ORIGINAL CONTRIBUTION

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Nasal lavage CCL24 levels correlate with eosinophils trafficking and symptoms in chronic sino-nasal eosinophilic inflammation*

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SUMMARY

***Objectives**: The aim of our study was to measure CCL24 (eotaxin-2) levels in nasal lavage fluid of patients with different forms of sinonasal chronic eosinophilic inflammation to verify the relationship with nasal hypereosinophilia and symptoms.*

***Methods**: Patients with nasal hypereosinophilia were randomly recruited and grouped in persistent allergic rhinitis, non-allergic rhinitis with eosinophilia syndrome (NARES) and chronic rhinosinusitis with polyps. Non rhinitic volunteers were recruited as controls. CCL24 concentration was measured by 'Quantikine Human CCL24 Immunoassay'. Differential cell counts were performed by microscopic cytological examination of nasal tissue scraped by inferior turbinate.*

***Results**: CCL24 levels measured in patient groups were significantly higher compared to control group with the highest levels in NARES patients. CCL24 levels were significantly correlated to severity of symptoms and to the percentage of eosinophils in nasal tissue.*

***Conclusions**: We revealed high levels of CCL24 in all patient groups showing a significant correlation with the degree of eosinophilia and clinical symptoms. A prolonged accumulation of CCL24 inside the nasal mucosa may sustain the process of unspecific self-perpetuating eosinophil recruitment pathognomonic of these patients.*

Key words: eosinophilic chronic rhinosinusitis, CCL24, eotaxin-2, NARES, nasal lavage, nasal polyps

INTRODUCTION

Common and chronic forms of sinonasal eosinophilic inflammation include persistent allergic rhinitis (PER), non-allergic rhinitis with eosinophilia syndrome (NARES), chronic rinosinusitis with polyps (CRS with NP) and allergic fungal rhino-sinusitis [1-3]. The pathogenesis of chronic hypereosinophilia in nasal mucosa is still unknown. Recent hypotheses focused on an increased transendothelial migration of eosinophilic granulocytes, attracted and activated by chemokines with less apoptosis [4]. An increased expression and production of several pro-inflammatory cytokines, responsible for guiding the inflammatory process, has been reported in chronic nasal disease [5-7]. Among those, the 'regulated upon activation normal T expressed and secreted' (RANTES/CCL5), 'monocyte chemoattractant proteins' (MCP-3/CCL7 and MCP-4/CCL13) are potent eosinophil attractants but are not selective. In contrast, eotaxin-1 (CCL11), eotaxin-2 (CCL24) and eotaxin-3 (CCL26) seem to be potent and selective attractants [8,9]. For this reason, eotaxins have been recently investigated in different chronic nasal diseases, with particular regard in nasal polyps [10-12].

Pods et al., [13] demonstrated elevated CCL24 mRNA expression, as well as protein-synthesis, in nasal polypous tissue specimens of patients suffering from Samter's triad. Confirming this data, Yao et al., [14] recently illustrated that eosinophilic chronic rhinosinusitis patients had a significant expression of eotaxins in nasal polyps and sinus effusion collected during endoscopic sinus surgery. The relevance of eotaxin proteins has well been demonstrated also in allergic patients. The intranasal challenge with eotaxins has been shown to induce nasal luminal eosinophils recruitment [15]. In non-allergic rhinitis, despite several studies [16,17], the mechanism remains unclear and the pathogenesis has not yet been well defined.

Nasal lavage fluid is widely used to determine mediators involved in the diseases. In contrast to biopsies, it is easy and non-invasive to collect, and it reflects local pathophysiology much better than systemic mediators [18].

The aim of our study was to investigate the presence of CCL24 in nasal lavage of patients with different forms of chronic eosinophilic inflammation, revealed by scraping of the nasal mucosa, to verify the relationship with nasal hypereosinophilia

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and symptoms.

MATERIALS AND METHODS

Patients

The study was performed in the Department of Otorhinolaryngology, Head and Neck Surgery of the Catholic University of the Sacred Heart, Rome, Italy. We randomly recruited 82 outpatients (37 males; 45 females; mean age 41.8 ± 14 years) with nasal symptoms such as rhinorrhea, nasal obstruction and sneezing, presenting themselves to our rhinology service, between November 2008 and January 2010. Patients with at least 12 weeks of rhinitic symptoms and with evidence of nasal hypereosinophilia, revealed by nasal scraping of mucosa at the inferior turbinate, were admitted to the study. Patients did not receive any treatment at the time of inclusion in the study. Healthy subjects (n = 20), without any nasal inflammation, no history of chronic rhinosinusitis or rhinitis, normal endoscopic findings, negative allergy test and negative X-rays of paranasal sinuses, served as controls. The protocol was approved by our institutional board and all subjects gave written informed consent.

All patients, on the same day of nasal lavage, were each asked to complete a rhinologic questionnaire. We scored in all patients the following symptoms: rhinorrhea, nasal obstruction, facial pain or pressure, sneezing, loss of smell, nasal itching, difficult sleeping, nocturnal awakenings, eye symptoms. Each symptom was scored by a visual analogical scale (VAS) from 0 to 10, in which 0 indicates no symptoms and 10 indicates severe symptoms. We obtained an individual symptom score adding scores of each symptom for every patient. Total symptom scores were assessed as the median value from individual symptom scores in each group.

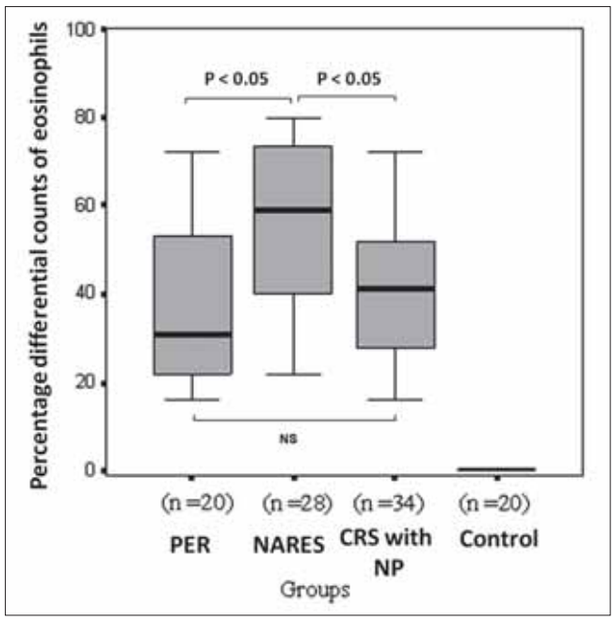

Figure 1. Percentage differential counts of eosinophils in all studied forms of eosinophilic chronic inflammation (PER: persistent allergic rhinitis; NARES patients: non allergic rhinitis eosinophilic syndrome; CRS with NP: chronic rhinosinusitis with nasal polyps). The box plots show the median and inter-quartile range and the error bars show the 5th and 95th percentiles.

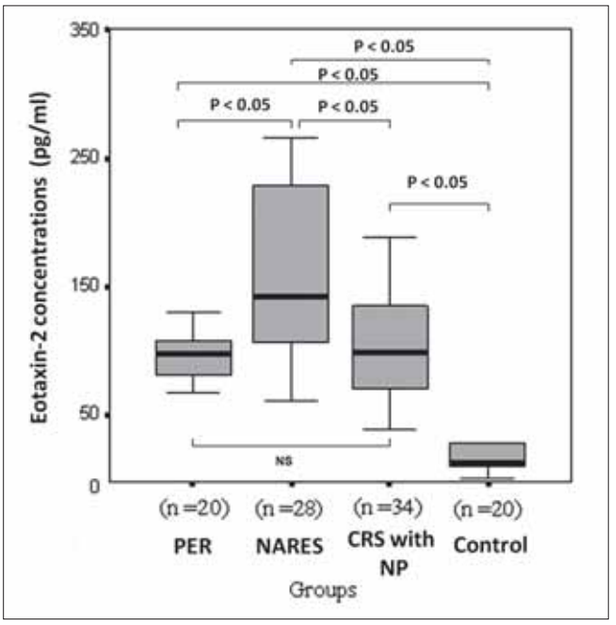

Figure 2. CCL24 concentration (pg/mL) in all studied forms of eosinophilic chronic inflammation (PER: persistent allergic rhinitis; NARES patients: non allergic rhinitis eosinophilic syndrome; CRS with NP: chronic rhinosinusitis with nasal polyps) and in the Control Group. The box plot show the median and interquartile range and the error bars show the 5th and 95th percentiles.

All patients were tested by nasal endoscopy and CT scan. Allergic status was confirmed by in vivo and in vitro tests, such as: total serum IgE, total serum eosinophil counts, skin prick test (with a series of 18 common inhalant allergens including house dust mites, major Italian pollens, fungus, dogs, and cats), allergen-specific IgE determination and intranasal allergen provocation.

According to the 'Allergic Rhinitis and its Impact on Asthma' report [19], we divided allergic patients into two categories: intermittent and persistent. We included in the study only the 'persistent' ones (more than 4 days a week and for more than 4 weeks) to avoid differences due to actual allergen exposure between co-seasonal and extra-seasonal patients. Chronic rhinosinusitis with nasal polyps was defined, according to 'European Position Paper on Rhinosinusitis and Nasal Polyps 2007 [20].'

Study Design

The study was an analytical cross sectional study with the level of evidence at 3b.

Disease groups

Patients were grouped according to the underlying forms of eosinophilic chronic nasal inflammation:

- *Persistent allergic rhinitis* [PER (n = 20)]: typical persistent symptoms (rhinorrhea, sneezing, itchy nose, nasal congestion and/or obstruction, watery or itchy eyes) and in vivo and/or in vitro confirmation of atopic status; negative nasal endoscopy

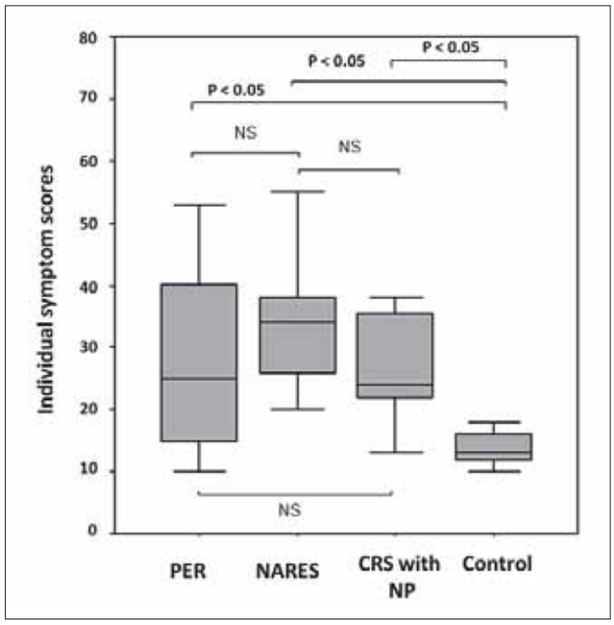

Figure 3. Symptom scores in all studied forms of eosinophilic chronic inflammation (PER: persistent allergic rhinitis; NARES patients: non allergic rhinitis eosinophilic syndrome; CRS with NP: chronic rhinosinusitis with nasal polyps) and in the Control Group. The box plot show the median and interquartile range and the error bars show the 5th and 95th percentiles. Total symptom scores were assessed as the median value from individual symptom scores in each group.

for polyps and negative CT scan of paranasal sinuses for nasal swelling;

- *Non-allergic rhinitis with eosinophilia syndrome* [NARES (n = 28)]: patients complained about typical symptoms of perennial allergic rhinitis (rhinorrhea, sneezing, itchy nose, nasal congestion and/or obstruction, watery or itchy eyes) but all in vitro and in vivo allergy tests failed to detect an atopy. Profound nasal eosinophilia was revealed by scraping of nasal mucosa and cytology examination (> 20% eosinophils in the total granulocytic or mononuclear cell population, excluding nasal epithelial cells). CT scan was negative in all patients.

- *Chronic rhinosinusitis with nasal polyps* [CRS with NP (n = 34)]: symptoms of chronic rhinosinusitis [nasal blockage, nasal discharge (anterior/posterior nasal drip), facial pain/pressure, reduction or loss of smell] and pathological mucosa swelling revealed by nasal endoscopy and CT scan. The allergic status was confirmed in 10 out of 34 patients based on allergic tests and clinical features.

We did not include allergic fungal rhino-sinusitis in this study because of its particular clinical features.

Epidemiology of the different groups is reported in Table 1.

Nasal cytology

Nasal leukocyte counts were performed on nasal scraped tissue obtained from the inferior turbinate bilaterally by rhinoprobe (Farmark s.n.c, Milan, Italy). The sample was gently spread on glass slides and immediately fixed in 95% ethylalcohol and stained with May-Grünwald-Giemsa. The percentage of eosinophils was assessed by microscopic cytological examination. The slides were examined under oil immersion by light microscopy at a magnification of x400. Eosinophil counts were expressed as a percentage of cells of the granulocytic or mononuclear cells, excluding nasal epithelial cells, at high power field, as the mean of at least 10 fields observed.

Nasal lavage fluid collection and processing

Nasal lavage fluid was obtained from subjects with the head bent down, based on methods previously described [21-22]. We washed each nostril instilling 5 ml of saline solution (NaCl 0.9%) pre-warmed to 35°C. The fluid was collected by asking the subjects to lean forward and blow the nasal contents gently into a funnel connected to a 30 ml universal container. The lavage fluid was filtered to remove any nasal mucus and centrifuged immediately at 4000 rpm for 5 minutes, then it was

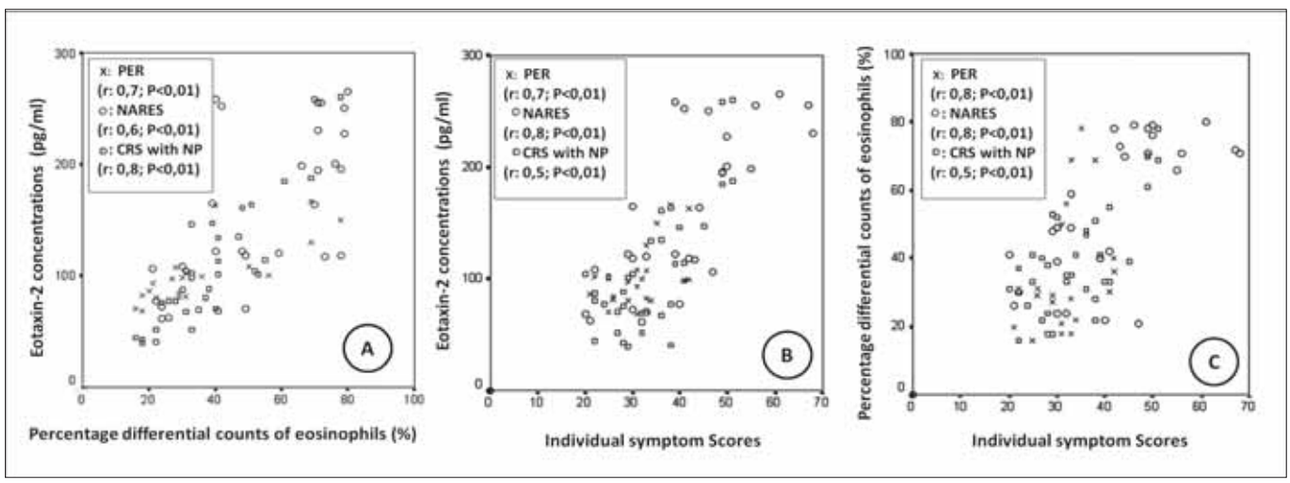

Figure 4. In 'A' we correlated nasal fluid CCL24 concentration (pg/mL) and eosinophils trafficking. In 'B' and 'C' we correlated the individual symptom score with CCL24 level (pg/mL) and percentage differential count of eosinophils for each patient. Individual symptom scores have been obtained adding scores of each symptom for every patient. Each symbol indicates measured values for different groups. Correlation coefficients (rs), assessed by Spearman's rank correlation test, are reported for different groups.

divided in aliquots and frozen at -80°C until the assays.

CCL24 (Eotaxin-2) assay

CCL24 was assayed according to the manufacturer's instructions (R&D Systems, Minneapolis, USA) by 'Quantikine Human CCL24 Immunoassay', an ELISA kit designed to measure CCL24 levels in cell culture supernatant, serum and plasma. The samples, analysed in a single session, were performed in duplicate and the mean value was calculated. The coefficient of variation (CV) of duplicates was always less than 3%. The sensitivity of the assay was 2.5 pg/ml and the measuring range was from 2.5-2500 pg/ml.

Statistical analysis

All statistical analyses were performed using SPSS for Windows. Continual variables have been expressed as mean ± SD and comparisons between groups were performed by means of Mann-Whitney U-test. The strength of the correlation was explored using the Spearman's rank correlation test. The level of significance was accepted as $p < 0.05$.

RESULTS.

The mean percentage differential counts of eosinophils observed in the group PER, NARES and CRS with NP were, respectively, 32.9 ± 18.6, 58.2 ± 20.9 and 39.8 ± 15.7. The highest values were observed in NARES patients with significant differences ($p < 0.05$) compared to groups PER and CRS with NP (Figure 1).

All patients were symptomatic and the mean concentration of CCL24 in their nasal lavage fluid was significantly increased compared to controls [128.9 ± 51.7 pg/mL vs 16.4 ± 10.7 pg/mL] ($p < 0.01$). The mean CCL24 concentrations measured in Group PER, NARES and CRS with NP were significantly higher compared to controls and were respectively 102.3 ± 36 pg/mL ($p < 0.05$), 149.1 ± 58.7 pg/mL ($p < 0.01$) and 103.8 ± 52.7 pg/mL ($p < 0.05$). Inter-group comparison revealed the highest levels of CCL24 in NARES, with significant differences as regards PER ($p < 0.05$) and CRS with NP ($p < 0.05$). No significant difference in CCL24 levels were observed between PER and CRS with NP (Figure 2). Total symptom scores obtained for PER, NARES and CRS with NP were, respectively, 25, 34 and 24. The values measured in all groups were significantly higher compared to controls ($p < 0.05$). The inter group differences instead did not reach values of statistical significance (Figure 3).

Table 1. Subjects' demographics.

	PER (n = 20)	NARES (n = 28)	CRS with NP (n = 34)	Control (n = 20)
Median age	33 (10-65)	43 (19-75)	47 (25-67)	41 (22-63)
Sex (M)	6 (30%)	8 (23.52%)	16 (47.05%)	8 (40%)
Asthma	7 (35%)	8 (28.5%)	11 (32.3%)	None
ASA intolerance	1 (5%)	6 (21.6%)	8 (23.5%)	None
Atopy	All	None	10 (29.4%)	None
CT scan (lund-mackay score)	0	0	15.35§	0

PER: persistent allergic rhinitis; NARES: non allergic rhinitis eosinophilic syndrome; CRS with NP: chronic rhinosinusitis with nasal polyps; Control: healthy subjects. §: median value

We found a significant correlation between CCL24 concentration and percentage differential counts of eosinophils ($p < 0.01$) with a Spearman's rank correlation coefficient of rs: 0.6. Figure 4A shows that the correlation was significant ($p < 0.01$), also measuring values for different groups.

Finally, a significant correlation was found between either rhino-sinusitis individual symptom score and nasal fluid CCL24 concentration (pg/mL) (Figure 4B), either rhino-sinusitis individual symptoms score and percentage differential count of eosinophils (Figure 4C). Spearman's rank correlation coefficient was respectively rs: 0.5 and rs: 0.6 ($p < 0.01$). Figure 4 shows that the correlation was significant ($p < 0.01$) also measuring values for different groups.

DISCUSSION

The underlying mechanisms of selective infiltration of eosinophils observed in some chronic forms of nasal inflammation, such as allergic persistent rhinitis, NARES and chronic rhinosinusitis with nasal polyps, are still partially unknown. A number of hypotheses have been formulated to explain the causes of eosinophil endothelial transmigration (TEM). Lately, some authors [23,24] have emphasized the importance of micro-environmental influences on airway inflammation; in particular, locally released chemokines might initiate and orchestrate the process of eosinophil trafficking, even though the exact order and sequence of the chemical signals are not yet understood.

Several chemical mediators are considered as eosinophil attractants in the airway mucosa; in particular, IL-5 and LTB4, members of the family of CC-chemokines, including CCL5, CCL7 and CCL13. and finally the recently discovered CCL11, CCL24 and CCL26. Several studies have established that eotaxins induce maximal transendothelial eosinophil migration, with respect to the others chemokines in the following order of strength: CCL24 (eotaxin) > CCL5 = CCL13 > IL-5 > LTB 4 [26-29]. Some chemokines operate by stimulation via several receptors, while all eotaxins act via the same receptor: CC chemokine receptor 3 (CCR3) [30]. CCL24 was identified having structural homologies to CCL11; both chemokines show the same efficacy in selective eosinophil chemo-attraction, but with a lower potency of CCL24 [31-33]. Increased expression of CCL11 and CCL24 has been reported in biopsies of atopic and non-atopic patients suffering from nasal polyps [34] and of asthmatic patients [35].

In our study, we used nasal lavage fluid because it has previously been used to analyse both mediator and cytokine levels as well as cell profiles [21-23]. It has several advantages: it samples a larger surface area in respect to biopsy and nasal scraping, it is a non-invasive, well tolerated and repeatable technique. Consistently with previous studies [11-13, 33], our results point out local high levels of CCL24 in patients with nasal polyps (CRS with NP) and a significant correlation with nasal tissue eosinophilia, confirming that CCL24 may play a role in recruiting inflammatory cells in this condition. In agreement with previous studies [36], we measured significantly increased levels of CCL24 also in nasal lavage of patients with persistent allergic rhinitis (PER). The tissue infiltration of eosinophils is certainly best described in allergic forms of rhinitis, supposing that it depends on the IgE mediated immune response. In contrast, in non-allergic rhinitis the mechanism remains unclear. Interestingly, we revealed the highest concentrations of CCL24 in nasal fluid of NARES patients compared to the other groups ($p < 0.05$). Such high levels are consistent with the excessive nasal eosinophilia, pathognomonic of NARES patients. It can be reasonably hypothesized that CCL24 in nasal lavage directly reflects the concentration of this chemokine in nasal mucous secretions. Since our data revealed a significant correlation between CCL24 levels and the percentage differential counts of eosinophils in the nasal mucosa in all patients ($p < 0.01$), we suggest that CCL24 may be in vivo a potent chemo-attractant of eosinophils in the nasal mucosa.

It is difficult to speculate on the pathophysiology of eosinophil recruitment in absence of information regarding the major cellular source of this CC-chemokine. It remains to be determined whether the high CCL24 levels could simply be because the eosinophils are able to generate it themselves or if other cells in the upper airways contribute to its production. Whatever means, the high CCL24 levels observed in nasal fluids in our series might at least explain the process of self perpetuating recruitment observed in chronic eosinophilic inflammation of nasal mucosa. Previous studies [33] revealed that quantitatively the epithelial and endothelial cells seem to be the main producers of CCL24 in nasal mucosa. The nasal epithelial cells have been considered for a long time a defense system that protects against the invasion of pathogenic organisms, by physiological barriers like tight junctions, mucociliary clearance and the production of enzymes and chemical mediators. Moreover, it has been demonstrated that epithelial cells release other factors, such as Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) that affect activation and survival of inflammatory cells [29,37,38]. We believe that further studies need to confirm the assumption that the epithelium may play a key role in the orchestration of local immune responses by releasing inflammatory mediators and expressing various types of cell adhesion molecules.

In conclusion, consistently with previous studies [21-23], demonstrating that nasal lavage is a useful sample for evaluating immunological mediators, we revealed in this study that CCL24 can be easily detected in nasal fluid of patients with allergy, non-allergic eosinophilic rhinitis and eosinophilic nasal polyps. Furthermore, we asked all patients to complete the symptom questionnaires to establish nasal levels of CCL24 in relation to the clinical findings. We found a significant correlation between CCL24 levels, eosinophilic infiltration and severity of symptoms. Therefore we suggest that the level of CCL24 measured in nasal fluid of patients with chronic eosinophilic inflammation correlates well with the severity of symptoms. We propose an important role of CCL24 in the pathophysiology of nasal hypereosinophilia sustaining the process of unspecific self- perpetuating eosinophil recruitment observed in different forms of chronic eosinophilic inflammation and particularly in NARES patients. The role of eosinophilic chemoattractants should be better investigated to understand the immunological mechanisms of eosinophilic nasal trafficking and to achieve new insights into the pathogenesis and treatment of nasal hypereosinophilia.

CONFLICT OF INTEREST

The authors have no financial disclosures. They have not actual or potential conflict of interest in connection with the submitted article.

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