

Expression of cysteinyl leukotriene receptor 1 and 2 (CysLTR1 and CysLTR2) in the lymphocytes of hyperplastic tonsils: comparison between allergic and nonallergic snoring children

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Background: Cysteinyl leukotriene receptor 1 and 2 (CysLTR1 and CysLTR2) are involved in allergic processes and play a role in adenotonsillar hyperplasia (AH). Clinically, only CysLTR1 may be blocked by montelukast. Our objective was to compare the expression of CysLTR1 and CysLTR2 in the B and T cells of hyperplastic tonsils of sensitized (SE) and control (NS) snoring children.

Methods: Sixty children, 5 to 10 years of age, referred for adenotonsillectomy, were divided into SE and NS groups, according to their responses to the skin-prick test. Cells from the removed tissues were stained for CysLTR1, CysLTR2, CD19, and CD3 and counted via flow cytometry. messenger RNA (mRNA) expression of the CysLTRs genes was measured real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR).

Results: The SE group showed reduced expression of the small CD3+/CysLTR1+ lymphocytes (4.6 ± 2.2 vs 6.5 ± 5.0 ; $p = 0.04$). Regarding the large lymphocytes, the SE group showed lower expression of CD3+/CysLTR1+ (40.9 ± 14.5 vs 47.6 ± 11.7 ; $p = 0.05$), CD19+/CysLTR1+ (44.6 ± 16.9 vs 54.1 ± 12.4 ; $p = 0.01$), and CD19+/CysLTR2+ (55.3 ± 11.3 vs 61.5 ± 12.6 ; $p = 0.05$) lymphocytes. Considering the total number of lymphocytes, the SE group had fewer CD3+/CysLTR1+ lymphocytes (11.1 ± 5.5 vs $13.7 \pm$

6.2 ; $p = 0.04$). All other cell populations exhibited reduced expression in the SE group without statistical significance. The expression of CysLTR2 was significantly higher ($p < 0.05$) than CysLTR1 in most studied cell populations. The mRNA expression did not show significant differences between the groups.

Conclusion: The expression of CysLTR is higher in the lymphocytes of the NS children, and CysLTR2 shows greater expression than CysLTR1. Respiratory allergies do not appear to be a stimulus for AH occurrence. Newer drugs capable of blocking both CysLTRs warrant further study. © 2016 ARS-AAOA, LLC.

Key Words:

cysteinyl leukotriene receptor; lymphocyte; tonsil; allergy; hyperplasia

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Adenotonsillar hyperplasia (AH) is the primary pathological cause of sleep respiratory disorders (SRDs) in children.^{1,2} The concentration of cysteinyl leukotrienes (CysLTs) in the blood and tonsil, as well as the expression of its biosynthetic enzymes, is higher in snoring children than in controls.³⁻⁵ These mediators are classically related to allergic diseases⁶⁻⁸ and activate CysLTR1 and CysLTR2.⁹ Recent studies have also demonstrated the role of these receptors in cell division in inflammatory and neoplastic diseases.¹⁰⁻¹⁷

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In AH, the role of the CysLTRs in the proliferation of tonsil lymphocytes has been reported,¹⁸ and 1 clinical trial reported a positive response to antileukotriene drugs, even in individuals without respiratory allergies.^{19,20} The adenoids underwent size decreases when those patients were exposed to montelukast therapy.^{4,21} Another in vitro experiment showed an evident decrease in tonsil lymphocyte proliferation when exposed to antileukotriene drugs.²⁰

Studies have been inconclusive regarding the question of whether allergic rhinitis (AR) is a cause of AH. Clinical surveys pointed to an inverted correlation between AR and adenoid^{22,23} and tonsil²⁴ hyperplasia. Other authors have shown that allergic diseases (rhinitis, asthma, and urticaria) may be considered risk factors for AH development^{23,25} and that the exposure to a specific allergen was related to increases in the size of the adenoid.^{26,27} Considering these data, the authors hypothesized that the pathophysiology of AH in children includes both activation of both CysLTRs as a key factor and that children with AR would have higher expression of these receptors than normal individuals.

Thus, the main objective of this study was to check the difference in the expression of CysLTR1 and CysLTR2 in the lymphocytes of tonsils and adenoids of sensitized and nonsensitized children with SRD secondary to AH. The secondary objective was to compare the expression of CysLTR1 with the expression of CysLTR2 in each cell population of these individuals.

Patients and methods

This work was performed at the Hospital das Clinicas of the University of Sao Paulo, Brazil.

Ethics statement

This study was approved by the Ethics Committee of the Hospital das Clinicas da Universidade de São Paulo (record number 10492/14). All guardians signed informed consent forms.

Sample

Male and female children aged 5 to 10 years old with AH and an indication for adenotonsillectomy were eligible for this study. The authors included children who had nighttime snoring with reported periods of apnea at least 4 nights per week, a Pediatric Sleep Questionnaire (PSQ) index greater than 8,^{28,29} and a persistently blocked nose. On physical examination, the tonsils were grade 3 or greater,³⁰ and the nasopharynx presented an occlusion of at least 60%^{31,32} by the adenoid (evaluated via flexible endoscopy). The body mass index (BMI) *z* score should be within the range of ± 2 according to the World Health Organization standards.^{33,34} We excluded patients with (1) craniofacial deformities; (2) cardiovascular, immunological, or genetic disorders; (3) related use of antileukotrienes, anti-inflammatory drugs, antihistamines, or antibiotics in the 60 days prior to surgery; (4) a history of recurrent

tonsillitis³⁵; (5) any current infection; (6) residence outside the region of Sao Paulo; or (7) non-respiratory allergies.

Skin-prick test

Patients underwent a bilateral skin-prick test (SPT) using disposable metallic lancets (Alergopricks[®]; Sao Paulo, Brazil), and each drop should have penetrated 0.1 to 0.2 mm into the skin on the anterior forearms. Patients were tested for *Lolium perenne*, *Aspergillus fumigatus*, *Penicillium notatum*, *Dermatophagoides pteronyssinus*, *Blomia tropicalis*, *Felis domesticus*, *Canis familiaris*, *Periplaneta americana*, *Blattella germanica*, a positive control with histamine (10 mg/mL), and a negative control with saline solution (antigens provided by IPI ASAC[®], Sao Paulo, Brazil). The reaction was positive when the diameter of the wheal was equal to or greater than 3 mm concordantly in both arms. Patients who presented (1) no response to the positive control, (2) dermatographism, or (3) disagreement between the sides were excluded from the study.

The subjects were then allocated to the sensitized (SE, positive SPT for at least 1 of the reactants) or nonsensitized (NS, negative in all tests) group.

Collection and processing of samples

The excised adenoids and tonsils were promptly transported to the laboratory. A fragment was fixed in paraffin (Sinth[®], Sao Paulo, Brazil), sliced in 4- μ m sections, stained with hematoxylin and eosin (Newprov[®], Sao Paulo, Brazil), and evaluated by the same pathologist to confirm tissue hyperplasia and exclude other abnormalities. The remaining tissue was mechanically dissociated in phosphate-buffered saline (PBS) (Laborclin[®], Sao Paulo, Brazil), and the cell suspension was used for flow cytometry and real-time quantitative reverse transcription polymerase chain reaction.

Flow cytometry

The cell suspension was divided into 2 tubes. Rabbit polyclonal primary antibody anti-CysLTR1 (Cayman Chemical[®], Ann Arbor, Michigan) and fluorescein isothiocyanate (FITC)-conjugated secondary polyclonal antibody goat anti-rabbit (BD[®], New Jersey, NJ) were sequentially added to the CysLTR1 tubes. The B and T lymphocytes were marked with a PerCP mouse antibody against human CD19 and with a PE mouse antibody against human CD3, respectively (Exbio[®], Prague, Czech Republic). Rabbit polyclonal antibody anti CysLTR2 (Cayman Chemical[®]) was initially added to the CysLTR2 tubes, and the other steps were repeated. After being labeled, the suspension was read on a FACSCalibur flow cytometer (BD[®], New Jersey, NJ) and the subpopulations of cells were analyzed according its size (Fig. 1).

RT-qPCR

The lymphocyte separation in the adenoid cell suspension was performed using Ficoll Paque solution

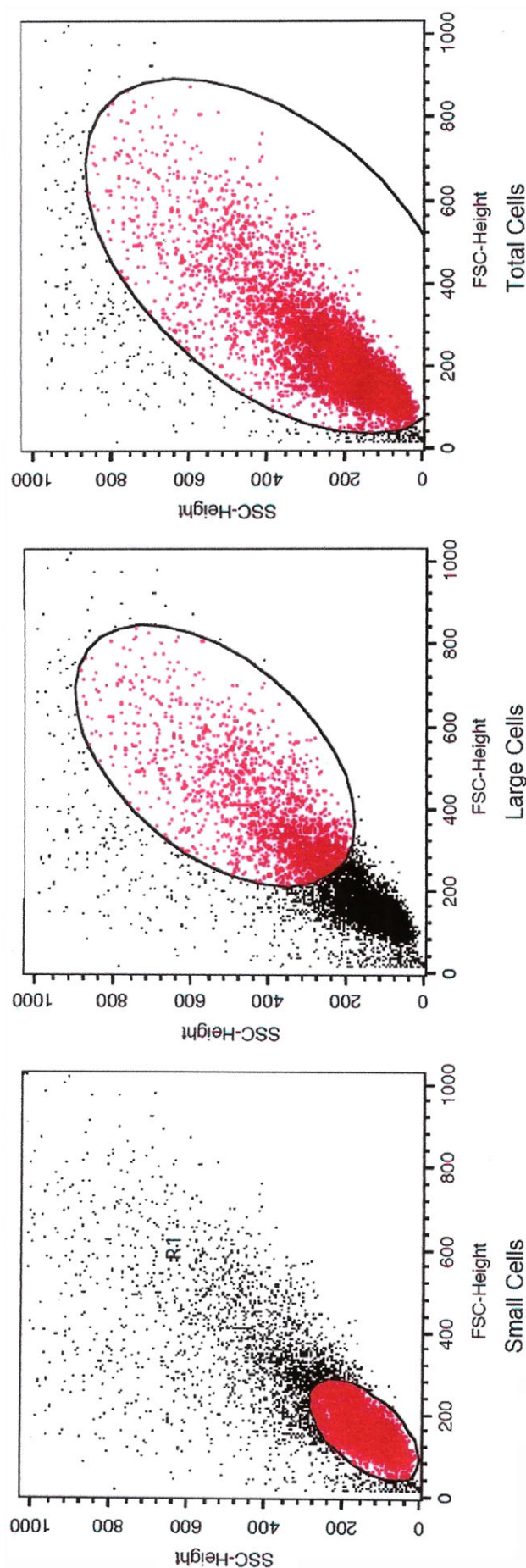


FIGURE 1. Analysis of adenoid cell populations via flow cytometry. Small, large, and general sets of cells were evaluated separately (within the oval zone). FSC = forward-scattered light; SSC = side-scattered light.

(GE Healthcare®, Little Chalfont, United Kingdom) according to the manufacturer's instructions. Total RNA was extracted from the obtained pellet with an RNeasy Mini Kit (Qiagen®, Venlo, Netherlands) according to the manufacturer's instructions. Aliquots of 1.0 μ g of RNA were reverse-transcribed into complementary DNA (cDNA) using Oligo(dT)20 primer (Invitrogen®, Carlsbad, CA) and Superscript III Reverse Transcriptase (Invitrogen®, Carlsbad, CA) according to the manufacturer's instructions. cDNA equivalent to 20 ng of the total RNA was subjected to RT-qPCR analysis in triplicate via real-time 7500 Fast PCR (Applied Biosystems®, Foster City, CA) according to the manufacturer's protocol (SYBR® Green PCR Master Mix; Invitrogen®, Carlsbad, CA). The PCR primers for CysLTR1, CysLTR2 and β -actin (Invitrogen®, Carlsbad, CA) were as follows: CysLTR1 forward primer 5'-TGACCGCTGCCTTTTATGTC-3' and reverse primer 5'-AGGAGAGGGTCAAAGCAACA-3', CysLTR2 forward primer 5'-TCCACTTGACGACATGGAAA-3' and reverse primer 5'-GGCCTTTTCTGAGTGCAGAC-3', and β -actin forward primer 5'-GGACTTCGAGCAAGAGATGG-3' and reverse primer 5'-AGCACTGTGTTGGCGTACAG-3'. The cycling conditions consisted of 1 cycle at 95°C for 3 minutes, 40 further cycles (95°C for 30 seconds and 60°C for 60 seconds), and a final dissociation cycle (95°C for 60 seconds and a stepwise increase from 55°C to 95°C). The CysLTR1 and CysLTR2 were expressed as ratios of the β -actin expression value. PCR results were analyzed using 7500 Software (Applied Biosystems®, Foster City, CA).

Data analysis

Clinical data were compared between groups using Student *t* test for continuous variables and the chi-square test for categorical variables. The correlations between data were measured by the Spearman's rank correlation coefficient (ρ). A *p* value <0.05 was adopted to indicate statistical significance.

Results

Clinical characteristics

Thirty children were included in each group. The clinical and demographic characteristics of the subjects are shown in Table 1. Children in the SE group had slightly higher PSQ scores.

Flow cytometry

Comparison between the SE and NS groups

The NS children showed higher expression of the CysLTR1 and CysLTR2 in all cell populations in the adenoid and tonsil tissues. In the adenoid cells, a significant difference existed in the expression of CD3+/CysLTR1+ small lymphocytes; CD3+/CysLTR1+, CD19+/CysLTR1+, and CD19+/CysLTR2+ large lymphocytes; and CD3+/CysLTR1+ in the total set of lymphocytes

TABLE 1. Clinical characteristics of the population under study

	SE (n = 30)	NS (n = 30)	p
Age (year)	7.5 ± 1.5	7.7 ± 1.4	ns
Sex, male, n (%)	18 (60)	11 (36)	ns
PSQ score	13.5 ± 2.2	12.1 ± 2.1	0.05
BMI	16.7 ± 1.6	17.0 ± 1.8	ns
BMI (z score)	0.8 ± 1.3	0.9 ± 1.2	ns
Tonsil grade	3.7 ± 0.6	3.9 ± 0.7	ns
Adenoid (% occlusion of rhinopharynx)	78.7 ± 9.1	78.3 ± 9.9	ns
Number of positive SPTs	1.7 ± 0.6	0 ± 0	<0.001

*Individuals were divided into SE and NS groups. Variables are presented as the means ± SD unless otherwise indicated.

BMI = body mass index; NS = nonsensitized group; ns = not significant; PSQ = Pediatric Sleep Questionnaire; SD = standard deviation; SE = sensitized group; SPT = skin-prick test.

(Table 2). In the tonsil cells, the difference was significant for the CD19+/CysLTR1+ small and large lymphocytes (Table 2).

Internal comparisons

A significantly greater expression of CysLTR2 compared with CysLTR1 was observed in all studied groups of adenoid cells, with the exception of small CD19 cells in the NS individuals (Table 3). The same pattern was observed in the tonsil cells (data not shown).

RT-qPCR

When all adenoid cells were considered, the number of messenger RNA (mRNA) copies of CysLTR1 and CysLTR2 normalized against β -actin was not significantly different between the groups (Table 4).

Clinical manifestation-to-cell correlations

When the percentages of adenoid cells expressing CysLTR1 and CysLTR2 were tested for correlations with the main clinical manifestations through Spearman's test, significant correlations were not observed (Table 5).

Discussion

The groups had similar clinical characteristics with the exception of the PSQ score that was higher among sensitized individuals, pointing to the greater severity of the SRD in these children. This finding corroborates the results of previous studies,²³ which indicated respiratory allergies as risk factors for obstructive sleep apnea syndrome (OSAS). However, these studies did not determine (by means of pathophysiology) whether such respiratory allergies are involved in adenoid hyperplasia.

The overexpression of CysLTs in the tonsil tissues of children with OSAS due to AH has been demonstrated.^{4,5,36} The enzymes 5-lipoxygenase, leukotriene A4 hydrolase, and leukotriene C4 synthase are highly expressed in tonsils, particularly in the mantle zone, where the expres-

sion of CysLTRs primarily occurs.^{18,37} Our results showed that CD19+ and CD3+ small cells, which are commonly present in the mantle zone, express CysLTRs at a high rate. An important effect of the activation of these receptors is an increase in the cell division rates.^{10–17}

Dayyat et al.³⁸ showed a reduction in tonsil cell division in cultures containing montelukast and BAY u9773. This effect was also associated with apoptosis induction in activated T lymphocytes exposed to crescent concentrations of montelukasts.¹² According to previously collected data and those found in this study, we suggest that, in tonsils, leukotriene synthesis and the activation of receptors culminate in cellular proliferation and hyperplasia.

SE children presented lower expressions of CysLTR1 and CysLTR2 than NS children, and no significant difference was observed in the adenoid and tonsil size between the 2 groups. Considering the pathway described above, this difference in expression may be explained by the greater concentration of cys-leukotriene in the mucosa of the allergic individuals.^{39,40} Thus, the balance between leukotriene release and the expression of its receptors reached an equilibrium in both groups. These data suggest a trend for a negative association between AH and AR and disagree with the authors' initial hypothesis.

A difference in expression has not been observed via RT-qPCR. This result may be attributed to the consideration of all cells as a whole; moreover, the main goal of this study was to quantify the expression of protein on the cell surface. The correlation tests did not point to a relation between the main clinical findings and any cell profile. These data suggest that all the subsets of cells, as well as both receptors, have similar roles in the pathophysiology of AH.

According to the results of the present study, virtually all SE and NS cell lines have higher expression of CysLTR2 than CysLTR1. Considering this finding and previous studies,^{18,38} we may suggest that both receptors are involved in cellular proliferation and hyperplasia. In clinical practice, drugs are only available to block CysLTR1, and these medications have shown effectiveness in improving

TABLE 2. Evaluation of adenoid cell or tonsil cell populations by flow cytometry*

	SE	NS	p
Adenoid cell population			
Small cells			
% of total adenoid cells	73.7 ± 6.3	71.4 ± 9.7	0.28
% CD3+ cells	32.5 ± 6.0	32.7 ± 3.1	0.87
% of CD3+ cells expressing CysLTR1	4.7 ± 2.3	6.6 ± 5.1	0.04
% of CD3+ cells expressing CysLTR2	9.6 ± 4.8	11.6 ± 8.0	0.23
% CD19+ cells	56.2 ± 11.4	54.7 ± 10.6	0.61
% of CD19+ cells expressing CysLTR1	30.6 ± 16.2	36.2 ± 13.8	0.15
% of CD19+ cells expressing CysLTR2	37.2 ± 17.2	42.2 ± 16.1	0.25
Large cells			
% of total adenoid cells	19.5 ± 4.4	20.6 ± 4.5	0.36
% CD3+ cells	20.5 ± 7.6	30.06 ± 8.6	0.09
% of CD3+ cells expressing CysLTR1	40.9 ± 14.6	47.6 ± 11.7	0.05
% of CD3+ cells expressing CysLTR2	52.2 ± 12.2	56.1 ± 11.3	0.20
% CD19+ cells	61.1 ± 8.0	61.5 ± 5.3	0.82
% of CD19+ cells expressing CysLTR1	44.6 ± 16.9	54.1 ± 12.4	0.01
% of CD19+ cells expressing CysLTR2	55.3 ± 11.3	61.5 ± 12.6	0.05
All cells			
% of total adenoid cells	93.7 ± 2.3	93.7 ± 2.8	0.97
% CD3+ cells	33.5 ± 6.6	36.4 ± 7.8	0.11
% of CD3+ cells expressing CysLTR1	11.1 ± 5.5	13.7 ± 6.2	0.04
% of CD3+ cells expressing CysLTR2	17.0 ± 7.5	19.2 ± 8.3	0.28
% CD19+ cells	53.7 ± 11.6	53.1 ± 11.2	0.82
% of CD19+ cells expressing CysLTR1	27.0 ± 12.0	32.2 ± 12.0	0.06
% of CD19+ cells expressing CysLTR2	36.2 ± 11.0	40.3 ± 15.0	0.23
Tonsil cell population			
Small cells			
% of total tonsil cells	67.7 ± 7.9	68.3 ± 6.3	0.20
% CD3+ cells	31.4 ± 6.0	39.3 ± 14.2	0.006
% of CD3+ cells expressing CysLTR1	4.8 ± 2.6	5.8 ± 5.0	0.30
% of CD3+ cells expressing CysLTR2	10.1 ± 4.2	10.5 ± 6.1	0.74
% CD19+ cells	50.6 ± 13.3	51.4 ± 9.2	0.79
% of CD19+ cells expressing CysLTR1	22.3 ± 13.0	28.2 ± 13.5	0.05
% of CD19+ cells expressing CysLTR2	34.0 ± 13.4	36.7 ± 13.0	0.43
Large cells			
% of total tonsil cells	22.1 ± 6.2	23.4 ± 6.7	0.43
% CD3+ cells	24.8 ± 7.4	25.7 ± 6.1	0.60
% of CD3+ cells expressing CysLTR1	34.8 ± 14.8	38.2 ± 9.8	0.30
% of CD3+ cells expressing CysLTR2	50.4 ± 13.5	52.1 ± 9.0	0.85

(Continued)

TABLE 2. Continued

	SE	NS	p
% CD19+ cells	64.2 ± 9.0	66.3 ± 6.4	0.66
% of CD19+ cells expressing CysLTR1	37.1 ± 17.4	44.6 ± 12.1	0.05
% of CD19+ cells expressing CysLTR2	50.4 ± 15.5	54.9 ± 10.9	0.20
All cells			
% of total tonsil cells	92.6 ± 2.9	92.2 ± 2.7	0.53
% CD3+ cells	33.2 ± 5.6	38.3 ± 8.0	0.005
% of CD3+ cells expressing CysLTR1	11.7 ± 6.4	13.0 ± 6.0	0.41
% of CD3+ cells expressing CysLTR2	19.2 ± 8.7	19.5 ± 7.5	0.88
% CD19+ cells	50.8 ± 12.0	51.3 ± 9.4	0.84
% of CD19+ cells expressing CysLTR1	23.7 ± 13.0	27.0 ± 12.0	0.30
% of CD19+ cells expressing CysLTR2	35.9 ± 13.4	37.6 ± 13.0	0.62

*Results are expressed as the means ± SD of percentages. The means were compared using Student t test.
NS = nonsensitized group; SD = standard deviation; SE = sensitized group.

TABLE 3. Comparison of receptor expression in each cell subgroup among the SE and NS individuals

	SE			NS		
	CysLTR1+	CysLTR2+	p	CysLTR1+	CysLTR2+	p
Small cells						
CD3+	4.6 ± 2.2	9.5 ± 4.8	<0.001	6.5 ± 5.0	11.6 ± 7.9	0.004
CD19+	30.6 ± 16.2	37.2 ± 17.1	0.07	36.2 ± 13.7	42.1 ± 16.1	0.12
Large cells						
CD3+	40.9 ± 14.5	52.2 ± 12.2	0.002	47.6 ± 11.7	56.2 ± 11.3	0.006
CD19+	44.6 ± 16.9	55.3 ± 11.3	0.005	54.1 ± 12.4	61.5 ± 12.6	0.002
Total cells						
CD3+	11.1 ± 5.5	17.0 ± 7.5	0.001	13.7 ± 6.2	19.2 ± 8.3	0.005
CD19+	27.04 ± 12.0	36.2 ± 11.0	0.003	32.2 ± 12.0	40.3 ± 15.0	0.002

*Results are expressed as the mean ± SD of percentages. Means were compared using Student t test.
NS = nonsensitive group; SD = standard deviation; SE = sensitive group.

TABLE 4. Relative genomic expression of CysLTR1 and CysLTR2*

	SE	NS	p
CysLTR1/ β -actin mRNA	1.17 ± 0.70	0.99 ± 0.62	0.37
CysLTR2/ β -actin mRNA	0.42 ± 0.78	0.91 ± 1.47	0.17

*Values are mean ± SD. Means were compared using Student t test.
mRNA = messenger RNA; NS = nonsensitive group; SD = standard deviation;
SE = sensitive group.

mild to moderate OSAS in children, even with the decreased expression of CysLTR1. BAY u9773 and BAY cysLT2 block CysLTR2 and are available exclusively for in vitro use, and their potential for inhibiting cell proliferation^{38,41} has been shown. Taking all these data together, we affirm

that the development of new drugs capable of blocking both CysLTR1 and CysLTR2 will have a positive impact in the nonsurgical treatment of AH.

The main limitation of this study is in regard to the SRD diagnosis; specifically, the authors used clinical questionnaires as references. However, the objectives were to study the cellular profile of symptomatic adenoid hyperplasia regardless of the severity. Additionally, the sensitivity and specificity of the PSQ justify its use in clinical studies, and according to Rosen et al.,⁴² this questionnaire has a sensitivity and specificity equivalent to those of polysomnography in diagnosing OSAS in children and is more encompassing due to its evaluation of SRD manifestations. The design used in our study (comparing allergic and nonallergic children) has not been previously used, and the number of individuals involved provides a relevant population

TABLE 5. Correlations between the clinical findings and the percentages of adenoid cells expressing CysLTRs

	SE			NS	
	PSQ score	NO (%)	Pricks+	PSQ score	NO (%)
Small cells					
CD3+/CysLTR1+	0.00	0.04	0.28	0.00	-0.14
CD3+/CysLTR2+	0.11	0.15	0.34	-0.09	-0.12
CD19+/CysLTR1+	-0.28	-0.23	0.13	-0.20	-0.36
CD19+/CysLTR2+	0.00	0.01	0.35	-0.16	-0.41
Large cells					
CD3+/CysLTR1+	0.02	0.02	0.06	-0.03	0.13
CD3+/CysLTR2+	0.24	0.01	0.23	0.13	-0.13
CD19+/CysLTR1+	-0.12	-0.14	0.02	-0.07	-0.28
CD19+/CysLTR2+	0.10	-0.05	0.16	0.05	-0.23
Total cells					
CD3+/CysLTR1+	-0.02	-0.01	0.16	-0.18	-0.25
CD3+/CysLTR2+	0.09	0.08	0.31	-0.14	-0.18
CD19+/CysLTR1+	-0.27	-0.17	0.16	-0.21	-0.36
CD19+/CysLTR2+	-0.07	0.01	0.25	-0.18	-0.36

*Values are the Spearman ρ with significance of 0.01.

NO = nasopharyngeal occlusion; NS = nonsensitive group; Pricks+ = number of positive skin-prick tests; PSQ = Pediatric Sleep Questionnaire; SE = sensitive group.

representation. Moreover, the use of flow cytometry provides a very precise measure of the cellular profiles.

Conclusion

In conclusion, this study showed that the expression of CysLTR1 and CysLTR2 is higher in the lymphocytes of hyperplastic tonsils in nonallergic children. Thus, respiratory allergies do not appear to be a stimulus for AH occurrence but may intensify OSAS symptoms.

Newer drugs capable of blocking both leukotriene receptors may provide benefits in the treatment of AH and SRD. 

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