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Perivascular Innervation in the Nasal Mucosa and Clinical Findings in Patients with Allergic Rhinitis and Idiopathic Rhinitis

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Abstract

Introduction The nonspecific hyperreactivity of rhinitis has been attributed to neurotrophins activating sensory nerves and inflammatory cells. The relationship between these markers and the intensity of the symptoms is not well established and few studies have evaluated individuals with idiopathic rhinitis.

Objective The present study aims to evaluate whether perivascular innervation and nerve growth factor (NGF) are related to the intensity of the clinical conditions in allergic rhinitis (AR) and idiopathic rhinitis (IR).

Methods A total of 15 patients with AR and 15 patients with IR with the indication for inferior turbinectomy (associated or not with septoplasty) were selected. The patients received a score according to their signs and symptoms. After the surgery, we quantified eosinophils, mast cells, NGF, and nerve fibers in the nasal turbinate.

Results The score of the signs and symptoms was higher in the AR group. Nerve growth factor was found in the cytoplasm of inflammatory cells in the submucosa in greater quantity in the AR group. The nerve fibers were distributed throughout the tissue, mainly in the subepithelial, glandular, and vascular regions, and there was no difference between the groups. Greater perivascular innervation was associated with a higher signs and symptoms score.

Conclusions We concluded that these findings suggest that the NGF produced by submucosal inflammatory cells stimulates increased perivascular innervation in rhinitis, thus directly reflecting in more intense clinical conditions, especially in AR.

Keywords
- allergic rhinitis
- hyperreactivity
- innervation
- nerve growth factor
- neurotrophins

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Introduction

Allergic rhinitis (AR) is one of the most prevalent respiratory chronic diseases and generates a considerable socioeconomic impact on the adult and pediatric population.[1] Its general repercussions, such as impaired sleep, decreased work productivity and school performance, behavioral changes, and psychological damage cannot be underestimated. Patients with severe rhinitis experience a significant worsening in quality of life.[2]

Allergic rhinitis is a type I hypersensitivity reaction characterized by an inflammation in which eosinophils, immunoglobulin E (IgE), and inflammatory mediators responsible for the symptoms prevail. When individuals are exposed to the allergen, reactions involving inflammatory cells and inflammatory mediators (mainly histamine) result in symptoms.[3,4] These symptoms are presented quite rapidly by the sensitized individuals when exposed to the allergen even in small concentrations.[5] However, hyperreactivity to nonspecific stimuli also occurs. Agents such as odors, climate change, and pollution can trigger all the symptoms of rhinitis without acting directly as allergens. Nonspecific hyperreactivity is present in both allergic and nonallergic rhinitis[6] (NAR) and is related to several neural changes in the nasal mucosa.[7]

In light of this, in addition to the inflammatory process, neural changes regulate the symptoms of AR and some types of NAR. The autonomic nervous system acts on nasal physiology by decreasing air resistance and increasing nasal secretion through its sympathetic and parasympathetic pathways, respectively.[7] Some studies suggest the existence of an autonomic imbalance in idiopathic or vasomotor rhinitis, with inhibition of the nervous system response in the nose and hyperreactivity of the parasympathetic system.[8–10] Chronic inflammation leads to up-regulation of the neural components of the nose, which presents as neural hyperresponsiveness.[2,11] This alteration may involve several aspects of the nervous system, such as the afferent function of the sensory nerve endings, neural efferences, parasympathetic reflexes (primarily controlling the nose glands), sympathetic tone (primarily controlling arteriovenous anastomoses and vascular engorgement),[7] and even central neural sensitization.[12] An additional aspect of neural hyperresponsiveness might be the release of increased amounts of neuroinflammatory peptides by the sensory nerve endings leading to neurogenic inflammation.[2]

Changes in the nerve structure and function (neuronal plasticity) may contribute to the pathophysiology of AR and some types of NAR. Some authors suggest the involvement of neurotrophins in the activation of sensory nerves and the main cells in the inflammatory response of the upper airways.[13,14] One of the most important neurotrophins involved in this process is the nerve growth factor (NGF), which produces several effects on the nerves, including increased innervation density, terminal axonal branching, and dendritic arborization, as well as its interaction with neuropeptides and neurotransmitters.[15] Furthermore, NGF can be produced by eosinophils,[16] interacts with inflammatory cells,[15] and participates in airway remodeling.[13] In summary, NGF has been shown to induce biochemical and structural changes that can lead to hyperreactivity.[13,15]

Some authors have found an increase in NGF in patients with rhinitis,[17,18] as well as an increase in the density of innervation.[18,19] Ubiquitin C-terminal hydrolase L1 (UCH-L1 or UCHL) or protein product of gene 9.5 (PGP 9.5) is a panneuronal marker and has been used to demonstrate the presence of neurons in tissues. In AR, the number of PGP 9.5/UCHL positive nerve fibers is increased in the epithelium, the subepithelium, deeper mucous glands, and blood vessel regions.[18]

The neural process underlying AR and NAR has not been clarified yet. Recent literature has shown an increase in neural activity in the nasal mucosa of individuals with AR through neurotrophins, neuropeptides, nerve fibers, and sodium channels, which relate it to nonspecific hypersensitivity. However, research relating the amount of these markers to the intensity of symptoms is scarce, and few studies have evaluated individuals with IR.

Objective

The present study aims to evaluate whether perivascular innervation and NGF are related to the intensity of the clinical findings in AR and IR.

Methodology

The proposal was designed and submitted for consideration by the Ethics Committee for the Analysis of Research Proposals (CAPPesq) and received the Certificate of Presentation of Ethical Appreciation number 08887712.0.0000.0068, online registration number 9215, being approved under the number 190.339. Informed written consent was obtained from each participant.

Initially, we selected patients between 18 and 50 years old diagnosed with persistent rhinitis for >3 years, confirmed by clinical history and physical examination, without satisfactory improvement after appropriate clinical treatment, maintaining grade 2 or 3 nasal obstruction, according to the score of symptoms and signs proposed by Mello et al.[5] detailed in ►**Table 1**.

Patients with an indication for bilateral partial inferior turbinectomy, with or without septoplasty, were selected to participate in the study. Patients were divided into two groups: with AR or IR, according to their clinical history, physical examination, nasal cytology, and immediate hypersensitivity skin test for inhalants. Patients were evaluated according to the score of symptoms and signs proposed by Mello et al.[5] as shown in ►**Table 1**. We considered the immediate hypersensitivity skin test for inhalants as being positive when the papule was ≥ 3 mm. Individuals with other airway comorbidities, systemic diseases, nasal trauma, nose surgery, smokers, or illicit drug users were excluded. Medications for rhinitis such as corticosteroids and antihistamines, for example, were discontinued at least 2 weeks prior to surgery.

Table 1 Score of nasal signs and symptoms

Symptoms	Signs
Sneezing / itching 0- Absent 1- 1 to 4 per day / occasional itching 2- 5 to 10 per day / sporadic itching for 30 minutes 3- $\geq$ 11 / interferes with sleep and / or concentration	Nasal secretion 0- Absent 1- The mucosa appears moist 2- Visible secretion in turbinates or nasal floor 3- Profuse/draining
Runny nose 0- Absent 1- Cleaning 1 to 4 times a day 2- Cleaning 5 to 10 times a day 3- Constant cleaning	Color of the nasal turbinates 0- Rosy 1- Reddish / pale pink 2- Red / pale 3- Anemic/ bluish
Nasal obstruction 0- Absent 1- Small and not disturbing 2- Mouth breathing most of the day 3- Does not breathe through the nose / interferes with sleep, smell, or voice	Volume of the nasal turbinate 0- Normal 1- Hypertrophy of the inferior or middle turbinate with a small nasal block 2- Congestion compromising breathing in one or both nasal cavities 3- Congestion preventing breathing in one or both nasal cavities
Retro-nasal secretion 0- Absent 1- Feeling secretion in the throat 2- Frequent throat cleaning 3- Cough and discomfort when speaking	Posterior wall of the oropharynx 0- Normal 1- Slightly red 2- Hyperemic and apparent lymphoid follicles 3- Visible mucus

Adapted from Mello Jr 2002.

The diagnosis of IR was given to those patients who had other etiologies of rhinitis excluded, such as nonallergic eosinophilic rhinitis, gustatory, drug, hormonal/pregnancy, and occupational causes through analysis of clinical history, physical examination, nasal cytology, and negative immediate hypersensitivity skin test for inhalants (papule $<$ 3 mm).

Thirty patients were selected, 15 from each group. Bilateral partial inferior turbinectomy was performed with scissors and the fragments were immediately fixated in 4% paraformaldehyde in pH 7.0 phosphate buffer for 24 hours and transferred to 70% alcohol, cleaned in xylene, and fixated in paraffin for further processing for morphological studies. Staining was performed by using the hematoxylin-eosin method for quantification of eosinophils, toluidine blue staining for mast cells, and immunohistochemistry for NGF and UCHL (nerve fiber marker). Antibodies were titrated with the immunohistochemical technique for NGF and UCHL, yielding the concentrations values of the respective antibodies which were described in ►**Table 2**. Details of the technical methodology were described on ►**Appendix 1**.

The images were then digitized and analyzed by a computer software to quantify each component studied. For the quantification of the inflammatory cells in the tissue, digital images were obtained with the NIS Elements F 3.0 software; in a computer coupled to a Nikon Opitphot light microscope (Nikon, Tokyo, Japan) with a Nikon DXM1200F digital video camera (Nikon, Tokyo, Japan). Images of 12 fields per slide were obtained by using a 40x objective. To quantify eosinophils and mast cells by area, the images obtained were analyzed by using Image J software (National Institute of Health, Bethesda, MD, USA). For immunohistochemical analysis, the slides were observed by using a Leica ICC50 HD optical microscope (Leica Camera AG, Wetzlar, Germany) and digitized with the Leica Acquire system (Leica Camera AG, Wetzlar, Germany).

Regarding the study of perivascular UCHL, five to seven cut crosswise vessels were selected and the area of their adventitial layers was measured, as shown in ►**Figure 1**. Immunohistochemical staining was quantified in area density by using Image J software (National Institute of Health, Bethesda, MD, USA). The viable tissue area of each slide was divided by the number of elements to be recorded and the element (field or vessel) closest to the center was selected. Finally, to analyze the slides, the evaluators did not access the clinical data on the subjects. As this was an exploratory study, the sample size calculation had not been previously performed. Shapiro-Wilk tests were used to verify the performance of quantitative data regarding normality.

Table 2 Antibodies used in immunohistochemistry

Antibody	Description	Manufacturer	Dilution
PGP9,5/UCHL	MAB6007 Human UCH-L1 MAb (Clone 671108)	Bio-Techne Minneapolis, MN	1:1000
NGF	AF-256-NA Human beta-NGF Affinity Purified Polyclonal Ab	Bio-Techne Minneapolis, MN	1:20

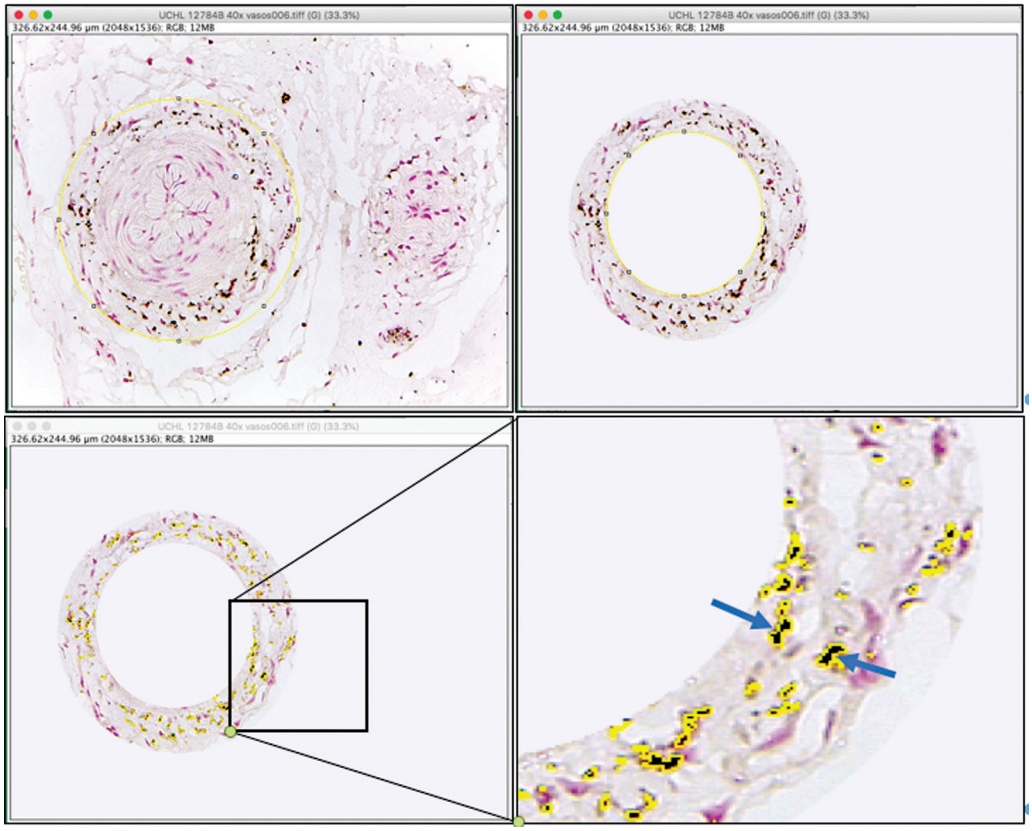

Fig. 1 Illustrative image of the quantification of perivascular innervation. Immunohistochemical stains are represented by dark areas highlighted in yellow, some others are by blue arrows.

When comparing the groups regarding the score of signs and symptoms, an extension of the Fisher exact test was used. To verify which variables would be associated with the severity of the disease, a multiple linear regression model was proposed. Among the existing independent variables, the computation of a new variable – named partial score – was proposed. For this variable, the values corresponding to the signs and symptoms that presented p-value ≤ 0.10 (after Bonferroni correction) were added to the individual association analyses. The purpose of computing this score was to verify the contribution of only those variables that had the biggest influence on the analyzed outcome. Therefore, the partial score was used as an outcome and the following variables, as initial predictors: gender, age, age at disease onset, types of rhinitis, tissue UCHL (density), perivascular UCHL (density), NGF (density), eosinophils per field, and mast cells per field. To choose the model predictors, the stepwise selection method was applied according to the Akaike Information Criterion (AIC).

Results

The groups of patients with IR and AR showed no statistical difference regarding gender, age, and age at onset of symptoms, as shown in ►**Table 3**. Nevertheless, patients with AR had a higher concentration of NGF, as shown in ►**Table 3**, whereas patients with IR had mast cells in greater quantity in the nasal turbinates. The other variables did not show significant differences between the compared samples.

When comparing the groups regarding the score of signs and symptoms, shown in ►**Table 4**, the sneezing/pruritus score was higher in the group of individuals with AR. This difference remained after Bonferroni correction ($p = 0.015$). Concerning runny nose, which was more prevalent in the group of individuals with AR, this difference was not sustained after correction for multiple tests.

Concerning the signs, the scores for the coloring of the turbinates and secretion were associated with the types of rhinitis, being higher in the group of individuals with AR. Both associations remained significant after Bonferroni correction ($p = 0.005$ and $p = 0.004$, respectively). The association between volume and types of rhinitis was not sustained after correction for multiple comparisons.

Nerve growth factor was observed in the cytoplasm of inflammatory cells distributed mainly across the submucosa, but some of it was present in the epithelial layer, as shown in ►**Figures 2** and **3**. The amount of UCHL positive nerve fibers, mainly arteries and arteriovenous anastomoses, was higher in the subepithelial region, close to the mucous glands and perivascular regions, as shown in ►**Figures 4** and **5**.

In the multiple linear regression model to verify which variables would be associated with disease severity, the predictors that remained in the regression model (AIC = 56.88) were gender, types of rhinitis, and perivascular

Table 3 Characterization of samples from patients with Idiopathic Rhinitis and Allergic Rhinitis

Variables	IR ($n = 15$)	AR ($n = 15$)	*p-value*
Gender[†]			
Women	7	6	1.000
Men	8	9	
Age[‡]	30.73 ± 8.61	26.13 ± 7.51	0.114
Age at onset[‡]	16.60 ± 12.25	9.27 ± 9.59	0.088
Tissue UCHL (density)[§]	0.004 ± 0.002	0.005 ± 0.002	0.143
Perivascular UCHL (density)[‡]	0.016 ± 0.007	0.024 ± 0.012	0.074
NGF (density)[‡]	**1.6e-03 ± 1.4e-03**	**2.0e-03 ± 6.4e-04**	**0.026***
Eosinophil per field[‡]	6.0e-06 ± 4.8e-06	8.1e-06 ± 6.1e-06	0.285
Mast cell per field[‡]	**3.0e-05 ± 1.8e-05**	**1.8e-05 ± 1.1e-05**	**0.026***

Abbreviations: AR, allergic rhinitis; IR, idiopathic rhinitis; NGF, nerve growth factor; UCHL, ubiquitin c-terminal hydrolase.
Quantitative variables are described as mean ± standard deviation
[†]Pearson chi-squared test
[‡]Wilcoxon test
[§]Student t-tests
*statistically significant p-value

Table 4 Association between symptoms and signs and types of rhinitis (IR and AR)

Variables	Fisher Exact Test	*p-value*	*p-value* (post-Bonferroni correction)
Symptoms			
Sneezing /itching	**13.975**	**0.002**	**0.015***
Runny nose	10.118	0.010	0.079
Nasal obstruction	–	1.000	1.000
Retronasal secretion	3.768	0.260	1.000
Signs			
Color	**14.618**	**0.001**	**0.005***
Secretion	**14.511**	**0.001**	**0.004***
Volume	8.785	0.011	0.090
Oropharynx	3.643	0.139	1.000

*statistically significant p-value after Bonferroni correction

UCHL (density). The partial score variable included the sum of the symptoms of sneezing/pruritus and runny nose and the signs of coloring, secretion, and volume. The betas and the respective p-values are shown in ►**Table 5**.

The effects of perivascular UCHL predictors and type of rhinitis on the partial score are shown in ►**Figure 6**. We can see that higher values of perivascular UCHL density are associated with higher partial scores. The regression showed an $R2 = 0.62$ and, in the verification of the dispersion of the residues, no problems of heteroscedasticity were found, as well as no outliers were detected by the analysis of RStudent residues.

Discussion

We have demonstrated in the present study that increased perivascular innervation in the submucosa of the turbinates is related to greater severity of rhinitis, both in IR and AR. This finding is corroborated by the presence of a higher concentration of NGF in the inferior turbinates of patients with AR, precisely the group with the highest score of signs and symptoms. Hence the relevance of the present study, which is the first one to correlate the innervation of the nasal mucosa with the clinical conditions of rhinitis.

Generally, immunohistochemical analysis is performed on fresh material submitted to freezing. In the present study, however, we used material previously collected in another study, which was embedded in paraffin. In such a process, the formalin fixation step may generate possible artifacts that cause a more fragmented appearance of the nerve fibers, even though it does not affect the measurement of innervation density.[20] Nerve fiber count is used in peripheral neuropathies and has a pre-established standard of

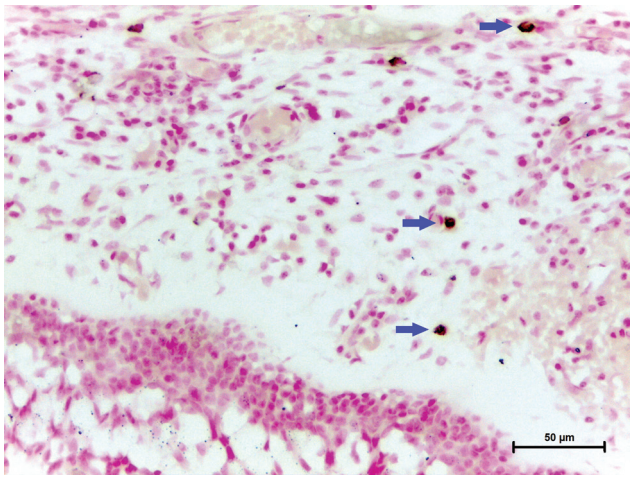

Fig. 2 Photomicroscopy showing immunohistochemical staining for NGF in the nasal turbinate of a patient with IR (40x objective). Inflammatory cells with immunohistochemical staining for NGF are marked with blue arrows.

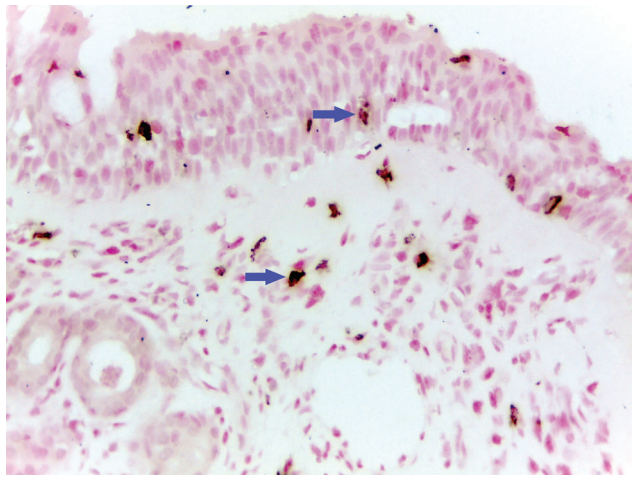

Fig. 3 Photomicroscopy showing immunohistochemical staining for NGF in the nasal turbinate of a patient with AR (40x objective). Inflammatory cells with immunohistochemical staining for NGF; some are marked with blue arrows.

normality.[20] As the objective of the present study was to verify the density of innervation, compare the groups, and correlate with the clinical findings, this methodology appeared to be more suitable.

The absence of statistical difference between the groups in terms of gender, age, and age at onset of symptoms allowed for a more suitable comparison between them. The onset of symptoms for AR is during childhood, while for NAR the onset is later on, usually, after 20 years old.[21] The averages found in the present study were 9.27 years old for the RA group and 16.6 years old for the IR group, but there was no statistical difference between them.

The clinical evaluation through the score of signs and symptoms made it possible to individually assess each clinical finding to compare the groups. Allergic patients had more intense symptoms.[21,22] According to the inclusion criteria, the patients had a high score for nasal obstruction (surgical indication), so it was decided to adjust the partial score to increase the sensitivity of the statistical tests. The high score for nasal obstruction (indication for surgery/inclusion

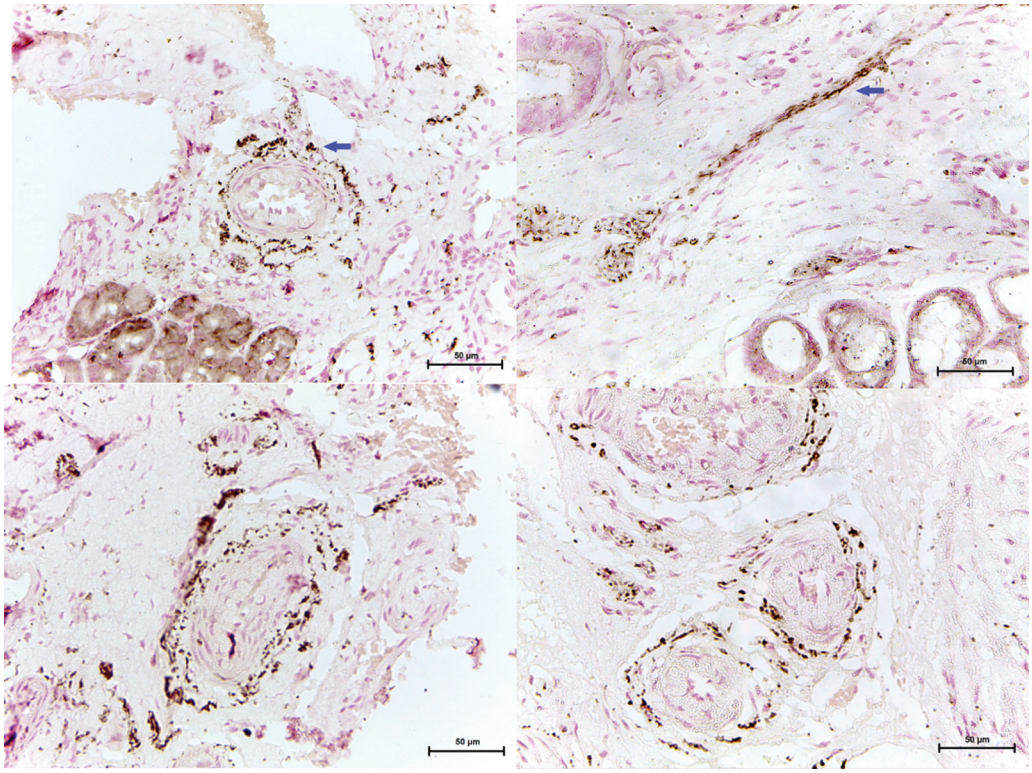

Fig. 4 Photomicroscopy showing the immunohistochemical staining for UCHL (high-density level) in the nasal turbinate of individuals with AR (40x objective). Immunohistochemical staining for UCHL is shown in the dark areas; some are marked with blue arrows.

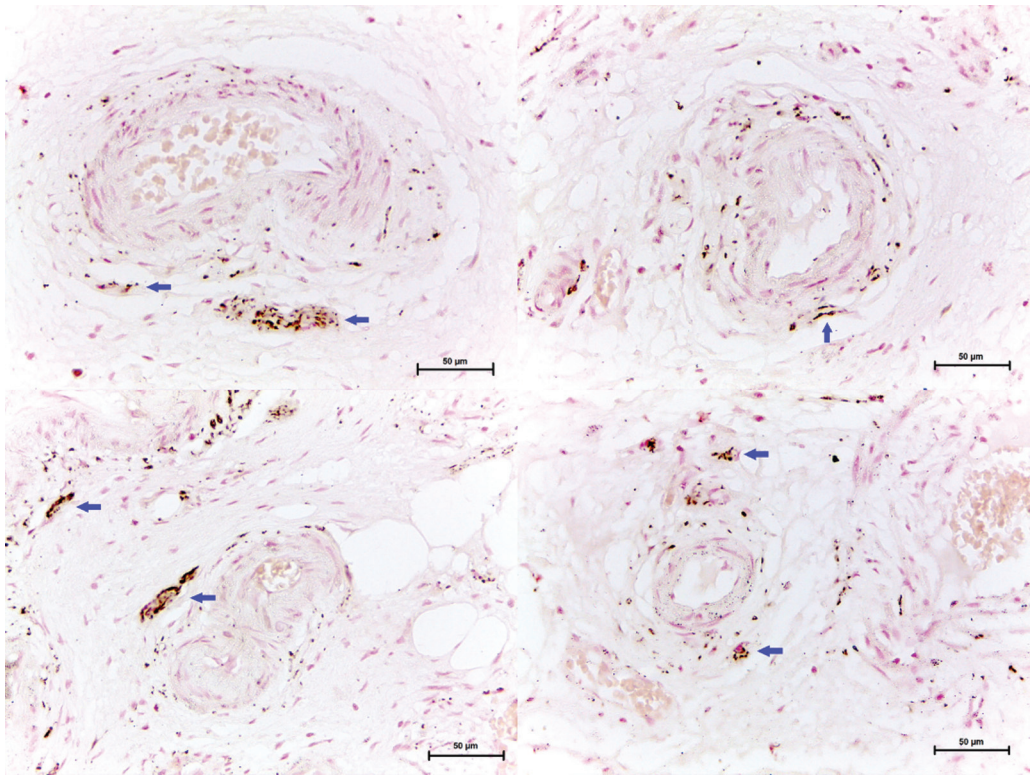

Fig. 5 Photomicroscopy of the immunohistochemical staining for UCHL (low-density level) in the nasal turbinate of individuals with IR (40x objective). Immunohistochemical staining for UCHL is shown in the dark areas; some are marked with blue arrows.

criteria) reflects the high score for turbinate volume in both groups. This fact may explain why the association between volume and types of rhinitis was not sustained after correction for multiple comparisons.

The presence of NGF in tissues regulates axonal growth, synapse formation, and neural plasticity; it also influences the synthesis of neurotransmitters and neuropeptides.[15] In addition, it has been shown to induce biochemical as well as structural changes in the nerves that can lead to hyperreactivity.[13,15] Human eosinophils are capable of producing, storing, and releasing NGF.[16] The literature presents some disputes regarding its location and predominance in allergic or nonallergic patients. For instance, NGF has been found in inflammatory cells in the submucosa and with higher values in allergic subjects.[17] However, another study found NGF in the epithelial layer and with higher values in nonallergic individuals.[18] Yet, in a third study, NGF was higher in the groups with AR and IR when compared with the control group, but they did not differ from each other.[23] Finally, in the present study, NGF was found in the cytoplasm of inflammatory cells in the submucosa of the turbinate and

Table 5 Multiple Linear Regression of disease severity (partial score) according to gender, types of rhinitis, and perivascular UCHL (density)

Variables	Beta	95%CI	*p-value*
Gender	-1.872	-3.71 – -0.03	0.047*
Types of rhinitis	4.222	2.25 – 6.19	0.0001*
Perivascular UCHL (density)	114.147	22.22 – 206.07	0.017*

Abbreviations: CI, confidence interval; UCHL, ubiquitin c-terminal hydrolase.
*statistically significant p-value

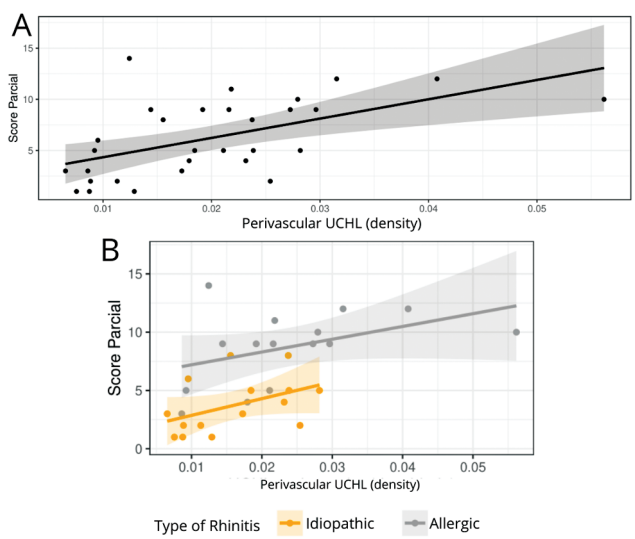

Fig. 6 Multiple Linear Regression for partial score. UCHL, *Ubiquitin C-Terminal Hydrolase.* Multiple Linear Regression for the partial score. **A** Marginal effect of perivascular UCHL (density) on the partial score. **B** Effect of perivascular UCHL (density) on the partial score by type of rhinitis.

with a larger value in allergic ones. We found few marked cells in the epithelial layer.

The literature has demonstrated the presence of NGF in the inflammatory cells of the nasal mucosa of patients with rhinitis. The local release of this neurotrophin may justify the increase in local innervation. Furthermore, it supports NGF as an important mediator between the nervous and immune systems. There is a greater vascular innervation of turbinates in children with symptoms of rhinitis when compared with children without rhinitis.[24] The group of patients with rhinitis was not evaluated for the presence of allergy or details of symptoms (predominant symptoms or intensity). The arteries and arteriovenous anastomoses of individuals with rhinitis had greater innervation than the blood vessels of those without rhinitis.[24]

Individuals with AR and controls without rhinitis have nerve fibers throughout the subepithelial region, predominantly in the glandular and vascular region, and some fibers penetrating the epithelial region.[19] Innervation is greater in subjects with AR when compared with healthy subjects.[19] A study comparing three groups (control, allergic, and nonallergic rhinitis) found the same distribution in patients with rhinitis; both allergic and nonallergic ones had greater innervation.[18] In the present study, we also found innervation following the same distribution and we did not find any differences between the groups.

The literature shows that the greater the nasal innervation, the greater the nasal sensitivity (nasal mucosa/inferior concha).[19] Consequently, finding augmented sensitivity and nerve fibers penetrating the epithelium[19] suggests greater afferent (sensory) innervation in AR. The presence of increased perivascular innervation in patients with rhinitis[24] suggests greater efferent innervation. In the present study, the increased perivascular innervation was related to greater disease severity. These findings corroborate that enlarged innervation of the nasal mucosa, including the efferent and afferent nerves, contributes to the symptoms of rhinitis.

The eosinophil by field variable was removed from the multiple linear and logistic regression analyses, since its inclusion before the application of the stepwise selection method generated a nonconvergence of the algorithm, taking all the probabilities of the covariates to 1. When analyzing the eosinophilic variable by field in the univariate regression models, it did not present significant p-values, neither in the multiple linear regression ($B = 3.89e+05$; $p = 0.350$) nor in the logistic regression ($B = 1.86e+04$; $p = 0.307$). Therefore, we decided not to include this variable in the complete models for the selection of predictors.

In the present study, no difference was shown in tissue eosinophils. Eosinophils are the main inflammatory cells involved in AR, but they can be present in other types of rhinitis. Gelardi et al.[25] described the subtypes of NAR based on the predominant cells found in the nasal cytological examination. They established three main types of NAR: eosinophil-infiltrated NAR (NARES), mast cell-infiltration (NARMA), and neutrophil-infiltration (NARNE). Additionally, a particular type has been characterized by infiltration by eosinophils and mast cells (NARESMA). Therefore, the large number of mast cells in the group with IR and the absence of difference in eosinophils could be explained by the possible presence of such subtypes of NAR not detected by the nasal cytology. Patients with a predominance of NARESMA had worse quality of life and reported greater nasal obstruction.[25] Due to the inclusion criteria used (persistent nasal obstruction) in the present study, we did not find a relationship between the number of inflammatory cells and the severity of the disease.

The limitation of the present study, for ethical reasons, was the need to include only patients with surgical indications of the turbinates. Therefore, they presented severe nasal obstruction, which makes it difficult to correlate this symptom with the histological findings. Research that can assess the histology of the turbinates of patients with different degrees of nasal obstruction may contribute to a better understanding of this relationship.

Conclusions

We conclude that the results suggest that NGF produced by the inflammatory cells of the submucosa stimulates the increase of perivascular innervation in rhinitis, directly reflecting in more intense clinical conditions, especially in AR.

Contributions of the Authors

Carvalho T.: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, visualization, writing – original draft preparation; Mello Jr J. F.: Conceptualization, project administration, supervision, writing – review and editing; Caldini E. T. E. G.: Conceptualization, Investigation, Methodology, Resources, Supervision; Salgado D. C.: Data Curation, resources; Carvalho N. M. G.: Data Curation, investigation; Damaceno-Rodrigues N. R.: Data curation, investigation, methodology, resources; Richard Louis Voegels R. L.: Funding acquisition, project administration, supervision, writing – review and editing.

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Conflict of Interests

The authors have no conflict of interests to declare.

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Appendix 1: Technical Methodology

The inferior turbinate fragments were immediately fixed after the surgery in 4% paraformaldehyde in pH 7.0 phosphate buffer for 24 hours and transferred to 70° alcohol for further processing for morphological studies. The materials already fixed were dehydrated in an alcohol gradient (95° to 100°), diaphanized in xylene and embedded in paraffin. 5-µm sections were obtained from the blocks containing the fragments in a Leica RM2245 microtome, stretched in a water bath at 37°C and collected on silanized slides.

For staining by the Hematoxylin-eosin method, the histological sections were deparaffinized in xylene, hydrated in an alcohol gradient (100° to 70°) and water, and stained for 1 minute with Harris Hematoxylin. Subsequently, the sections were washed in running water and counterstained with eosin for 10 minutes; then, they were washed in running water, dehydrated in an alcohol gradient (95° and 100°), diaphanized in xylene and mounted with a coverslip and *entellan* for microscopic analysis.

Immunohistochemistry

In order to obtain the best staining, antibodies were titrated using the immunohistochemistry technique. The knowledge of the titers allows a more accurate and detailed analysis of slides.

Nasal turbinate sections from patients with rhinitis were placed on silanized slides (Sigma Chemical Co. St. Louis, MO, USA) on appropriate support. The deparaffinization process was carried out by placing the slides in hot xylene in an oven at 60 to 65°C for 5 minutes and then in 3 cold xylene baths. For hydration of the sections, the slides were placed in 2 baths of absolute alcohol, a bath of alcohol 95° and a bath of alcohol 70°. Then, they were washed in running water and deionized water and left in phosphate buffer pH 7.4 (PBS).

The recovery of antigenic sites was performed at high temperature in citrate pH 6 for Beta NGF and UCHL. Blocking of endogenous peroxidase was performed with 10v hydrogen peroxide (3%), then the slides were washed in running water, distilled water and left in tris-phosphate buffer pH 7.4 (TBS). Blocking of nonspecific proteins was performed with Ultra V Block (THERMO Scientific) for 30 minutes at room temperature. Antibodies were diluted at the following concentrations: UCHL 1:1000 and Beta NGF 1:20.

The slides were incubated overnight at 4oC in a humid chamber. Subsequently, the incubation was performed with the secondary antibody Polymer ImPRESS HRP Polymer (Vector Laboratories, Burlingame, CA, USA) for 30 minutes in an oven at 37°C. Diaminobenzidine (DAB) (Sigma-Adrich Steinheim, Germany) was used as the chromogen. Subsequently, counterstaining with Harris Hematoxylin (Merck, Darmstadt, Germany) was performed.

