 400-µm-long dermal nerve branch (comprising the dermal nerve in the center of the inflammatory infiltrate plus the surrounding tuberculoid granulomatous infiltrate) is irrefutable evidence that the patient should be classified as having polar tuberculoid HD.

Another more recent investigation[10] addressed the differential diagnosis of HD with other inflammatory diseases. The authors remarked that the cell composition of the inflammatory infiltrate (presence of plasma cells, formation of palisading), the lichenoid distribution of the inflammatory process, epidermal changes such as spongiosis and acanthosis, and the presence of viral-induced cytopathic alteration are contributive to distinguish HD from other diseases, despite the presence of inflammatory infiltrate along the trajectory of dermal nerve in non-HD specimens.

In the present study, perineurial enlargement, a nonspecific morphological change, was frequently observed in the HD group, but it was also observed in the INC group, albeit less frequently. This finding is important, particularly in the INC group, as the presence of morphological alterations in dermal nerves suggests that clinical monitoring of these individuals should be established to detect potential explicit manifestations of HD that may arise.

The perineurium and endothelial cells of endoneurial capillary vessels compose the blood-nerve barrier.[17] These concentric layers of flat cells respond to injury by increasing the number of layers and by proliferating into the inflammatory endoneurial environment, forming microfasciculation. Therefore, perineurial enlargement can be considered a response to nerve injury.[18]

The higher frequency of samples with inflammatory infiltration of the sweat glands in the HD group is consistent with the areas of hypohidrosis, dry skin, and hair loss observed in patients with HD. This can be used as additional histopathological evidence in the differential diagnosis of HD,[11] or as an indication for the prolonged follow-up of patients with an inconclusive diagnosis.

The absence of a difference in the increased endoneurial ECM parameter among the HD, INC, and OD groups (►**Table 4A**) is in agreement with the equivalent AQI in the dermal nerves of the three groups (►**Table 4C**), because increased endoneurial ECM is usually a consequence of endoneurial axonal loss.

We did not find fibrosis of dermal nerves in the present study, only nerves exhibiting increased ECM. Endoneurial fibrosis manifests as a compact hyaline ECM, with few or no cell nuclei in the matrix. According to Antunes et al,[19] nerve fibrosis is an end-stage event in the pathogenesis of HD neuropathy; therefore, absence of nerve fibrosis in the sections may be an indication that the nerve lesions of the selected samples had not evolved to fibrosis yet; thus, they were not in the advanced stage.

As the AQI values in the HD group were not significantly different from those of the INC and OD groups, we deduced that the localized cutaneous nerve dysfunction observed in the patients diagnosed with HD was probably a result of local functional impairment of nerve fibers due to the HD skin inflammatory process without detectable axonal loss. Initiation of multidrug therapy (MDT) may enable the recovery of nerve function once the inflammatory process decreases.[20]

The OD group also exhibited samples that showed nerve damage besides the specific alteration characteristic of their non-HD diagnosis. The present findings indicate that non-HD diseases may also cause nerve damage distinctive from HD; thus, special attention is required in the differential histological diagnosis.

It is worth mentioning that other inflammatory diseases such as granuloma annulare are also treated with dapsone[21] or clofazimine.[22] Therefore, cases of these diseases misdiagnosed as HD may be apparently cured by MDT, as it includes either drug alone or both drugs. Nevertheless, special attention is necessary to avoid a misdiagnosis since patients diagnosed with HD are prone to be socially stigmatized and discriminated in endemic countries.

The presence of nerve alterations and inflammatory involvement of sweat glands in the INC group increased the suspicion of HD. This was also an indication for the clinical follow-up of these patients. It is worth commenting that one patient returned to the clinic sixteen years after the inconclusive diagnosis presenting with a different clinical profile and was diagnosed with HD. None of the other patients included in the present study returned to the clinic.

We conclude that the importance and efficacy of the histopathological examination of HD-suspected skin lesions can be enhanced by the detection of nonspecific inflammatory and noninflammatory alterations IN dermal nerve branches. Since the diagnosis of HD in the INC and OD groups could not be totally ruled out, we recommend that patients

with these nerve changes (particularly those that exhibit more than one), which do not enable a definitive HD diagnosis, should undergo close clinical monitoring to detect future clinical and/or laboratory manifestations of HD.

It is worth commenting that HD may exist as a comorbidity with other cutaneous diseases; therefore, the diagnosis of any other disease from a HD-suspected skin lesion does not completely rule out a concomitant HD diagnosis.

The value of the histopathological examination of skin lesions as a decisive tool in the diagnosis of HD was confirmed in the HD group, as clinical and laboratory changes alone could not enable diagnostic conclusion.

Authors' Contributions

EAFC: conceptualization, data curation, formal analysis, investigation, methodology, project administration, supervision, validation, visualization, writing of the original draft, and writing – review and editing. TPA: formal analysis and writing – review and editing. XI: conceptualization, formal analysis, funding acquisition, methodology, project administration, and writing – review and editing. BP: data curation, formal analysis, investigation, methodology, and software. JACN: data curation, investigation, methodology, and resources. AMS: data curation, investigation, resources, and visualization. SLGA: conceptualization, project administration, writing, analysis, supervision, review, and data curation.

Support

The authors declare that the present study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)/ Programa de Ações Estratégicas para o Desenvolvimento e Fortalecimento dos Laboratórios Credenciados e das Áreas de Apoio à Pesquisa (PAEF), Instituto Oswaldo Cruz.

Conflict of Interest

The authors have no conflict of interest to declare.

Acknowledgments

The authors would like to thank Amy Goundry for proofreading and editing in English, and Mariana Hacker for the statistical analysis.

References

1 Alves ED, Ferreira TL, Ferreira IN. Neuropatia da hanseníase. In: Hanseníase: avanços e desafios. Brasília: NESPROM; 2014:492
2 White C, Franco-Paredes C. Leprosy in the 21st century. Clin Microbiol Rev 2015;28(01):80–94. Doi: 10.1128/CMR.00079-13
3 Illarramendi X, Carregal E, Nery JA, Sarno EN. Progression of acral bone resorption in multibacillary leprosy. Acta Leprol 2000-2001–2001;12(01):29–37
4 Scollard DM, Truman RW, Ebenezer GJ. Mechanisms of nerve injury in leprosy. Clin Dermatol 2015;33(01):46–54. Doi: 10.1016/j.clindermatol.2014.07.008
5 Rambukkana A, Zanazzi G, Tapinos N, Salzer JL. Contact-dependent demyelination by Mycobacterium leprae in the absence of immune cells. Science 2002;296(5569):927–931. Doi: 10.1126/science.1067631
6 Rambukkana A. Usage of signaling in neurodegeneration and regeneration of peripheral nerves by leprosy bacteria. Prog Neurobiol 2010;91(02):102–107. Doi: 10.1016/j.pneurobio.2009.12.002
7 Antunes SL, Chimelli L, Jardim MR, et al. Histopathological examination of nerve samples from pure neural leprosy patients: obtaining maximum information to improve diagnostic efficiency. Mem Inst Oswaldo Cruz 2012;107(02):246–253. Doi: 10.1590/S0074-02762012000200015
8 Jessen KR, Mirsky R, Lloyd AC. Schwann cells: development and role in nerve repair. Cold Spring Harb Perspect Biol 2015;7(07):a020487. Doi: 10.1101%2Fcshperspect.a020487
9 Antunes SLG, Fazan VPS, Jardim MR, et al. Morphometric analysis of nerve fibers in neural leprosy. Muscle Nerve 2021;63(04):593–599
10 Abbas O, Bhawan J. Cutaneous perineural inflammation: a review. J Cutan Pathol 2010;37(12):1200–1211. Doi: 10.1111/j.1600-0560.2010.01614.x
11 Kotteeswaran G, Chacko CJ, Job CK. Skin adnexa in leprosy and their role in the dissemination of M. leprae. Lepr India 1980;52(04):475–481
12 Ridley DS. Histological classification and the immunological spectrum of leprosy. Bull World Health Organ 1974;51(05):451–465
13 Illarramendi X, Costa EA, Miranda AM et al. Small Nerve Fiber Evaluation to Aid the Early Diagnosis of Leprosy. In: Book of Abstracts of the 18 th International Leprosy Congress Hidden Challenges; 16 th -19 th , September of 2013; Brussels, Belgium. P.136. Available at. *http://www.leprosyila.org/arquivos/leprosy_congress.pdf*
14 Birke JA, Brandsma JW, Schreuders TAR, Piefer A. Sensory testing with monofilaments in Hansen's disease and normal control subjects. Int J Lepr Other Mycobact Dis 2000;68(03):291–298
15 Antunes SL, Chimelli LM, Rabello ET, et al. An immunohistochemical, clinical and electroneuromyographic correlative study of the neural markers in the neuritic form of leprosy. Braz J Med Biol Res 2006;39(08):1071–1081. Doi: 10.1590/s0100-879x2006000800010
16 Davison AR, Kooij R, Wainwright J. Classification of leprosy. 1. Application of the Madrid classification of various forms of leprosy. Int J Lepr 1960;28:113–125
17 Richard L, Védrenne N, Vallat JM, Funalot B. Characterization of endoneurial fibroblast-like cells from human and rat peripheral nerves. J Histochem Cytochem 2014;62(06):424–435. Doi: 10.1369%2F0022155414530994
18 Vallat JM, Leboutet MJ, Henry P, Millan J, Dumas M. Endoneurial proliferation of perineurial cells in leprosy. Acta Neuropathol 1991;81(03):336–338. Doi: 10.1007/BF00305877
19 Antunes SLG, Jardim MR, Vital RT, et al. Fibrosis: a distinguishing feature in the pathology of neural leprosy. Mem Inst Oswaldo Cruz 2019;114:e190056. Doi: 10.1590/0074-02760190056
20 Illarramendi X, Machado AM, Magalhaes GO, Castro AC, Antunes SL. Recovery from sensory impairment and cutaneous lesion nerve regeneration in cutaneous lesions leprosy patients. Mem Inst Oswaldo Cruz 2012;107:68–73. Doi: 10.1590/S0074-02762012000900012
21 Martín-Sáez E, Fernández-Guarino M, Carrillo-Gijón R, Muñoz-Zato E, Jaén-Olasolo P. [Efficacy of dapsone in disseminated granuloma annulare: a case report and review of the literature. Actas Dermosifiliogr 2008;99(01):64–68
22 Mensing H. [Clofazimine–therapeutic alternative in necrobiosis lipoidica and granuloma anulare]. Hautarzt 1989;40(02):99–103. Doi: 10.4081%2Fdr.2015.5749