

Cysteinyl-leukotrienes in nasal lavage fluid in children with asthma

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The aim of this study was to investigate repeatability of cysteinyl-leukotrienes (cys-LT) measurements in nasal lavage fluid (NLF) and to determine if cys-LT levels in NLF are related to asthma severity in children. As a second outcome, we investigated if cys-LT in NLF reflect lower airway inflammation as assessed by exhaled NO measurement. To assess the repeatability of cys-LT measurements, two NLF samples were obtained from eight healthy controls 24 h apart. Sixty-nine asthmatic children (mean age; range: 12.8; 7.3–17.7 yr), which were grouped according to asthma severity were studied cross-sectionally on one occasion. Cys-LT in NLF were analyzed using a specific enzyme immunoassay, exhaled NO, and pulmonary function parameters were measured. The coefficient of repeatability for the repeated cys-LT measurements was 1.45 pg/ml. Cys-LT levels in NLF differed significantly between asthma severity groups ($p < 0.001$): mild intermittent: [median (IQR)] 6.88 pg/ml (2.00–27.87); mild persistent: 21.09 pg/ml (4.50–84.67); and moderate persistent asthmatics: 36.41 pg/ml (11.03–118.40). Concentration of cys-LT in NLF and exhaled NO was positively correlated ($r = 0.85$; $p < 0.001$). In conclusion, concentration of cys-LT in NLF correlates with asthma severity in children and is related to lower airway inflammation.

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While Cysteinyl-leukotrienes (cys-LT: LTC₄/D₄/E₄) are pro-inflammatory mediators in asthma pathophysiology, leukotriene receptor antagonists (LTRA) were shown to improve control of asthma in children (1). On the contrary, subgroup analyses of clinical baseline characteristics in asthmatic children aged 2–14 yr were not able to predict, which patients are more likely to successfully respond to LTRA therapy (2).

Measurement of an inflammatory marker that can predict the response of asthmatic children to LTRA treatment would be helpful in the management of childhood asthma. It has been shown that LTRA treatment reduces the concentration of cys-LT in exhaled breath condensate (EBC) (3) and in nasal lavage fluid (NLF) (4) of children with asthma. Investigating nasal lavage (NL) can provide an insight into disease-related upper airway changes, and is well tolerated in children

and infants (5, 6). Sampling NLF is a simple and non-invasive technique allowing not only measurement of non-volatile molecules but also analysis of cellular components (5, 7). As sampling duration is generally much shorter (10 s) than, i.e., sampling of EBC (usually 10 min), the technique of NL could be more feasible for young children. Inflammatory markers in nasal lavage fluid (NLF) have been shown to reflect to some extent lower airway inflammation (7–10), and to correlate with asthma phenotypes (11, 12). Furthermore, cys-LT have been detected in NLF in atopic (13) and asthmatic children (4).

The aim of this study was to determine whether measurement of cys-LT in NLF is reproducible and related to asthma severity in children. As a second outcome, we investigated if cys-LT in NLF are correlated to exhaled NO (eNO) reflecting lower airway inflammation.

Materials and methods

Study design

The study was divided into two parts. First, we assessed repeatability of measurements of cys-LT in NLF 24 h apart in a group of healthy children with no respiratory diseases. Secondly, levels of cys-LT in NLF were measured cross-sectionally in children with clinically stable asthma. Furthermore, in a randomly selected subgroup of these children, we measured eNO and correlated eNO to cys-LT in NLF.

Subjects

Repeatability study. Eight healthy control subjects (mean age; range: 12.7; 7.9–16.3 yr) were recruited from the general outpatient clinic; they had no history of asthma, no history of allergic rhinitis, and atopic dermatitis, respectively, and no respiratory tract infection in the last 2 wk as evidenced by interview and physical examination. Allergic sensitization was not assessed in healthy subjects.

Cross-sectional study. Asthmatic patients were recruited from the asthma outpatient clinic at the Department of Pediatric and Adolescent Medicine of the Medical University Vienna. Inclusion criteria were: age between 6 and 18 yr and clinically stable asthma. Children were excluded if they had other severe diseases, a respiratory tract infection and allergic rhinoconjunctivitis symptoms in the last 2 wk or treatment with oral steroids, nasal steroids, and LTRA, respectively, in the last 4 wk prior to the study.

Sixty-nine children (mean age; range: 12.8; 7.3–17.7 yr) with clinically stable asthma were studied. Asthma was diagnosed according to American Thoracic Society (ATS) guidelines (13) and severity was classified according to Global Initiative for Asthma (GINA) guidelines (14) into three asthma severity groups: mild intermittent, mild persistent, and moderate persistent asthma. Allergic sensitization was assessed in all asthmatic children by skin prick test to common airborne allergens, performed annually in the outpatient clinic. Inhaled steroids were prescribed to 47 of 69 asthmatics. None of the patients received treatment nasal steroids at the time of the study.

As the chemoluminescence analyzer used for eNO measurements was erratically available because of servicing throughout the study period, eNO measurements were performed only in a random subgroup of 23 asthmatic children. The study protocol was approved by the Ethics

Committee of the Medical University of Vienna. Informed written consent was obtained from both children and parents.

Methods

Exhaled NO. Fractional eNO was measured using a chemoluminescence analyzer (NIOX: Aerocrine, Stockholm, Sweden) according to published European Respiratory Society (ERS)/ATS guidelines (16). Exhaled NO measurements were always performed before nasal lavage and spirometry.

Nasal lavage. Nasal lavage was performed as previously described (17). Briefly, NLF was obtained by instilling 5 ml of lukewarm saline solution (NaCl 0.9%) with a syringe into each nostril. The child sitting with the head bent to the neck was instructed to hold its breath for 10 s and then expel the fluid into a specimen cup. NLFs recovered from each nostril were pooled and centrifuged (500 *g* for 15 min) at +4°C. The supernatant was purified with an affinity resin (Cayman Chemical, Ann Arbor, MI, USA) and stored at –80°C for later analysis. Concentrations of cys-LT in NLF were analyzed with a specific enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA). To halve the detection limit samples were concentrated. One milliliter of supernatant was resuspended in 0.5 ml of buffer solution. For samples below the lowest limit of detection (2 pg/ml), data was expressed as 1 pg/ml (18).

Spirometry. Spirometry was performed with a spirometer (Masterlab, Jaeger, Wuerzburg, Germany) according to ATS standards (19). Forced expiratory volume in 1 s (FEV₁) and forced vital capacity (FVC) were expressed as percent predicted from published normative data (20).

Statistical analysis. The data were statistically analyzed by non-parametric tests. Within-group comparisons were performed by the Wilcoxon signed-rank test, between-group comparisons by the Mann–Whitney *U* test. Kruskal–Wallis one-way analysis of variance was used to test for differences of concentrations of cys-LT in NLF between subjects in three asthma severity groups. Correlations between cys-LT in NLF and exhaled NO were determined by Spearman correlation analysis. Results were expressed as means and s.d. or medians and interquartile ranges (IQR), as appropriate. Bland and Altman methods (21) were used to graphically assess repeatability and to calculate the limits of

agreement between repeated measurements of cys-LT in NLF (mean $\pm$ 2 s.d. of differences) and the coefficient of repeatability (twice the s.d. of differences). A p -value < 0.05 was considered statistically significant. All data were analyzed using SAS (Version 8; SAS Institute Inc., Cary, NC, USA).

Results

Repeatability study

Mean concentrations of cys-LT in NLF samples of healthy controls ($n = 8$) on day 1 and on day 2 were 6.48 pg/ml (range: 1.84–17.84) and 6.38 pg/ml (1.00–16.66), respectively. Mean difference between eight pairs of repeated measurements was -0.09 pg/ml and the limits of agreement (1.41 to -1.60 pg/ml) were small (Fig. 1). The coefficient of repeatability (CR) for the repeated measurements cys-LT in NLF was 1.45 pg/ml. Concentrations of cys-LT in NLF were below the detection limit in four NLF samples of control subjects.

Cross-sectional study

Main subject characteristics are shown in Table 1. In asthmatic subjects median NLF retrieval was 5.40 ml (IQR, 1.4–7.4). There was no significant difference in NLF yield between asthma severity groups. NLF retrieval significantly correlated with subject age ($r = 0.59$; $p < 0.001$) but not with levels of cys-LT in NLF ($r = -0.10$; $p = 0.460$) in asthmatic

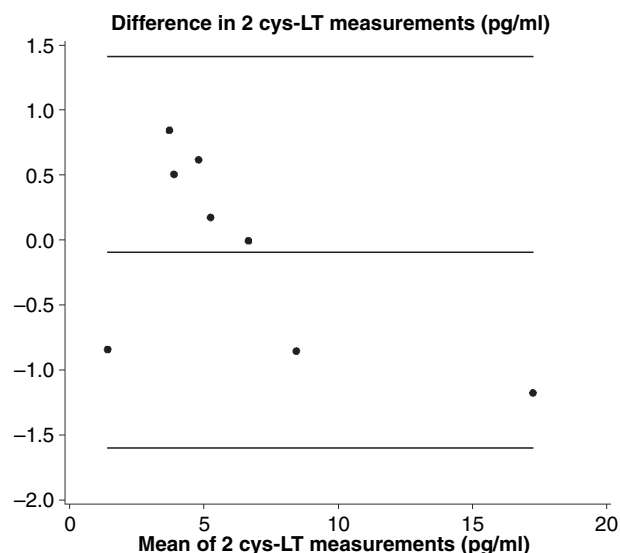


Fig. 1. Repeatability of cys-LT measurements in nasal lavage fluid samples of healthy controls ($n = 8$) within 24 h, Bland and Altman plot. Lines represent mean difference and upper and lower limits of agreement.

Table 1. Subject characteristics

	Subjects with asthma		
	Mild intermittent	Mild persistent	Moderate persistent
N (male)	26 (17)	34 (20)	9 (5)
Age, yr*	13.4 $\pm$ 2.3	12.9 $\pm$ 3.3	11.1 $\pm$ 2.3
FEV ₁ % predicted*†	103.6 $\pm$ 10.6	100.0 $\pm$ 11.1	75.3 $\pm$ 4.0
FVC % predicted*‡	105.4 $\pm$ 10.6	101.4 $\pm$ 12.1	90.1 $\pm$ 11.5
Allergic sensitization (n)	22	34	8
EIB§ (n)	0	14	4
Inhaled steroids (n)	16	24	7
Low dose¶	13	16	4
Intermediate dose**	3	7	3
High dose††	0	1	0

*Data are presented as means $\pm$ s.d.

†Forced expiratory volume in 1 s as percent predicted.

‡Forced vital capacity as percent predicted.

§Exercise induced bronchoconstriction symptoms during the last 2 months.

¶Low dose: 100–200 μ g fluticasone or budesonide daily.

**Intermediate dose: >200–400 μ g fluticasone or >200–600 μ g budesonide daily.

†† High dose: >400 μ g fluticasone or >600 μ g budesonide daily.

subjects. Levels of cys-LT in NLF did not correlate with subject age ($r = 0.08$; $p = 0.468$). Concentrations of cys-LT in NLF were below the detection limit in three samples of asthmatic children ($n = 69$).

Cys-LT in NLF and asthma severity

Levels of cys-LT in NLF differed significantly ($p < 0.001$) between the asthma severity groups (Fig. 2). Cys-LT in NLF of children with moderate persistent asthma were significantly increased compared with children with mild persistent and mild intermittent asthma [median (IQR): 36.41 pg/ml (11.03–118.40) vs. 21.09 pg/ml (4.50–84.67) and 6.88 pg/ml (2.00–27.87); Fig. 2]. Children with mild persistent asthma had significantly higher concentrations of cys-LT in NLF compared with children with mild intermittent asthma (Fig. 2). Cys-LT in NLF correlated with GINA guidelines (15) severity scale ($r = 0.55$; $p < 0.001$) and to a lesser extent with parameters of spirometry [FEV₁ % predicted ($r = 0.31$; $p = 0.013$), and FVC % predicted ($r = 0.28$; $p = 0.027$)]. Doses of inhaled steroids used in asthmatic children did not differ significantly between the three severity groups (Table 1). There were no significant differences in concentrations of cys-LT in NLF between ICS-treated and steroid-naïve asthmatic children.

Asthmatic children who reported exercise induced bronchoconstriction (EIB) symptoms during the last 2 months had higher concentrations of cys-LT in NLF compared with asthmatic

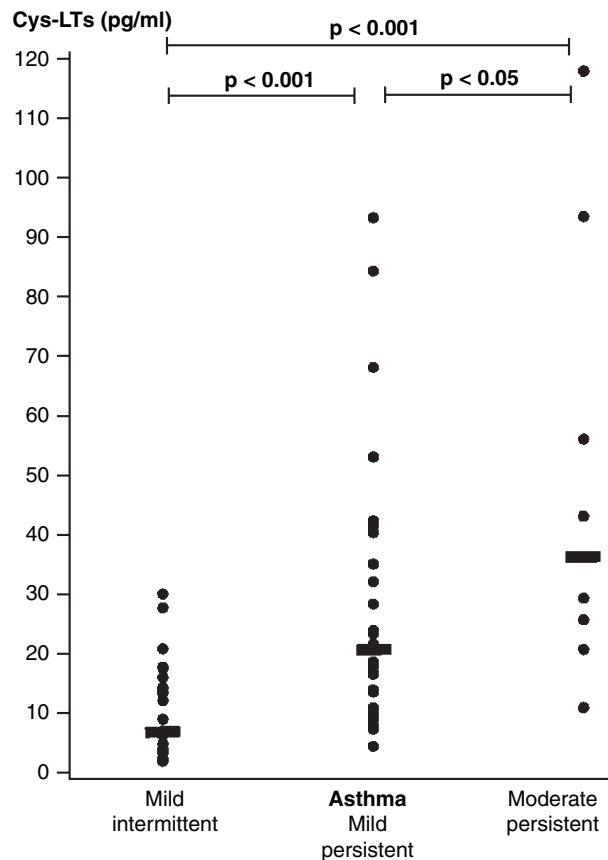


Fig. 2. Concentrations of cys-LT found in nasal lavage fluid from clinically stable asthmatic children plotted against Global Initiative for Asthma (GINA) (14) scale of severity. Individual data and medians are shown. P values were calculated by Mann-Whitney *U*-test.

children without EIB symptoms during the last 2 months [median (IQR): 36.42 pg/ml (7.51–93.87) vs. 13.82 pg/ml (2.00–35.23); $p < 0.001$].

Cys-LT in NLF and exhaled NO

Exhaled NO was measured in 23 asthmatic children (seven mild intermittent, 14 mild persistent and two moderate persistent asthmatics). All subjects but mild intermittent asthmatics received ICS treatment. Two children with mild intermittent asthma were non-atopic.

Median (IQR) eNO was 16.2 ppb (7.20–92.60), median concentration of cys-LT in NLF was 9.55 pg/ml (3.29–35.23). There was a significant strong positive correlation between cys-LT in NLF and eNO ($r = 0.85$; $p < 0.001$; Fig. 3). This correlation was still significant when assessed only in ICS-treated children ($n = 16$; $r = 0.82$; $p < 0.001$) but not in steroid-naïve asthmatic children ($n = 7$; $r = 0.33$, $p = 0.381$). No significant difference in eNO between ICS-

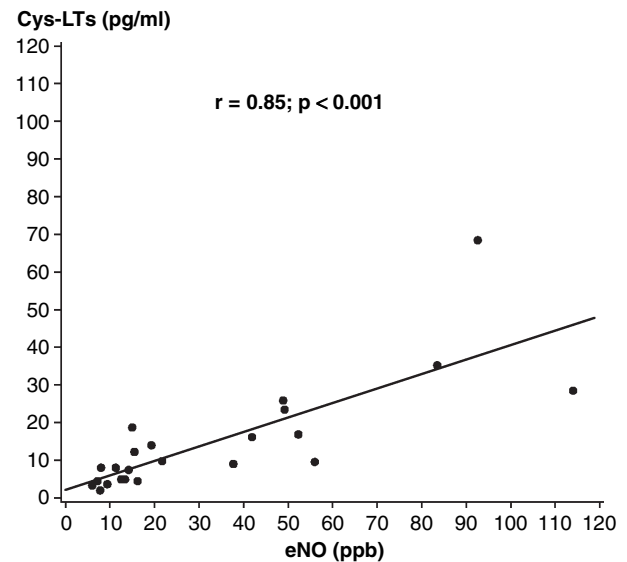


Fig. 3. Correlations between cys-LT in nasal lavage fluid and fractional exhaled NO (eNO) from children with mild-to-moderate asthma. Individual values and Spearman correlation coefficient are shown.

treated and steroid-naïve asthmatic children was detected. Exhaled NO did not correlate with FEV₁ % predicted ($r = -0.37$, $p = 0.085$) and FVC % predicted ($r = -0.05$; $p = 0.816$), respectively.

Discussion

In this study, we have investigated, if measurements of cys-LT in NLF are repeatable and related to asthma severity in children. Repeated measurements of cys-LT in NLF exhibited good repeatability within 24 h. The Bland-Altman plot shown in Fig. 1 demonstrates the agreement between the first and the second measurement of cys-LT in NLF. The repeatability over 24 h of measurements of cys-LT in NLF (CR = 1.45 pg/ml) was at least as good as the repeatability over 24 h reported of measurements of cys-LT in EBC of healthy children (CR = 3.2 pg/ml) (18). We did not investigate if shortened dwell time of lavage fluid in the nose is feasible for measurements of cys-LT in NLF. In our study, the vast majority of the subjects was able to produce a 10-s breath hold with water in the nose. However, a shortened dwell time for sampling NLF might be more eligible for younger children (6). Dalt et al. (6) has recently shown that sampling NLF by instillation and immediate aspiration of normal saline solution is feasible in infants with respiratory syncytial virus (RSV) bronchiolitis and healthy controls. The shorter protocol for sampling

NLF can certainly provide an advantage in young children not cooperating with other non-invasive methods such as EBC collection, which usually takes about 10 min. Moreover NLF sample volumes and cys-LT concentrations in our study were larger compared with standard EBC samples (18, 22) facilitating the quantification of low cys-LT levels.

In our study, asthma severity was associated with the concentration of cys-LT in NLF. Consistent with our findings several studies showed that cys-LT in EBC are increased in children with asthma (18, 24), levels of cys-LT in EBC are related to disease severity (18, 23, 24) as well as exercise induced asthma (25) and are correlated with reticular basal membrane thickness, a marker for airway remodeling in asthma (3). Elevated levels of cys-LT have also been found in bronchoalveolar lavage fluid of children with recurrent wheeze (26) and asthma (27). These findings support that cys-LT measured in different physiological fluids are related to different pediatric asthma phenotypes.

Our finding that there is no difference in concentrations of cys-LT in NLF of stable ICS-treated asthmatic children and stable steroid-naïve asthmatic children is in line with previous studies (23, 24) showing that levels of cys-LT in EBC did not differ significantly from ICS treated and untreated asthmatic children.

Our study also showed for the first time that levels of cys-LT in NLF may reflect lower airway inflammation in steroid-treated asthmatic children as assessed by eNO. Zanconato et al. (24) found a positive correlation between cys-LT in EBC and eNO in steroid-naïve asthmatic children. However, others have reported a lack of correlation between eNO and cys-LT in EBC (18, 23) in steroid treated, and between eNO and urinary cys-LTE₄ (28) of steroid-naïve asthmatic children. Concerning steroid-naïve children, our study was underpowered (n = 7) to show a significant correlation between eNO and cys-LT in NLF.

Because of the heterogeneity of the clinical response to LTRA therapy (2) additional phenotype information by measuring cys-LT in physiological fluids may enable better prediction of treatment response. Szeffler et al. (29) have recently shown that the response to montelukast, a specific LTRA, is related to concentrations of urinary cys-LTE₄ in steroid-naïve children with mild-to-moderate asthma. As in our study cys-LT in NLF were correlated with asthma severity and represented lower airway inflammation, this method might be suitable to design a RCT with the aim to identify children with asthma most

likely to exhibit beneficial treatment response when taking a LTRA.

Limitations of the study

Allergic rhinitis is a common concomitant disease in asthmatic children. We tried to minimize the influence from allergic nasal inflammation by excluding children with allergic rhinoconjunctivitis symptoms in the last 2 weeks and children with nasal steroid use in the last 4 weeks prior to the study. Nevertheless, we could not completely exclude that nasal inflammation contributed to some extent to the levels of cys-LT in NLF measured. As most of the children were sensitized to different allergens, the analyses could not be stratified for atopy.

Furthermore, we did not assess reproducibility of the NLF retrieval rate in our study, however, potentially differing NLF retrieval rate did neither seem to affect repeatability of cys-LT measurements in healthy children nor did NLF yield correlate with cys-LT in NLF of asthmatic children. Of importance is that NLF retrieval rate correlated with subject age but not with cys-LT in NLF of asthmatic and healthy children. These findings suggest that cys-LT in NLF may be reliably quantified in children as young as 7 yr irrespective of NLF retrieval rate. In summary, measurements of cys-LT in NLF show good repeatability. Cys-LT in NLF correlate with asthma severity and lower airway inflammation in asthmatic children.

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