

# Leprosy patients: neurotrophic factors and axonal markers in skin lesions

Pacientes com hanseníase: fatores neurotróficos e marcadores axonais em lesões de pele

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## ABSTRACT

Neurotrophins are growth factors with crucial roles in neural pathophysiology. These mediators functionally modulate nociceptive fibers, and changes in neurotrophins expression have been correlated with early loss of nociception in leprosy. This study investigated the expression of NGF, BDNF, and NT3 in dermal nerves of leprosy patients. Characterization of Remak bundles was achieved by p75<sup>NTR</sup>, and axonal markers NF-L and PGP 9.5 immunostaining. Clinical parameters of neural impairment have been evaluated by Semmes-Wenstien monofilaments. Our findings demonstrated decrease of NGF in borderline leprosy, when compared to control specimens. Similar results were observed in PGP 9.5 expression (borderline:  $p < 0.001$  and lepromatous:  $p < 0.05$ ) and NF-L (lepromatous:  $p < 0.05$ ), suggesting advanced Remak bundles degeneration in multibacillary leprosy. It has also been observed positive correlation between p75<sup>NTR</sup> and PGP 9.5, indicating association between Schwann cells and axons in Remak bundles. Present data indicate that neurotrophins imbalance may participate in the establishment of peripheral nerve damage.

**Key words:** peripheral nerves, leprosy.

## RESUMO

Neurotrofinas são fatores de crescimento com papel fundamental na fisiopatologia neural. Esses mediadores modulam funcionalmente fibras nociceptivas. Mudanças em sua expressão têm sido relacionadas à perda precoce da nocicepção na hanseníase. Este estudo investigou a expressão de NGF, BDNF e NT3 em nervos dérmicos de pacientes hansenianos. A caracterização de fibras nervosas não mielinizadas foi feita por p75<sup>NTR</sup> e marcadores axonais NF-L e PGP 9.5. Os parâmetros clínicos de dano neural foram avaliados por monofilamentos Semmes-Wenstien. Nossos achados demonstram diminuição de NGF nos pacientes dimorfos em comparação aos controles. Resultados similares foram observados para PGP 9.5 (dimorfos:  $p < 0,001$ ; virchowianos:  $p < 0,05$ ) e NF-L (virchowianos:  $p < 0,05$ ), sugerindo degeneração avançada das terminações nervosas na hanseníase multibacilar. Foi observada correlação positiva entre p75<sup>NTR</sup> e PGP 9.5, indicando associação entre células de Schwann e axônios em fibras nervosas não mielinizadas. Os resultados indicam que o desequilíbrio na expressão das neurotrofinas pode participar do dano neural periférico.

**Palavras-Chave:** nervos periféricos, hanseníase.

The main principles of leprosy control consist of early detection of new cases, proper treatment by multidrug therapy (MDT), prevention of disabilities and rehabilitation<sup>1</sup>. In spite of worldwide efforts, endemic countries still have leprosy control as an unreached goal. The prevention and recovery of peripheral neuropathy are among the hardest challenges of leprosy. In Brazil, approximately 5.9% of new cases of leprosy present grade II of disability at the moment of first diagnosis<sup>1</sup>. Unfortunately, the established neural impairment tends to persist as lifelong sequelae in these patients<sup>2</sup>.

*Mycobacterium leprae* (*M. leprae*) has tropism for the peripheral nervous system, where it infects Schwann cells and eventually endothelium<sup>3</sup>, generating polymorphic skin lesions with variable granulomatous infiltrate according to the clinical form of the disease<sup>4</sup>. This tropism is attributed to the binding of *M. leprae*-related glycolipid PGL-1 to the complex formed by the molecule dystroglycan- $\alpha$  from Schwann cells and the G domain of the  $\alpha 2$  chain of laminin-2, located in the extracellular basal lamina<sup>5</sup>. The colonization of peripheral nerves may also be mediated by the binding of *M. leprae* to Erb2 receptor of neuregulin-1<sup>6</sup>, as well as adhesins exposed on the bacillus

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surface<sup>7,8</sup>. The contact and invasion of *M. leprae* lead to antigen presentation by Schwann cells, which secrete inflammatory mediators and chemotactic factors for macrophages, thus perpetuating the immune response<sup>9</sup>.

In leprosy, nerve damage produces functional changes due to unsuccessful attempts of regeneration<sup>10</sup>. Microscopic characteristics of neural lesions include degeneration of Schwann cells, loss of the myelin sheath, axonal retraction, periaxonal fibrosis and a wide range of immunopathological responses. Paucibacillary forms of leprosy (TT and BT) demonstrate intense inflammatory infiltrate which leads to tissue destruction and Wallerian degeneration<sup>11</sup>. When regeneration is still feasible after nerve damage, the axon endings seek out a suitable microenvironment for reinstatement<sup>12</sup>. However, without neurotrophic factors, the neurites do not extend back to the perineural space<sup>13</sup>. If the axonal atrophy cannot be reversed in time due to a neural scar, Schwann cells become increasingly less responsive, and potential regeneration is progressively lost<sup>14</sup>.

Neurotrophic factors represent a group of biochemical mediators classified on the bases of their properties including regulation of neural development, neuroprotection and reduction of neural degeneration in central and peripheral nervous system (PNS and CNS). The participation of these growth factors in regeneration of CNS and PNS provide promising perspectives for therapeutics in neurodegenerative diseases. Neurotrophins correspond to a specific family of neurotrophic factors synthesized as inactive precursors and processed inside both cell bodies and extracellular environment<sup>15</sup>.

Once processed, they generate active pro-peptides and mature proteins. This group of growth factors comprises NGF, NT3, NT4/5, BDNF and GDNF. Two classes of receptors bind to neurotrophins: neurotrophic receptor p75 (p75<sup>NTR</sup>) and tropomyosin-related kinase receptors (TrkA, TrkB, TrkC). Each neurotrophin binds specifically to a Trk receptor, whereas all of them bind equally to p75<sup>NTR</sup>. P75<sup>NTR</sup> has an intracellular type II death domain, which distinguish it as a member of tumor necrosis factor family. Additionally, p75<sup>NTR</sup> is able to modify the binding affinity of neurotrophins to Trk receptors<sup>16,17</sup> and participates on inhibition of axonal outgrowth<sup>18</sup>. The interaction between NGF and p75<sup>NTR</sup> results in the blockage of this receptor in terms of binding with other mediators and indirectly allows the axons to extend back to the place of origin. These examples underscore the pivotal role played by p75<sup>NTR</sup> and NGF in neural plasticity<sup>19</sup>. In functional studies, p75<sup>NTR</sup> can be adopted as a marker of phenotype since this receptor is expressed in immature and non-myelinating Schwann cells<sup>19</sup>.

Previous studies have demonstrated that in leprosy the correlation of cutaneous NGF expression to functional evaluation of autonomic and sensorial fibers have been associated with decreasing of NGF in Schwann cells, which may participate of early loss of nociception and paradoxically

related to neuropathic pain<sup>20-22</sup>. In the pathogenesis of leprosy, inflammation plays a crucial role in the development of neuropathy, but the non-inflammatory mechanisms that participate in axonal degeneration and demyelination should be better surveyed.

In this study, we proposed to investigate circulating and immunohistochemical expression of neurotrophins (NGF, BDNF and NT3) in dermal nerves (Remak bundles) of non-reactional leprosy patients. Remak bundles have been demonstrated by immunohistochemical staining of p75<sup>NTR</sup> - adopted here as a marker of unmyelinated nerve fibers, and axonal markers NF-L and PGP 9.5. Clinical sensory testing has been performed by evaluation with Semmes-Wenstien monofilaments in order to investigate the correlation between clinical parameters of neural impairment and expression of neurotrophic factors.

## METHODS

### Sampling

Paraffin embedded specimens have been selected from Pathology Laboratory of Lauro de Souza Lima Institute (ILSL) – Bauru, São Paulo, Brazil. The specimens were obtained from skin biopsies of leprosy patients referred to Dermatology Clinic of ILSL. The medical files of patients were accessed to survey their MDT status, data on sensitivity tests and laboratorial parameters. Samples were collected under approval of Human Research Ethics Committees and patients signed informed consent. Patients were grouped according to classification of Ridley and Jopling (1966) into: Tuberculoid Leprosy (TT) → n=10; Borderline Leprosy (BB) → n=10; Lepromatous Leprosy (LL) → n=10. The control group was composed of skin specimens without inflammatory changes, obtained for diagnostic investigation of non-infectious dermatosis or neuropathic disorders (C) → n=08.

### Immunohistochemistry

Neurotrophins, p75<sup>NTR</sup>, S100 and axonal markers were evaluated in non-reactional leprosy lesions and skin samples from donors without leprosy. The following antibodies were used: monoclonal rabbit anti-human NGF antibody (Abcam, Cambridge, UK); monoclonal mouse anti-human BDNF antibody (R&D Systems, Minneapolis, MN, USA); polyclonal rabbit anti-human NT3 antibody (Abcam); monoclonal mouse anti-human p75<sup>NTR</sup> antibody (R&D Systems); polyclonal rabbit anti-human S100 antibody (Dako Corporation, Carpinteria, CA, USA); polyclonal rabbit anti-human PGP 9.5 antibody (Dako Corporation, Carpinteria, CA, USA) and monoclonal mouse anti-human NF-L antibody (Dako Corporation). Five micrometer sections of paraffin-embedded biopsies were deparaffinized and hydrated by passing them through xylene and alcohol gradients. Specimens were kept in a water

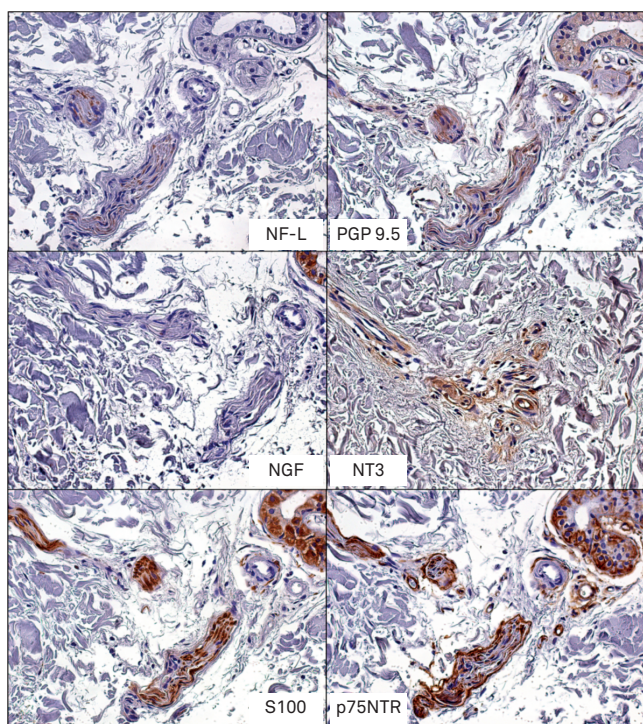
bath for 20 minutes at 95° C on 0.01 M sodium citrate buffer pH 6.0 for antigen retrieval. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide in methanol. Then, sections were incubated overnight with primary antibodies. The detection was achieved by the EnVision Dual Link System-HRP (Dako Corporation). Sections were counterstained with Harry's hematoxylin, dehydrated, cleared and mounted with Permount (Fischer Scientific, Pittsburgh, USA).

### Scoring of neurotrophic factors

Each microscopic field has been analyzed as a whole in all sections by two independent observers. The immunohistochemical presentation has been evaluated in Remak bundles of dermis (Fig 1). Positive Remak bundles were counted for each marker employed and the results obtained for different groups were compared by using non-parametric Kruskal-Wallis test and Dunn's multiple comparison post-test (GraphPad InStat Version 4.00, San Diego, CA, USA);  $p < 0.05$  was considered significant.

### ELISA

The detection of NGF and BDNF in serum samples was performed by ELISA using commercial kits NGF Emax® ImmunoAssay System (Promega, Madison, WI, USA) and ChemoKine Brain Derived Neurotrophic Factor (BDNF) Sandwich ELISA (Millipore Corporation, Billerica, MA, USA) according to manufacture instructions.



**Fig 1.** Immunohistochemical expression of NF-L, PGP 9.5, NGF, NT3, S100 and p75<sup>NTR</sup> in Remak bundles of leprosy patient (LL).

### Light touch threshold

Skin sensory tests have been performed by using Semmes-Weinstein monofilaments, varying in strength in a range of 0.05, 0.2, 2.0, 4.0, 10.0 and 300 g. To each monofilament it has been attributed a score ranging from 6 to 1. For correlation purposes, the higher results for each patient have been included in body location closer to sites where biopsies were undertaken to histopathology and immunohistochemistry.

### Statistical analysis

The data were expressed as mean and standard deviation values, and analyzed by non-parametric Kruskal-Wallis test and Dunn's multiple comparison post-test, using Graph Pad Prism 4.00 software for Windows. Correlations were carried out by Spearman non-parametric test. A  $p$ -value  $< 0.05$  was considered to be statistically significant.

## RESULTS

### Immunohistochemical analysis

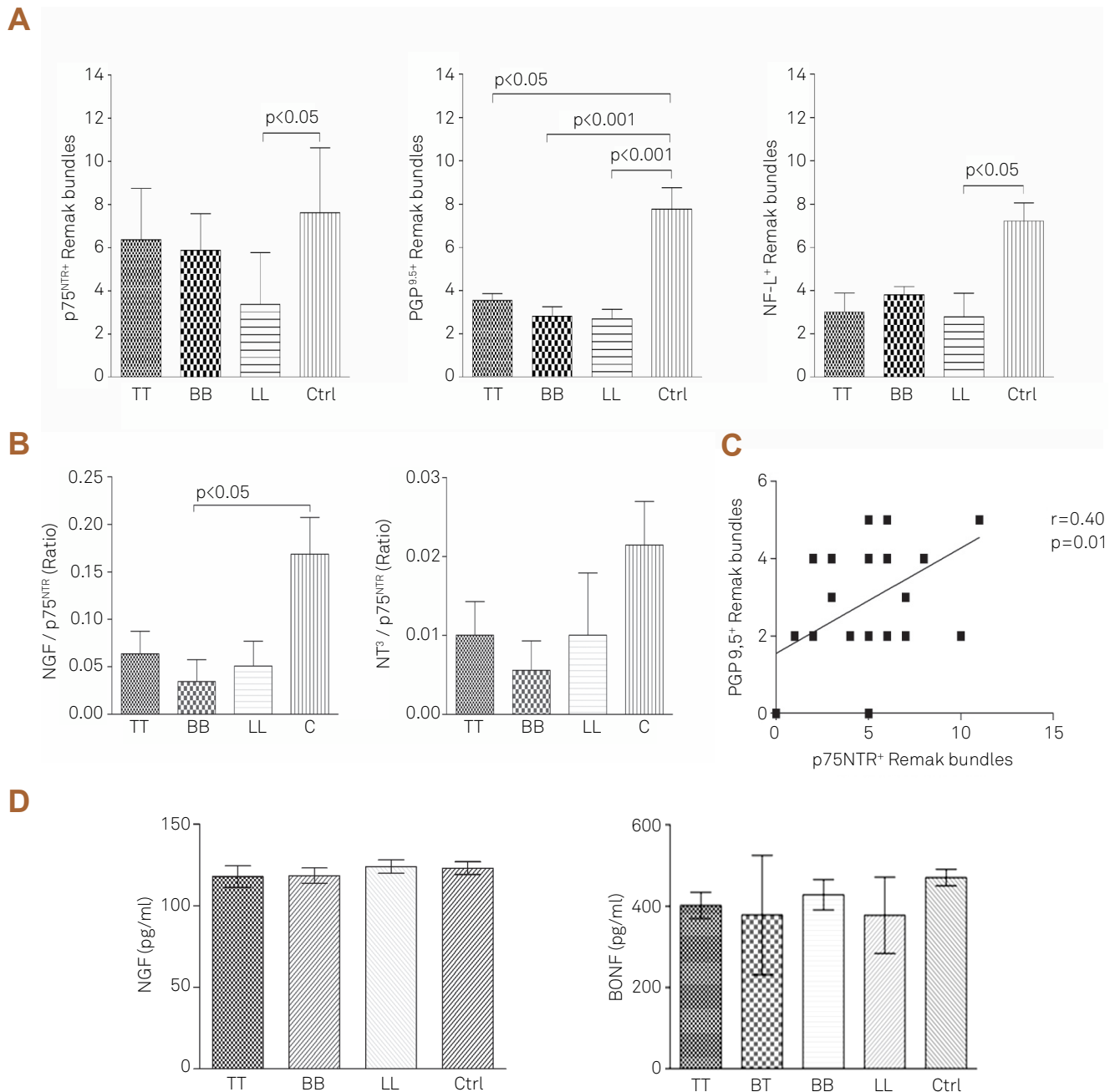
Samples and controls were analyzed by immunohistochemistry to detect neurotrophic factors and axonal markers. In this study, p75<sup>NTR</sup> was adopted as a marker of non-myelinating Schwann cells phenotype. p75<sup>NTR</sup> decreased significantly ( $p < 0.05$ ) in LL in comparison to controls, indicating reduction of detectable nerve branches of the skin of leprosy patients, mainly LL (Fig 2A). Similar results were observed regarding expression of axonal markers PGP 9.5 (TT:  $p < 0.05$ ; BB:  $p < 0.001$  and LL:  $p < 0.001$ ) and NF-L (LL:  $p < 0.05$ ) (Fig 2A). BDNF did not demonstrate any immunohistochemical positivity on samples and controls. The evaluation of Remak bundles demonstrated decreasing of NGF and NT3 expression in leprosy lesions. The ratio between NGF and p75<sup>NTR</sup>, as well as NT3 and p75<sup>NTR</sup>, was established in order to evaluate expression of neurotrophins independently of nerves branches amount in the skin (Fig 2B). Results indicated a statistically significant reduction of NGF ( $p < 0.05$ ) expression in borderline leprosy (BB) when compared to control specimens. For NT3, no significant difference was observed. It has also been observed positive correlation between p75<sup>NTR</sup> and PGP 9.5 ( $r = 0.40$ ;  $p = 0.01$ ), indicating the association between Schwann cells and axons in Remak bundles (Fig 2C).

### ELISA

The serum concentration of NGF and BDNF in leprosy patients did not differ significantly from control subjects (Fig 2D).

### Light touch threshold

Evaluation of skin sensitivity among TT, BB and LL leprosy patients did not show significant differences

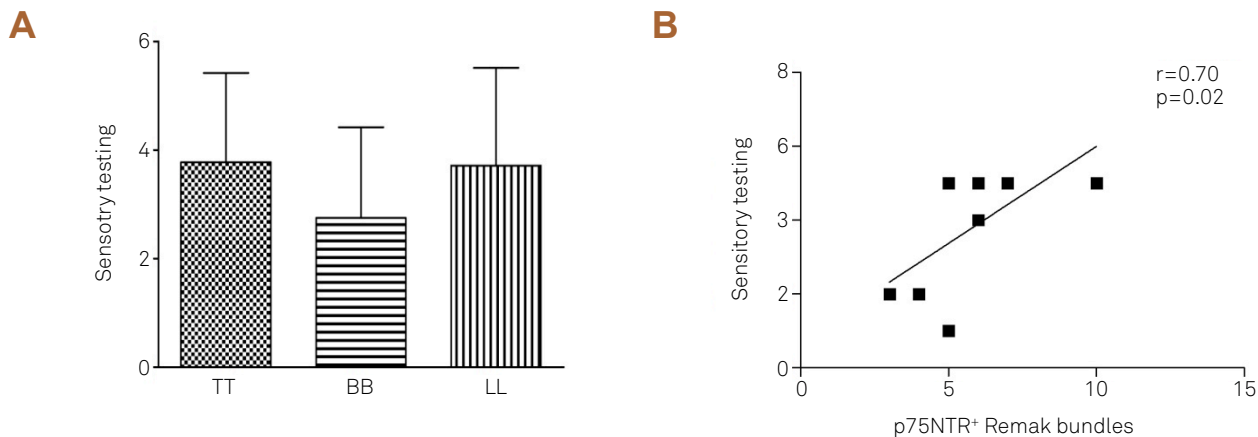


**Fig 2.** Morphometric evaluation of Remak bundles. (A) Count of positivity to p75<sup>NTR</sup>, NF-L and PGP 9.5. (B) Ratio between neurotrophins and p75<sup>NTR</sup>, revealing decrease of neurotrophins expression in leprosy. (C) Spearman correlation between p75<sup>NTR</sup> and PGP 9.5. (D) Serum concentration of NGF (pg/ml) and BDNF (pg/ml) in leprosy and controls, no differences were detected among groups. TT: tuberculoid leprosy patients; BB: borderline leprosy patients; LL: lepromatous leprosy patients.

(Fig 3A), whereas BB has presented lower light touch response than other groups. Additionally, tuberculoid leprosy patients (TT) presented positive correlation between sensory results and expression of p75<sup>NTR</sup> ( $r=0.70$ ;  $p=0.04$ ), indicating preservation of protective sensitivity in this group (Fig 3B). Apart from TT patients, it was not possible to establish correlation between *in situ* expression of neither neurotrophins, nor axonal markers and the results of sensory testing.

## DISCUSSION

Neurotrophins are key mediators in the process of myelination of peripheral nervous system and has been shown that myelin formation can be inhibited by functional changes in neurotrophic mediators. Neurotrophins are characterized as a family of growth factors with crucial roles in neural pathophysiology. These mediators may, among other activities, regulate nociceptive fibers. In leprosy patients, changes in the expression of some neurotrophins



**Fig 3.** Light touch threshold evaluation. (A) Score for sensibility test by using Semmes-Weinstein monofilaments. (B) Spearman correlation between sensory results and expression of p75<sup>NTR</sup> in TT patients. No significant differences were observed between different groups of leprosy patients. TT: tuberculoid leprosy patients; BB: borderline leprosy patients; LL: lepromatous leprosy patients.

were correlated with the early loss of nociception<sup>22</sup>. Previous studies demonstrate a correlation between nerve dysfunction on clinical tests and morphological changes in skin, regardless the type of leprosy<sup>23</sup>. The present study aimed to investigate the involvement of neurotrophic mediators in the alterations of peripheral nerve branches of non-reactional leprosy skin lesions, based on a comparison between clinical tests of skin sensitivity and expression of neurotrophins, phenotypic and axonal markers.

Neurotrophin family was characterized after pioneer studies on NGF, the first well established neural growth factor<sup>24</sup>, and plays key roles in the initiation of axon growth, synaptic rearrangement, neurite sprouting, besides preventing sympathetic and sensory neuronal death<sup>25</sup>. NGF deprivation participates in development of peripheral neuropathies, including leprosy and diabetes<sup>26</sup>. Accordingly, the decrease in endogenous levels of NGF and TrkA has been demonstrated in skin lesions of leprosy patients<sup>20</sup>. Our results agreed with former studies; *in situ* expression of NT3 and NGF decreased in leprosy lesions when compared to control skin samples, suggesting that the imbalance of neurotrophic factors might be involved in impairment of peripheral nerve regeneration after nerve damage. In spite of antibodies against NGF have also been implicated as indicators of leprosy neuritic status<sup>27</sup>, serological data in our study did not demonstrate any differences between leprosy and control serum samples concerning circulating neurotrophins, suggesting that it is not a representative method to evaluate neural damage in non-reactional leprosy. In accordance, Costa et al.<sup>28</sup> did not find differences in BDNF serum levels in healthy control and leprosy patients before, during and after multidrug therapy. We also investigated alterations in clinical parameters, such as loss of protective sensitivity, and its possible correlation with tissue expression of neurotrophins. The perception of touch is determined by myelinated nerves, as well as Meissner and Pacinian corpuscles of the skin, which provide light touch and pressure perception respectively. The test with Semmes-Weinstein monofilaments evaluates touch sensation, being therefore related to

myelinated fibers. However, when there is no response to 4 to 300 g monofilaments, loss of protective pain sensation can be established. Pain perception is evoked by nociceptors and involves unmyelinated fibers of the skin (type C fibers). Therefore, Semmes-Weinstein monofilaments provide a liable alternative to prediction of early leprosy neuropathy. Accordingly, we compared results of Semmes-Weinstein monofilaments evaluation in TT, BB and LL patients with the expression of NF-L and PGP 9.5, in order to estimate the axonal degeneration in skin lesions of leprosy patients. Our findings did not allow us to correlate sensory evaluation with *in situ* expression of axonal markers and neurotrophins. However, immunohistochemistry demonstrated statistically significant reduction of NGF ( $p<0.05$ ) expression in borderline leprosy (BB) when compared to control specimens. Similar results were observed regarding the expression of PGP 9.5 (BB:  $p<0.001$  and LL:  $p<0.05$ ) and NF-L (LL:  $p<0.05$ ), suggesting higher Remak bundles degeneration in multibacillary leprosy. Likewise, Karanth et al.<sup>29</sup> observed impairment in PGP 9.5 and neurofilament immunoreactivity in leprosy lesions, mainly in tuberculoid patients. Moreover, the authors reported lack of neuropeptides in most of leprosy lesions. Reduction of neurofilament and PGP 9.5 immunostaining in nerve biopsies from pure neuritic leprosy was also demonstrated by Antunes et al.<sup>30</sup>, as well as its correlation with reduced potential amplitudes in electroneuro-myographic evaluation. Our results have shown positive correlation between p75<sup>NTR</sup> and PGP 9.5 ( $r=0.40$ ;  $p=0.01$ ), indicating the association between Schwann cells and axons in Remak bundles. Present data indicate alterations in neurotrophins, along with inflammatory process, as additional mechanisms involved in the establishment of peripheral nerve damage.

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