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## The human leukocyte antigen G molecule (HLA-G) expression in patients with nasal polyposis<sup>☆</sup>

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### KEYWORDS

Nasal polyps;  
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### Abstract

**Introduction:** Sinonasal polyposis (NP) is a chronic inflammatory pathology of the nasal/paranasal cavities which affects from 1%-4% of the population. Although polyps seem to be a manifestation of chronic inflammation in both allergic and non-allergic subjects, the pathogenesis of nasal polyposis remains unknown. HLA-G molecules are a kind of no classic class I antigen with anti-inflammatory and tolerogenic properties. Little attention has been paid to the role of HLA-G chronic inflammatory disorders.

**Objective:** The aim of this study is to investigate the expression of HLA-G in the NP.

**Materials and methods:** Prospective study involving samples of patients presenting with nasal polyposis that were subjected to the immunohistochemistry technique. After a skin prick test, all patients were divided into atopic and nonatopic groups and classified as asthmatic or non-asthmatic.

**Results:** Immunohistochemical staining demonstrated a higher expression of the HLA-G molecule in samples from nonatopic than in those from atopic patients, and was significantly lower in the non-asthmatic patients.

**Conclusion:** These results indicate that HLA-G may play an important role in the pathology of nasal polyposis. Considering the anti-inflammatory properties of HLA-G, this study suggests that it could reduce susceptibility to atopy and asthma.

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### PALAVRAS-CHAVE

Pólipos nasais;  
Alergia e imunologia;  
Antígenos HLA-G

### A expressão do antígeno humano leucocitário G (HLA-G) em pacientes com polipose nasossinusal

### Resumo

**Introdução:** Polipose nasossinusal (PNS) é uma patologia inflamatória crônica das cavidades nasais/paranasais que afeta 1%-4% da população. Embora os pólipos pareçam ser uma manifes-

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tação de inflamação crônica em ambos os indivíduos alérgicos e não alérgicos, a patogênese da polipose nasal permanece desconhecida. Moléculas HLA-G são antígenos não clássicos da classe I com propriedades anti-inflamatórias e tolerogênicas. Pouca atenção tem sido dada ao papel do HLA-G em doenças inflamatórias crônicas.

**Objetivo:** Investigar a expressão de HLA-G na PNS.

**Materiais e métodos:** Estudo prospectivo de pacientes com polipose nasal que foram submetidas à técnica de imuno-histoquímica. Após realizarem teste cutâneo, os pacientes foram divididos em grupos atópicos e não atópicos e classificados como asmáticos ou não asmáticos.

**Resultado:** A coloração imuno-histoquímica mostrou uma maior expressão da molécula HLA-G em pacientes não atópicos do que naqueles atópicos e foi significativamente inferior nos pacientes não asmáticos.

**Conclusão:** Os resultados indicam que o HLA-G pode ter um papel importante na patologia da polipose nasal. Considerando as propriedades anti-inflamatórias do HLA-G, este estudo sugere que ele poderia reduzir a susceptibilidade a atopia e asma.

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## Introduction

Sinonasal polyposis (NP) or chronic rhinosinusitis with nasal polyps is a chronic inflammatory pathology of the nasal and paranasal cavities,<sup>1,2</sup> which affects from one to four per cent of the population and has a clear association with asthma, aspirin sensitivity and cystic fibrosis.<sup>3</sup>

Patients with NP typically present with nasal obstruction, rhinorrhea, hyposmia and reduced quality of life.<sup>3,4</sup> Although polyps seem to be a manifestation of the chronic inflammation of nasal/paranasal sinus mucosa in both allergic and non-allergic subjects, the pathogenesis of nasal polyposis remains unknown,<sup>5,6</sup> but it is probably a multifactorial disease with several different etiological factors, and chronic persistent inflammation is undoubtedly a major factor irrespective of the etiology.<sup>5</sup> Chronic inflammation of the mucous represents a challenge for the otolaryngologist.

The diagnosis of NP is confirmed by nasal endoscopy or a computed tomography (CT) scan.<sup>1</sup> Despite the major impact on quality of life,<sup>7</sup> in the literature little attention has been paid to the biomarkers involved in the pathogenesis of nasal polyps and their possible contributions to the prognosis of NP.

The HLA-G is a nonclassical MHC class I molecule characterized by its restricted expression in tissues (cytotrophoblast, amnion epithelial cells of the thymus, pancreas, and in the proximal nail matrix), its low polymorphism as well as its seven isoforms (HLA G1 -G7).<sup>8,9</sup> The expression of HLA-G is induced by interleukin-10 or gamma interferon and exerts a negative immunoregulatory effect by inhibiting the proliferation of allogeneic T cells, natural killer cells and antigen-specific cytotoxic T cells. Because of its effects on the immune system, this expression can have beneficial as well as harmful consequences for the organism.<sup>9,10</sup>

Little attention has been paid to the role of HLA-G in autoimmune and chronic inflammatory disorders, but evidence shows that the expression of HLA-G in inflammatory skin diseases including psoriasis and atopic dermatitis<sup>11</sup> is associated with a better clinical course of the disease.<sup>3,9</sup> Another study suggests that serum HLA-G might be considered a biomarker for atopic asthmatic patients.<sup>12</sup> In cancer, HLA-G expression has been associated with metastasis development.<sup>13</sup>

Considering this lack of understanding of the mechanism which triggers inflammation and which hinders the devel-

opment of new treatments for this disease, and the importance of the characterization of inflammatory mediators involved in the pathogenesis of NP, the objective of this study was to investigate the HLA-G expression in NP in atopic and non-atopic patients. There are no studies about the HLA-G *in situ* expression in the NP lesions.

## Methods

### Study population

This was a historical cohort with cross-sectional study in which twenty-five patients, who presented with sinonasal polyposis and who had been submitted to surgery for polyp resection, were evaluated. We also evaluated five normal mucosa samples from the middle meatus of patients without symptoms of atopy that were submitted to rhinoseptoplasty. All patients underwent clinical otorhinolaryngologic evaluation with a nasal endoscopy, computed tomography (CT) scan, lung function test (spirometry) and the skin prick test before surgery. The preoperative CT scans were graded according to the Kennedy classification, to evaluate the extent of patients with NP,<sup>10</sup> and all were investigated for allergy and positive history for asthma. They were divided in two groups: atopic (positive skin prick test) and non-atopic patients (negative skin prick test). The skin prick test was performed with 10 extracts: histamine (positive control), saline solution (negative control), *D. pteronyssinus*, *D. farinae*, *Blomia tropicalis*, *Felix domesticus*, *Canis familiars*, *P. Americana*, *Aspergillus fumigatus* and *Alternaria alternate*. The response was considered positive when there was a halo of 3 mm larger than that of the negative control.

The NP patients were classified as having positive or negative history for asthma, which was confirmed by spirometry. This study was approved by the Research Ethics Committee under the protocol 0711/11 and approved in 06/02/11. All patients signed a term of informed consent for participation in the study. The financial support of this study was National Counsel of Technological and Scientific Development (CNPq) and Foundation for Research Support of the State of Goiás (FAPEG).

## Immunohistochemistry

After resection surgery, the polyps were subjected to the immunohistochemistry technique. Four-micrometer sections were cut from paraffin-embedded specimens. The Universal HRP-Polymer MACH 4 detection system (Biocare Medical, Concord, CA, USA) was used. In summary, after rinsing the sections in phosphate buffered saline with 0.1% saponin, endogenous peroxidases were inhibited using  $H_2O_2$ . Samples were initially incubated with specific or irrelevant antibodies for 1 hour at room temperature and subsequently with a solution containing a MACH 4 Mouse Probe for 15 minutes. Diaminobenzidine plus a chromogen-substrate was used to develop antibody fixation. The specific monoclonal antibodies MEM-G/2 (Exbio, Prague, Czech Republic) recognize the free heavy-chain of all HLA-G isoforms. An identical IgG1 isotype anti-desmin antibody which was run simultaneously with each sample served as a negative control.

## Evaluation of stained sections

The paraffin-embedded polyp specimens were stained with haematoxylin-eosin-safranin for histological examination. The percentage of eosinophilic inflammatory infiltrate was scored as follows: 0, no inflammatory cells; 1, < 25% inflammatory cells; and 2,  $\geq$  25% inflammatory cells.

The immunohistochemical analysis was carried out on polyp tissue. Immunoreactivity was scored using a semi-quantitative scoring method by evaluating the percentage of positive cells. The cut-off scores for determining the positivity of HLA-G detected by immunohistochemistry were obtained by the receiver operating characteristic (ROC) curve analysis. ROC curve analysis was performed for HLA-G expression. This was the first study to demonstrate the expression of HLA-G in polyposis nasinal. There are no references in the specialized literature about the value to be considered as positive and negative in ROC curve. However, as is well established in studies on various types of cancers, any rate of expression of this molecule has consequences in the tumor microenvironment, inducing immunomodulation. Our goal was to calculate the ROC curve, so we used standard statistical test to establish the cut-off to produce greater sensitivity and specificity, although we know that even an expression less than 20% of cells has the potential to provide immunomodulatory effects.

All sections were blindly analyzed using a light microscope with high-power fields ( $\times 400$ ). Ten random fields were chosen. Labeling of cells and other structures, as well as some of the epithelial cells and inflammatory cells were not observed. It was considered, to establish the percentage of positive cells, the average HLA-G positive cells among the total epithelial cells per field. The average HLA-G positive cells in the ten fields analyzed was calculated to obtain the final value.

## Statistical analysis

The ROC curve of staining performance for the determination of HLA-G cut-off expression was performed. The immunostaining scores were compared to the Mann-Whitney U test, and the correlations of the immunostaining scores were tested with Spearman correlation analysis.

Comparative analysis between the groups were performed by the two-sided Fisher exact test. A p-value of less than 0.05 was considered significant. All statistical analysis were performed using the GraphPad InStat (version 5.0).

## Results

### Clinical and epidemiological findings

The results included 25 patients with sinonasal polyposis submitted to nasal endoscopy and CT scan to confirm the disease. The patient group consisted of 13 (52%) males and 12 (48%) females aged between 35 and 83 (mean: 48.8 years). The patients are divided into two groups: the group with a negative prick test (non-atopic group) consisted of 13 patients and the other group consisted of 12 patients with positive prick test (atopic group). Among these patients, 17 presented asthma.

Using CT-scan evaluation, patients were classified according to the criteria described in Kennedy et al. to evaluate the extent of NP. Most patients were classified as grade II.

### The expression of HLA-G

Following histological examination, HLA-G staining was observed in epithelial and inflammatory cells in NP samples (Fig. 1). HLA-G was not seen in normal mucosa samples.

On ROC curve analysis, the cut-off for positive results was 20% or greater (AUC = 0,750;  $p = 0,011$ ; 95% CI 0,516 to 0,910). Considering that, HLA-G molecules could be detected in 9 out the 25 specimens evaluated (36%).

HLA-G expression in NP specimens was associated with atopy, asthma and the presence of eosinophilia on polyps.

Immunohistochemical staining demonstrated a higher expression of HLA-G molecule in samples of non-atopic patients when compared with atopic patients ( $p = 0.028$ ). It was observed that among the HLA-G positive specimens, only 2 patients presented atopy ( $p = 0.0154$ ; RR = 0.2727; 95% CI, 0.07606 to 0.9779).

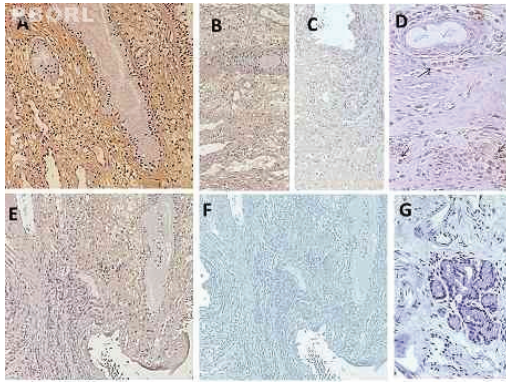
The HLA-G expression was significantly higher in the group of non-asthmatic patients ( $p = 0,005$ , and 95% CI 0,01304 to 0,6791). Only 1 of the 9 HLA-G + patients presented asthma.

The patients whose prick test was positive had lower percentage of HLA-G positive cells when compared with patients whose prick test was negative ( $p = 0.03$ ) (Fig. 2).

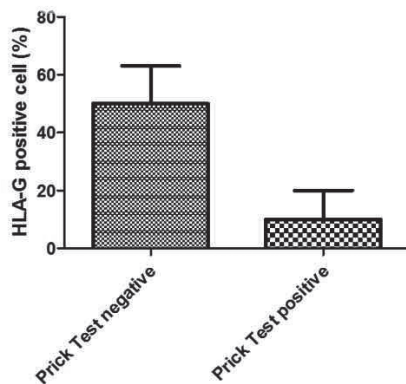
Finally, comparing the expression of HLA-G with eosinophilia found in SN, we observed that HLA-G positive patients presented lower eosinophilia (Fig. 3).

## Discussion

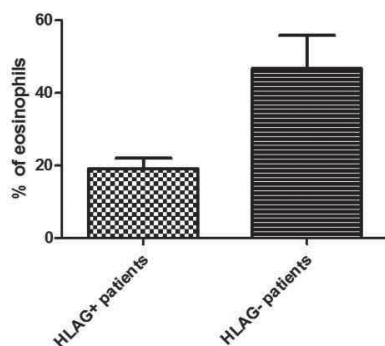
NP is a chronic inflammatory condition associated with substantial impaired quality of life, reduced workplace productivity, and considerable medical treatment costs.<sup>7,14</sup> Despite recent research evidence contributing to further



**Figure 1** HLA-G protein detection SNP biopsies. Photomicrograph showing different categories of immunolabeling intensity. A, Strong staining by epithelial cells; B, Moderately and C, weak staining; D, Expression of HLA-G by immune-inflammatory cells; E, Positive SNP patient sample to HLA-G expression and F, the isotype control (irrelevant antibody); G, Negative sample to HLA-G expression. Original magnification 10 $\times$  (B,C and F), 20 $\times$  (A) and 40 $\times$  (D and G).



**Figure 2** HLA-G expression and Prick Test positivity. Prick Test positive patients presented lower percentage of HLA-G positive cells when compared with patients whose Prick test result was negative ( $p = 0.03$ ).



**Figure 3** Eosinophilia and HLA-G expression. Inverse relationship between eosinophilia and expression of HLA-G. HLA-G positive patients presented lower eosinophilia (Mann Whitney test,  $p = 0.0330$ ; Spearman correlation  $r, p = 0.0126$ ).

comprehension of the pathophysiology of this chronic airway condition, the pathogenesis of SNP remains poorly understood and appears to be multifactorial, being associated with conditions such as atopy, asthma, cystic fibrosis, aspirin sensitivity and chronic rhinosinusitis.<sup>15-17</sup>

A diverse spectrum of alterations involving T-cell patterns, cytokine profiles, IgE production, microorganisms and immune system malfunctions should be associated with NP pathogenesis.<sup>9,10</sup> In this context, this study evaluated the HLA-G expression in NP lesions for the first time.

This analysis showed that the HLA-G was expressed in 9 patients with NP. There was no HLA-G expression in normal mucosa. Interestingly, the HLA-G expression tended to be significantly more frequent in patients with negative prick test, or in other words, non-atopic patients ( $p = 0.028$ ). In contrast to these results, Ciprandi et al. showed that serum HLA-G (s-HLA-G) molecules are significantly increased in allergic rhinitis, but they conducted their study with allergic adults and children without polyposis, and worked with the serum expression.<sup>18,19</sup> More recently, HLA-G has again been shown to have increased expression in several immunological diseases including asthma and allergic rhinitis.<sup>20</sup> Based on the contrasting results of this study, it can be speculated that the expression of HLA-G in NP may contribute to decreased susceptibility to atopy in NP, a factor which aggravates the disease.

A significant association between HLA-G expression and the absence of asthma was also seen, which could reinforce the thesis that the presence of this HLA-G could be a marker for a better prognosis of SNP. Although it has been hypothesized that HLA-G is associated with the pathogenesis of asthma, these results remain controversial. Zhen et al. have suggested that sHLA-G might be considered as a biomarker for atopic asthmatic patients, and have shown that an increase of sHLA-G with decreased IL-10 levels may have implications for the pathogenesis of atopic asthma.<sup>20,21</sup> But the authors of this study saw that non-asthmatic and non-atopic patients presented a significantly higher expression of HLA-G. It must be considered that the groups were very different, because all the patients in this study had SNP, and the presence of atopy and asthma or otherwise are adjuvant factors which can aggravate the disease.

A role for HLA-G in asthma pathogenesis was further suggested by the demonstration of the expression of a soluble isoform of HLA-G, sHLA-G5, in airway epithelial cells.<sup>22</sup> Localization of HLA-G in airway epithelium suggests that its deregulation could contribute to airway inflammation in chronic asthma. The results of this study are consistent with previous reports by Rizzo et al. of the defective production of soluble HLA-G molecules by peripheral blood monocytes in patients with asthma.<sup>23</sup> HLA-G could regulate the inflammatory activity of inflammatory cells found in asthmatic airways and influence asthma susceptibility through atopic pathways.

Histologically, nasal polyps can be classified into eosinophilic and non-eosinophilic. Eosinophilic NP represents 80% to 90% of SNP,<sup>23,24</sup> characterized by massive edema and the accumulation of eosinophils.<sup>25</sup> The abundance of eosinophils in nasal polyps would seem to be explained by the increased migration of eosinophils into tissues and increased survival of these cells,<sup>26</sup> but the pathological mechanism through which these eosinophils contribute to tissue damage, to the

inflammatory process and the formation of polyps is not well understood.<sup>27</sup>

Eosinophils may contribute to nasal polyp formation and growth, not only through inflammation, but also by exerting their effects on extracellular matrix including the stimulation of collagen synthesis. In a similar vein, another interesting finding of this study was the inverse relationship between eosinophilia and HLA-G expression.<sup>28,29</sup> HLA-G positive polyps had a lower eosinophilia when compared with HLA-G negative polyps. This suggests that HLA-G has anti-inflammatory properties.

To sum up, this study has demonstrated, for the first time, the expression of HLA-G in NP. It was significantly higher in non-atopic and non-asthmatic patients, suggesting the HLA-G could inhibit the inflammatory response in NP.

Further investigations would provide deeper insights into the role that the HLA-G expression plays in the NP pathogenesis.

## Conclusion

The results of this study indicated that HLA-G may play an important role in the pathology of sinonasal polyposis. After considering the anti-inflammatory properties of HLA-G, these authors suggest that HLA-G could decrease susceptibility to atopy and asthma in NP.

## Conflicts of interest

The authors declare no conflicts of interest.

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