Angiogenesis and eosinophilia in the nasal mucosa of patients with different clinical phenotypes of chronic rhinosinusitis

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Abstract

Introduction: Dense inflammatory cell infiltration and vascularization of the nasal mucosa are histological characteristics of chronic rhinosinusitis (CRS). We aimed to evaluate the association between eosinophilia and vascularization in the stroma of mucosal layer/nasal polyps (NP) and clinical parameters in patients with different phenotypes of CRS.

Methodology: This cross-sectional study involved 33 patients who had CRS with NP without aspirin sensitivity (CRSwNP), 20 NP patients as a part of aspirin-exacerbated respiratory disease (AERD), and 10 patients who had CRS without NP (CRSsNP), selected for surgery. Control group consisted of 31 subjects without nasal/sinus inflammation, selected for surgery of pneumatized middle turbinate. All patients were clinically scored before surgery for nasal symptoms, quality of life (QoL) outcome and findings from computed tomography scans. NP/nasal mucosa samples of participants were immunohistochemically stained for eosinophil infiltration marker BMK13 and angiogenesis markers CD31 and CD34.

Results: AERD patients had the highest level of immunoexpression for BMK13. The strongest staining pattern of CD34 was found in AERD group and the highest expression level for CD31 in CRSwNP group. We found a positive correlation between BMK13, impaired QoL and radiologically evaluated disease extent in patients with CRSwNP. Excepting CRSsNP patients, no correlation was found between the marker of tissue eosinophilia and markers of vascular proliferation.

Conclusions: Patients from AERD phenotype have the highest degree of stromal eosinophilic infiltration and endothelial proliferation in comparison to other CRS phenotypes. Eosinophil infiltration marker BMK13 correlates better with the clinical parameters of CRS in comparison to the vascular proliferation markers.

Key words: endothelium; eosinophils; immunohistochemistry; inflammation; nasal polyps; sinusitis.

J Infect Dev Ctries 2023; 17(10):1480-1488. doi:10.3855/jidc.18166

(Received 03 March 2023 – Accepted 21 March 2023)

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Introduction

Chronic rhinosinusitis (CRS) is inflammation of the mucous membrane of the nose and paranasal sinuses with symptoms and local findings lasting longer than twelve weeks [1,2]. Etiology and pathogenesis have not been sufficiently investigated, although various factors including repeated bacterial, viral and fungal infections, staphylococcal enterotoxins as superantigens, biofilms, allergies, air pollution, impaired arachidonic acid metabolism, impaired mucociliary transport and weakened immune mechanisms are still the subject of research [1,2]. The results of numerous histological studies indicate the presence of a tissue remodeling process, including hypertrophy of the respiratory epithelium, thickening of the basement membrane, and advanced stromal edema followed by fibrosis and dense inflammatory cell infiltrate [1-3]. This disease manifests itself through several clinical phenotypes, but according to the dominant type of immune response, the two most important are CRS without nasal polyps (CRSsNP), in which the T1 immune response predominates, and CRS with NP (CRSwNP), in which there is a predominance of T2 immune response. In the phenotype of CRS with the formation of NP, histological studies showed the features of damaged nasal and sinus mucosa mediated by eosinophils in more than 90% of European and North American population [1,2]. Within the NP form, we distinguish a special clinical phenotype in which CRSwNP is associated with hypersensitivity to non-steroidal anti-
inflammatory drugs (NSAIDs) and non-allergic asthma. This aspirin-exacerbated respiratory disease (AERD) was caused as a result of disturbances in the metabolism of arachidonic acid and is characterized by a particularly severe clinical feature with rapid disease progression and frequent relapses, relatively soon after endoscopic surgical treatment [1-5].

Eosinophils play a significant role in the pathogenesis of most cases of CRS. After activation of eosinophils by T2 cytokines, such as interleukin-4 (IL-4), IL-5 and IL-13, their cytoplasm deposits granules of basic proteins such as major basic protein (MBP) and eosinophil cationic protein (ECP) [6,7]. These toxic proteins play the role of enzymes that damage the epithelium and lamina propria of the nasal and sinus mucosa. In addition, activated eosinophils release mediators such as platelet-activating factor (PAF) and eotaxin, which increase vascular permeability and attract new eosinophils to the site of inflammation [6,7]. Activated eosinophils that release MBP are immunohistochemically stained with the BMK13 antibody that specifically binds to MBP [6]. During the remodeling of the nasal mucosa, the process of vascularization takes place. The blood vessels that form in pathologically altered mucosa in CRS are described as ‘immature’ and the edema that occurs in the stroma is, among other things, a consequence of increased plasma leakage [8,9]. CD31 and CD34 may serve as markers of angiogenesis [8-10]. CD31 is a pan-endothelial marker and a member of the immunoglobulin superfamily. As an adhesion molecule, it plays an important role in the transendothelial migration of leukocytes, including eosinophils [8]. CD34 is also an intercellular adhesion molecule and a cell surface glycoprotein, expressed on endothelium, hematopoietic progenitor cells, and fibroblasts. It is believed that CD34-positive cells play a role in the pathophysiological mechanisms that result in tissue remodeling in CRS patients [8-12].

Although the eosinophilic infiltration of the nasal mucosa in patients with CRS have been relatively well investigated, only a few studies have examined vascularization in these patients [8-12]. Also, the studies did not include different clinical phenotypes within CRS, nor was the relationship between stromal vascularization and clinical characteristics of these patients investigated. So, the aim of this study was to evaluate, based on immunohistochemical staining of BMK13, CD31 and CD34, the degree of association of eosinophil infiltration and endothelial proliferation in the stroma of the nasal and sinus mucosa with clinical parameters in patients with different phenotypes of CRS, as well as in subjects without inflammation of the nasal mucosa. We also wanted to examine whether AERD is a separate entity within CRS with the formation of NP according to its histological features related to angiogenesis and eosinophil infiltration.

**Methodology**

**Ethical consideration**

This cross-sectional investigation was conducted in accordance with the Helsinki Declaration. The protocol and methods of the study were approved by Ethics Committee of our tertiary care institution (IRB Approval No 21/2022). A written informed consent was obtained from all patients. The study was performed at the Department of Otorhinolaryngology and Institute for Pathology of our tertiary care hospital, between March 2020 and October 2022.

**Study population**

The study included patients who were surgically treated in our hospital during the above-mentioned period. They were diagnosed with CRS in accordance with the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) 2020 [2] and the American Academy of Otolaryngology – Head and Neck Surgery (AAO-HNS) [13] guidelines. Some of the patients were without (CRSsNP) and some with polyps (CRSwNP). Among patients with polyps, we distinguished a group of patients with AERD. The criteria for the inclusion of AERD patients were the diagnosis of CRSwNP, mild persistent asthma, confirmed by an experienced pulmonologist, and information from the medical history about the worsening of respiratory symptoms after taking one of the NSAIDs. The other patients with CRS were without asthma and without sensitivity to NSAIDs. The control group consisted of patients without symptoms and signs of inflammation of nasal mucosa, selected for surgical treatment of nasal obstruction due to pneumatization of the middle turbinate (concha bullosa).

Exclusion criteria for the study: people younger than 18 and older than 65 years, pregnant women, nursing mothers, patients with systemic diseases affecting the nasal cavity/sinuses (Churg-Strauss syndrome, granulomatosis with polyangiitis, sarcoidosis, etc), patients with choanal polyps, hamartomas and fungal rhinosinusitis, with a congenital disorder (cystic fibrosis, primary ciliary dyskinesia, etc), smokers, patients with previous surgery of the nose/sinuses, subjects who took topical and/or systemic corticosteroids, antihistamines and antibiotics within a month before the start of the study.
Clinical evaluation

All the patients were preoperatively scored according to the intensity of nasal/sinus symptoms and quality of life (QoL) assessment, while patients with CRS were additionally scored according to the extent of the disease on computed tomography (CT) scans, according to the Lund-Mackay score (LMS) [14]. To assess the intensity of symptoms (nasal obstruction, nasal secretion, postnasal discharge, a feeling of fullness in the sinuses, weakened sense of smell, headache), we used a visual analogue symptom (VAS) score (from 0 – no symptom to 10 – maximum intensity of symptom) [15,16]. To assess the QoL, we used the sino-nasal outcome test 22 (SNOT-22) questionnaire, as previously described [16].

Tissue sampling, histopathological and immunohistochemical analysis

All patients were operated under general anesthesia. Tissue samples of polyps in CRSwNP/AERD patients as well as hypertrophic mucosa of patients with CRSSNP were taken from the ethmoidal labyrinth during endoscopic sinus surgery (ESS). Tissue samples of the nasal mucosa of the control subjects were taken by lateral resection of the pneumatized middle turbinate (concha bullosa). Tissue specimens have been fixed for 24 hours in 4% buffered formaldehyde solution. Then, they were washed with water and dehydrated with concentrated ethanol (70% up to absolute), then treated with xylene and embedded in paraffin. The paraffin blocks were sectioned to a thickness of 3-5 micrometers. The sections were stained with hematoxylin and eosin (H&E).

Immunohistochemical staining included deparaffinization after cutting the sections of 3-4 micrometers form paraffin mold and drying following soaking in xylene, alcohol and distilled water. Deparaffinized sections were heated twice for five minutes in a microwave oven in a cuvette with 250 mL of citrate buffer solution (10 mmol/L). After that, they are cooled in a citrate buffered solution at the room temperature for 30 minutes and washed with distilled water two times for 30 seconds. The next phase involves the blocking of endogenous peroxidase: tissue sections were soaked 3% hydrogen peroxide for five minutes; then washed with distilled water, overlaid with a phosphate buffer three times for two minutes, as previously described [17]. Immunohistochemical staining for MBP was performed with human BMK13 antibodies (Santa Cruz Biotechnology, Inc. Dallas, Texas, USA), while staining for CD31 and CD34 was performed with human anti-CD31 and anti-CD34 antibodies (Elabscience, Houston, Texas, USA).

Analysis of immunohistochemical findings was performed with a digital light microscope. The immune-reactivity score obtained was compared in relation to the CRS patients’ groups and in relation to the control group. Immunoreactivity of BMK-13 was recorded as staining of degranulated eosinophils, namely: grade 0 no positive cells, grade 1 - few positive cells (less than 5 cells), grade 2 - moderate number of positive cells (5-10 cells), grade 3 - moderate number of single and grouped cells (5-10 single and grouped cells), grade 4 - a large number of positive cells, including grouped cells (more than 10 cells), as previously described [6]. Positive immunoreactivity to CD31 and CD34 was recorded as membrane staining of endothelial cells in the continuity of the lumen of the vascular space.

The microvascular density quantification method involved determining the number of vascular spaces in the tissue part per square millimeter of surface, on scanned preparations stained by immunohistochemical methods. Briefly, this method involved initial scanning of the entire section under a low-power microscopic field to identify a few sites with the highest density of blood vessels, followed by counting individual new microvessels under a high-power microscopic field. Each positive cluster of endothelial cells of immunoreactivity in the selected field was counted as an individual vessel, in addition to morphologically identified vessels with a lumen. The intensity of CD31 and CD34 staining was evaluated as absence of staining - value 0, weak intensity - value 1, moderate intensity - value 2 and strong intensity - value 3, as previously described [8].

Statistical analysis

The data normality was tested using Shapiro-Wilk’s test. Student’s t-test for independent samples and One-way ANOVA was used to assess differences in patient’s age. One-way ANOVA was used to explore the differences between patient groups relative to the values of their clinical parameter scores followed by post-hoc testing using Tukey’s test. To test the differences in tissue staining intensity distribution between patient groups, Chi-square test and Fisher’s exact test were used. Correlation testing between these parameters was done using Spearman’s rank correlation coefficient. A p value less than 0.05 was considered statistically significant. Results related to clinical parameters in the figures and tables are represented as mean ± standard deviation (SD).
Results

A total of 94 patients were included in the present study. They were divided into 4 groups as follows: CRSwNP (without NSAID sensitivity) (n = 33; 35.1%), AERD (n = 20; 21.3%), CRSsNP (n = 10; 10.6%) and control (C) group consisting of patients without inflammatory disease of the nasal mucosa, selected only for nasal septum and nasal turbinate surgery (n = 31; 33%).

Of the 94 patients, 53 (56.4%) were male and 41 (43.6%) were female. The mean age of male patients was statistically higher than female patients [43.9 ± 12.8 vs. 38.8 ± 8.6; t = 2.299, p < 0.05]. No statistical difference was found in the mean age of patients between different patient groups (F (3.90) = 1.6, p > 0.05). Statistical difference was found in the gender distribution between four groups of patients [χ² (3) = 8.128, p < 0.05] (Figure 1), with male patients predominating in CRSwNP and C groups and female patients predominating in AERD group.

Comparison of clinical parameters (VAS, SNOT-22 and LMS), between experimental patient groups and control group showed a significant difference for all three investigated parameters as determined by one-way ANOVA [VAS: F (3, 90) = 9.495, p < 0.001; SNOT-22: F (3, 90) = 5.471, p < 0.01; LMS: F (2, 60) = 12.603, p < 0.001] (Figure 2). Post-hoc testing was done for each of the three tested parameters, which yielded differences between groups. In the case of VAS, significant statistical difference was detected between C and CRSwNP (p < 0.01), C and AERD (p < 0.001), C and CRSsNP (p < 0.05). In the case of SNOT-22, significant statistical difference was detected between C and CRSwNP (p < 0.01), C and AERD (p < 0.01). In

![Figure 1. Gender distribution between patient groups.](image1)

![Figure 2. Comparison of different clinical parameter scores between patient groups.](image2)
the case of LMS, significant statistical difference was detected between CRSwNP and AERD ($p < 0.01$) and CRSsNP and AERD ($p < 0.001$) (Figure 2).

Representative examples of immunohistochemical staining for all four groups of patients are shown in Figure 3. All three groups of CRS patients showed significant statistical difference in the values of investigated immunohistochemical markers: CD31 ($p < 0.05$); CD34 ($p < 0.01$); BMK13 ($p < 0.001$). We did not find a complete absence of CD31 and CD34 immunoreactivity in any subjects. Concerning CD31, the highest frequency of the strongest staining was observed in tissue samples obtained from CRSwNP patients, while the strongest staining pattern of CD34 was found in AERD patients. BMK13 expression was almost entirely absent in C group of patients, which is in complete contrast to patients from CRSwNP group, which showed various levels of BMK13 staining. The highest level of BMK13 expression (score 4) was detected in patients with AERD (Figure 4). Numerical data of clinical and immunohistochemical parameters are presented in Table 1.

CD31, CD34 and BMK13 were assessed in tissue samples obtained from all four patient groups. Using Fisher’s exact test, a significant statistical difference in the distribution of staining intensity for all three immunohistochemical markers was detected between four groups of patients. CRSwNP: chronic rhinosinusitis with nasal polyps; AERD: aspirin-exacerbated respiratory disease; CRSsNP: chronic rhinosinusitis without nasal polyps.
Table 1. Clinical and immunohistochemical parameters in different clinical phenotypes of chronic rhinosinusitis (CRS) and controls.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CRSwNP</th>
<th>AERD</th>
<th>CRSsNP</th>
<th>Controls</th>
</tr>
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<tr>
<td>VAS *</td>
<td>6.2 ± 1.6</td>
<td>7.1 ± 2.2</td>
<td>5.0 ± 2.1</td>
<td>4.3 ± 1.9</td>
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<td>SNOT-22 *</td>
<td>49.3 ± 15.3</td>
<td>52.25 ± 20.6</td>
<td>40.1 ± 21.9</td>
<td>33.7 ± 19.4</td>
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<td>LMS *</td>
<td>14.36 ± 4.4</td>
<td>18.45 ± 3.1</td>
<td>11.1 ± 4.1</td>
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<tr>
<td>BMK13 values</td>
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<tr>
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<td>0 – 0.0%</td>
<td>0 – 30.0%</td>
<td>0 – 90.3%</td>
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<td>2 – 20.0%</td>
<td>2 – 3.2%</td>
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<tr>
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<td>4 – 10.0%</td>
<td>4 – 0.0%</td>
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<tr>
<td>CD34 values</td>
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<td>3 – 40.0%</td>
<td>3 – 20.0%</td>
<td>3 – 12.9%</td>
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</table>

*Results are presented as mean ± SD (standard deviation). VAS: visual analogue score; SNOT-22: Sino-nasal Outcome Test-22; LMS: Lund-Mackay score; CRSwNP: chronic rhinosinusitis with nasal polyps; AERD: aspirin-exacerbated respiratory disease; CRSsNP: chronic rhinosinusitis without nasal polyps.

Figure 5. Heatmap of correlation between immunohistochemical markers and clinical score parameters.

Colours in the heatmap correspond to the strength and direction of correlation, where red colour denotes a positive correlation, blue colour denotes negative correlation and white colour denotes a correlation coefficient of 0. Values in the heatmap boxes represent the Spearman’s correlation coefficient. * - denotes a p < 0.05; ** - denotes a p < 0.01; *** - denotes a p < 0.001. CRSwNP: chronic rhinosinusitis with nasal polyps; AERD: aspirin-exacerbated respiratory disease; CRSsNP: chronic rhinosinusitis without nasal polyps; VAS: visual analogue score; SNOT-22: Sino-nasal Outcome Test-22; LMS: Lund-Mackay Score.
The relationship between immunohistochemical markers and clinical parameter scores of patients included in the present study showed a moderate positive correlation between BMK13 and SNOT-22 (rs = 0.46) and between BMK13 and LMS (rs = 0.58) in CRSwNP group (Figure 5). In the CRSSsNP group, a borderline statistical significance in correlation was only shown for CD31 and BMK13, which showed a moderate positive correlation (rs = 0.65). LMS was shown to correlate with both VAS and SNOT-22 in CRSwNP (rs = 0.53; rs = 0.58, respectively) and AERD (rs = 0.55; rs = 0.6, respectively) groups of patients (Figure 5). In control subjects, we found positive correlation between SNOT-11 and VAS (rs = 0.77) and between CD31 and CD34 (rs =0.47) (Figure 5).

Discussion

Chronic inflammation and angiogenesis are two processes that run in parallel during tissue remodeling in CRS, with numerous mutual interactions. Many mediators of inflammation that accumulate at the site of chronic inflammation stimulate pro-angiogenic signaling molecules, including growth factors, adhesion molecules, cytokines and chemokines [18,19]. These pro-angiogenic mediators stimulate endothelial cells to proliferate and form new blood vessels [20]. Despite numerous studies related to chronic inflammatory disorders in the body, only a few of them deal with these complex processes in patients with CRS. Hirshoren et al. [12], based on a significantly higher level of immunoexpression for CD34, concluded that the process in angiogenesis is far more intense in the nasal mucosa of patients with CRSwNP compared to antrochoanal polyps. Khurana et al. [21] found significantly higher expression for pro-angiogenic genes and a higher level of blood flow in the mucosal tissue of patients with CRSwNP and CRSSsNP compared to the mucosa of healthy subjects. In another study, the quantification of microvessels showed higher expression of CD34 in type 2 CRS than in non-type 2 CRS [22]. However, while reviewing the literature, we found no studies investigating the relationship between eosinophilic infiltration and vascularization of subepithelium of the nasal mucosa, particularly in patients with AERD.

Our study is the first one to investigate eosinophilic infiltration and vascularization in the nasal/sinus mucosa of patients with AERD, as a distinct clinical phenotype within CRS. The results of the present study indicate that patients with AERD and CRSwNP without NSAID-sensitivity have more pronounced symptoms and a more impaired QoL compared to patients with CRSSNP and subjects with non-inflamed nasal mucosa selected for surgical treatment of nasal obstruction. Also, patients with AERD have more intense extension of sinus disease on CT scans compared to patients with CRSwNP and CRSSsNP. A relatively good correlation of clinical parameters was observed in all examined groups. While BMK13 as a marker of eosinophilic infiltration and CD34 as a marker of endothelial proliferation were best expressed in the group of patients with AERD, CD31, also a marker of angiogenesis, was best expressed in the group with CRSSNP without aspirin sensitivity. The correlation of BMK13 with clinical parameters is best manifested in the group of patients with CRSwNP, which suggests that the intensity of eosinophilic infiltration of the nasal and sinus mucosa is directly related to the intensity of the inflammatory process and the spread of the disease. On the other hand, it is unusual that in patients with AERD this correlation is not significant, even though eosinophilic infiltration is the densest in them. This points to the need to conduct new studies with larger number of participants.

One of the most significant findings of this research is the lack of correlation between BMK13, as a marker of infiltration of the nasal mucosa by activated eosinophils, and CD31 and CD34, as markers of angiogenesis in patients with NP. Although CD31 and CD34 play the roles of adhesion molecules, important in the process of transendothelial migration of eosinophils, they are not the main adhesion molecules in that phase of the eosinophil life [8,10]. Besides being adhesion molecules for eosinophils, they are also important in the passage of other leukocytes between endothelial cells [8,10]. After the phases of attraction and activation of eosinophils, where the main roles have eosinophil chemokines eotaxin and RANTES (regulated on activation, normal T-cell expressed and secreted), as well as cytokines IL-4, IL-5 and IL-13, the most important role in the transendothelial migration of eosinophils is played by vascular cell adhesion molecule-1 (VCAM-1) [23]. Without the binding of eosinophils to VCAM-1, there is no accumulation of them in the stroma and epithelium of the nasal/sinus mucosa, where they release their enzymes during chronic inflammation, including MBP [23]. It is interesting that the significant correlation of BMK13 and CD31 was found only in patients with CRSSNP, while the positive correlation of angiogenesis markers CD31 and CD34 was manifested only in the mucosa of the control subjects. These findings could theoretically indicate the fact that in the nasal mucosa where eosinophilic inflammation is not significantly
expressed and the tissue remodeling process is not so much intense, the process of normal angiogenesis is not significantly impaired. However, this is only our subjective conclusion, without the evidence in the results and it requires further investigation.

Our study has limitations. The number of patients with CRSSNP and AERD was relatively small. On the other hand, that number reflected the real influx of patients who came to our institution for surgical treatment. It is known that patients with CRSSNP are less often referred for surgical treatment, considering that good therapeutic effects are achieved with medical treatment. Only AERD patients with mild persistent asthma were included in our study, since more severe forms of asthma require the use of systemic corticosteroid therapy, which can affect the manifestation of inflammation in the tissue of the nasal and sinus mucosa in those patients. This means that the intensity of BMK13, CD31 and CD34 immunooexpression in patients with a more severe form of AERD would be even higher than what was observed in our patients.

Conclusions
The results of our study showed an almost complete absence of activated eosinophils in the mucosa of participants without nasal inflammation. On the other hand, the immunooexpression of BMK13, as a marker of eosinophilic infiltration is extremely high in the sinus mucosa of patients with NP, especially in ones with AERD. Angiogenesis markers are significantly more expressed in AERD and CRSwNP patients. Among the different phenotypes of CRS, the intensity of eosinophilic infiltration correlates better with the clinical parameters (radiologically estimated extent of the disease, impaired QoL) than the intensity of vascularization in the lamina propria. We found no correlation between markers of tissue eosinophilia and markers of vascular proliferation among patients with NP.

Acknowledgements
This investigation was conducted as a part of a scientific project of the Faculty of Medicine of the Military Medical Academy, Belgrade, Serbia (MFVMA02/23-25/).

Ethics committee approval
This investigation was approved by Ethics Committee of the Military Medical Academy, Belgrade, Serbia (Approval Number IRB 21/2022).

References


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Conflict of interests: No conflict of interests is declared.