Functional Analysis of Airway Remodeling is Related with Fibrotic Mediators in Asthmatic Children

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Abstract

Background: Asthmatic children present variable degrees of airway inflammation, remodeling and resistance, which correlates with disease control and severity. Chronic inflammatory process of the airways triggers airway remodeling, which reflects the degree of airway resistance. Pro-inflammatory and pro-fibrotic mediators are centrally involved in this process. This study has investigated for the first time, whether the levels of pulmonary and systemic pro-inflammatory and pro-fibrotic mediators present correlation with the resistance of respiratory system and of proximal and distal airways. Methods: 24 asthmatic children (persistent mild and moderate) and 24 non-asthmatic children (both between 6-13 years old) were evaluated for anthropometric characteristics, lung function and mechanics, pulmonary and systemic immune response. Results: Asthmatic children showed an increased number of blood eosinophils ($p<0.04$), basophils ($p<0.04$), monocytes ($p<0.002$) and lymphocytes ($p<0.03$). In addition, asthmatic children showed an impaired lung function, as demonstrated by FEV1%pred. ($p<0.0005$) and FEV1/FVC ($p<0.004$), decreased total resistance of respiratory system (R5Hz; $p<0.009$), increased resistance of proximal airways (R20Hz; $p<0.02$), increased elastance (Z5Hz; $p<0.02$) and increased reactance (X5Hz; $p<0.002$). Moreover, the following inflammatory factors were significantly higher in asthmatic than non-asthmatic children: GM-CSF in the breath condensate (BC) ($p<0.0001$) and in the serum ($p<0.0001$); TGF-beta in the BC ($p<0.0001$) and in the serum ($p<0.004$); IL-5 in the BC ($p<0.02$) and in the serum ($p<0.01$); IL-4 in the serum ($p<0.0002$). Conclusions: Impulse oscillometry is a sensitive method to detect airway resistance in asthmatic children, reflecting airway remodeling, an event followed by increased levels of pro-inflammatory and pro-fibrotic mediators.

1. Introduction

Asthma is a heterogeneous disease and is defined as a chronic airway inflammation, leading to a variable airflow limitation, resulting in respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity [1]. The disease affects more than 334 million people worldwide and, according to the world health organization, 14% of children have ever suffered from any asthma symptom [1].

This high rate of asthma prevalence in children, as well as the rates of uncontrolled asthma and asthma exacerbation leads high rates of school absenteeism [2]. In addition, acute exacerbations remain one of the main causes of emergency department visits in childhood asthma [3]. In this context, innovative methods that could precisely detect the possibility of exacerbations is highly needed. Thus, impulse oscillometry system (IOS) had demonstrated to be useful technique to detect possible exacerbations, since IOS revealed to be much sensitive than traditional lung function test, spirometry [3]. In addition, some parameters measured by IOS, such as total airway resistance (R20Hz), proximal airway resistance (R5Hz), distal airway resistance (R5Hz – R20Hz) can reflect, when increased, some degree of airway remodeling, as compared with multidetector computed tomography (MDCT) imaging [4,5]. In fact, airway remodeling is a central feature of asthma and is directly related to disease control and progression [4].

Several inflammatory and fibrotic mediators such as Th2 cells related cytokines, eicosanoids and growth factors are released in the lungs as well as in the systemic circulation [6]. In this context, nitric oxide (NO) is a noninvasive biomarker which is most widely used in the assessment of airway inflammation. In the respiratory tract NO is released by different type of cells (epithelial cells, airway nervous, inflammatory cells and from vascular endothelium). A high concentration of NO is observed in asthmatics when compared with normal individuals [6]. In addition, high levels of NO have been associated with loss of control in asthma, hyperresponsiveness and airway remodeling [7]. Furthermore, high levels of NO are believed to trigger the release of pro-inflammatory mediators and growth factors, being related to more severe forms of asthma, such as corticosteroid resistant asthma [8]. Airway remodeling is known to be mediated by both pro-inflammatory and pro-fibrotic mediators [9].

Therefore, the present study investigated, for the first time, if asthmatic children present impaired lung mechanics associated with increased accumulation of pro-inflammatory and pro-fibrotic growth factors.

2. Material and Methods

2.1. Study design

All procedures performed in this study have been approved by local ethical committee from *Universidade Brasil* (registration number 2.939.688).

Twenty-four asthmatic children and twenty-four non-asthmatic children were recruited and took part in the study, after parents' agreement and signature of the term of consent. To be include into the study, children must had at least [?] 6 months in clinical treatment and be clinically stable, that is, at least 2 months without exacerbation. The exclusion criteria were to be non-obese [(BMI [?]20) evaluated by BMI and multi-frequential octopolar bioimpedance Bioscan 920 II-S (% Body Fat [?] 20%) Maltron Inc, Essex, England], non-hypertense, non-dyslipidemic, without cardiovascular diseases, never smokers (even not passive smokers), previous respiratory diseases (except asthma and bronchitis) and neurological diseases. The anthropometric characteristics of children enrolled in the study is presented in Table 1.

2.2. Lung Function and Mechanics

Spirometry and impulse oscillometry were performed to measure pulmonary function by using Jaeger Masterscreen pulmonary function instrument (Masterscreen IOS, Erich Jaeger, Hoechberg, Germany) in strict accordance with the American Thoracic Society/European Society of Respiratory Diseases guidelines [10]. The reference values for spirometry were specific for the Brazilian population. Total respiratory impedance (Z5Hz), total resistance of respiratory system (R5Hz), resistance of proximal airways (R20Hz), resistance of distal airways (R5-R20Hz), and pulmonary reactance (X5Hz), resonance frequency (Fres), and respiratory impedance (Z5Hz) were recorded by impulse oscillometry as described previously [11].

2.3. Exhaled Nitric Oxide

The levels of nitric oxide in the exhaled air was measured by chemiluminescence by using the NOBreath monitor (Bedfont Scientific) [12]. The results were expressed in parts per billion (ppb).

2.4. Systemic and Pulmonary Immune Response

Five milliliters of venous blood were collected by using vacuum tubes containing EDTA K2 as anticoagulant. The whole blood analysis (white and red series) was performed using the automated system Sysmex 800i (Sysmex Europe GmbH, Germany). Immediately after whole blood analysis, the blood tubes were centrifuged at 1000g, for 7 minutes at 4°C. The serum was stored until the measurements of pro-inflammatory and pro-fibrotic mediators.

For analysis of pulmonary immune response, the levels of pro-inflammatory and pro-fibrotic mediators were measured in breath condensate, which has been collected using RT Tube (Respiratory Research, TX, USA) according to the manufacturer's recommendations.

Thus, the levels of GM-CSF, TSLP, IL-4, IL-5 and TGF-beta was measured both in the serum as well as in the breath condensate by DuoSet ELISA kits (R&D Systems; MN, USA) through using a microplate reader Spectramax I3 (Molecular Devices, CA, USA).

2.5. Statistical Analysis

The graphs were built and the data were analyzed by using SigmaStat 5.0 software (California, USA). Normality of the data was evaluated by the Kolmogorov-Smirnov test. The data were submitted to an unpaired t test for a comparison between the groups. Statistical significance was set at p-value < 0.05.

3. Results

3.1. Volunteers Anthropometric Characteristics

Table 1 shows the anthropometric characteristics of non-asthmatic and asthmatic children. Except for age ($p<0.04$), other physical characteristics were not significantly different between asthmatic and non-asthmatic with asthmatic children ($p>0.05$) (Table 1).

3.2. Lung Function

Figure 1 shows the lung function parameters analyzed (Figure 1A and 1B, FVC; Figure 1C and 1D, FEV1; Figure 1E and F; FEV1/FVC; respectively absolute and % of predicted values) comparing non-asthmatic with asthmatic children. The results demonstrated that asthmatic children present impaired FEV1%pred. ($p<0.0005$) and FEV1/FVC ($p<0.004$) when compared with non-asthmatic children.

3.3. Lung Mechanics

Figure 2 shows the lung mechanics parameters analyzed (Z5Hz, X5Hz, R5Hz, R20Hz, R5Hz-R20Hz) comparing non-asthmatic with asthmatic children. Asthmatic children showed impaired impedance of respiratory systems (Z5Hz; $p<0.02$), respiratory reactance (X5Hz; $p<0.002$); proximal airways resistance (R20Hz; $p<0.02$), total resistance of respiratory system (R5Hz; $p<0.009$) and resistance of peripheral/distal airways (R5Hz-R20Hz; $p<0.01$).

3.4. Cellular and Humoral Systemic Immune Response

Figure 3 shows the cellular systemic immune response between non-asthmatic and asthmatic children, which has been evaluated by the whole blood analysis. Asthmatic children demonstrated increased density of lymphocytes (Figure 3D; $p<0.02$ and 3E; $p<0.003$), monocytes (Figure 3F; $p<0.002$ and 3G; $p<0.001$), eosinophils (Figure 3H; $p<0.008$ and 3I; $p<0.04$) and basophils (Figure 3J; $p<0.04$ and 3K; $p<0.01$).

Figure 4 shows the humoral systemic immune response between non-asthmatic and asthmatic children, which has been evaluated by humoral mediators' measurement in the blood serum. The results showed increased levels of GM-CSF (Figure 4A; $p<0.0001$), TGF-beta (4B; $p<0.004$), IL-4 (Figure 4C; $p<0.0002$), IL-5 (Figure 4D; $p<0.01$) in asthmatic children as compared with non-asthmatic children, while for TSLP no differences were found ($p>0.05$).

3.5. Pulmonary Immune Response

Figure 5 shows the humoral pulmonary immune response between non-asthmatic and asthmatic children, which has been evaluated in the breath condensate (BC). There was an increase in the levels of pulmonary GM-CSF (Figure 5A; $p<0.0001$); TGF-beta (Figure 5B; $p<0.0001$) and IL-5 (Figure 5D; $p<0.02$) in asthmatic compared to non-asthmatic children, while no differences were found for IL-4 (Figure 5C; $p>0.05$) and TSLP (Figure 5E; $p>0.05$).

3.6. Pulmonary Levels of Nitric Oxide

Figure 5F shows that asthmatic children demonstrated an increased level of exhaled nitric oxide compared with non-asthmatic children (Figure 5F; $p<0.02$). The results were expressed in parts per billion (ppb).

4. Discussion

This study shows for the first time that asthmatic children presenting increased airway resistance together with the increased levels of pro-inflammatory and pro-fibrotic factors, beyond increased levels of exhaled nitric oxide.

Systemic eosinophilc inflammation has been considered as an important cellular biomarker to classify different asthma phenotypes and to predict response to treatments [13]. In summary, blood eosinophils above 300 cells/μl is an important cellular biomarker to predict severe asthma and/or corticosteroid resistant asthma [13]. In the present study, it was observed that the group of asthmatic children enrolled in the study have values ranging 200 cells/μl until nearly 500 cells/μl, corresponding to values above 6% of eosinophils. In this context, it has been established that such profile of patients presents more severe hyperresponsiveness, bronchospasm and airflow limitation [14]. However, in the present study, while we only measure the hyperresponsiveness indirectly using pre- and post-bronchodilator for spirometry, without a confirmation of hyperresponsiveness, the susceptibility to bronchospasm and airflow limitation were additionally accessed by pre- and post-bronchodilator response to impulse oscillometry, which has been done for the first time. In this context, the present study found that asthmatic children presented not only impaired FEV1%pred and FEV1/FVC response, as classically found, but also impaired lung mechanics, as denoted by impaired impedance of respiratory systems (Z5Hz), respiratory reactance (X5Hz); proximal airways resistance (R20Hz), total resistance of respiratory system (R5Hz) and resistance of peripheral/distal airways (R5Hz-R20Hz). In addition, a slight but novel mechanistic investigation was performed, as demonstrated by increased levels of systemic and pulmonary pro-inflammatory humoral mediators (GM-CSF, IL-4, IL-5) and pro-fibrotic mediators (TGF-beta).

Increases in the levels of Th2 pro-inflammatory (GM-CSF, IL-4, IL-5) and pro-fibrotic mediators (TGF-beta) are key players in the process of airway remodeling, stimulating hypertrophy and hyperplasia of airway smooth muscle and of airway epithelial cells, leading to exaggerated mucus and extracellular matrix proteins production and accumulation [15]. In this view, the present study shows for the first time that increased airway resistance in asthmatic children, which reflect functionally the airway remodeling, is followed by increased levels of Th2 pro-inflammatory mediators as well as the growth factor TGF-beta, not only in the systemic circulation, but also into the lungs, as demonstrated in the breath condensate.

Beside Th2 mediators and growth factors, nitric oxide (NO), has also been linked to asthma development, severity and exacerbations [6, 7, 8, 9]. NO is an instable gas, considered a highly reactive nitrogen specie, which present a very short half-life. NO can be measured noninvasively in the exhaled air by chemiluminescence, by using bench [16] or portable [12] NO detecting devices. In fact, increased levels of NO have been associated not only with several aspects of asthma [6, 7, 8, 9], but also with exercise-induced bronchoconstriction, which is a highly prevalent characteristic of asthmatic patients [16, 17]. In the present study, it was found that asthmatic children presented high levels of exhaled NO, ranging among 25-30 ppb, which clearly indicates an inflammatory process of the airways. However, although these levels of NO already indicate airway inflammation, it was not enough to predict asthma exacerbation or even hyperresponsiveness [18], as observed in the present study (no response [?] 10% in the FEV1%pred after bronchodilator). On the other side, the increased levels of NO were associated with increased levels of systemic and pulmonary Th2 cytokines (GM-CSF, IL-4 and IL-5) as well as with growth factor TGF-beta. In fact, pre-clinical studies

have classically reported that high levels of NO is involved in exacerbated Th2 cell related airway inflammation, remodeling and hyperresponsiveness, while the blocking of inducible nitric oxide synthase (iNOS), responsible for very high levels of NO synthesis, can prevent or even revert this asthmatic phenotype (Th2 airway inflammation, remodeling and hyperresponsiveness) [19, 20].

Conclusions

We conclude that impulse oscillometry is a very sensitive method to detect airway resistance in asthmatic children, which reflect the airway remodeling, an event followed by increased levels of pro-inflammatory and pro-fibrotic mediators, which has been also observed in the lungs, in the breath condensate, as well as systemically, in the present study.

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Figure legends

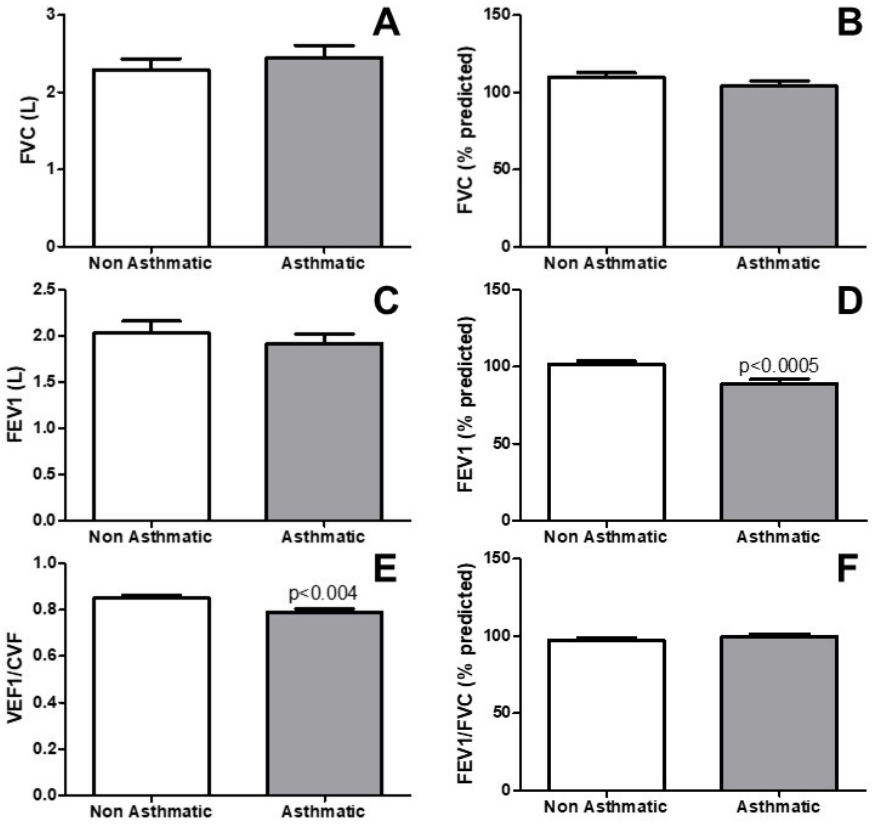
Figure 1 . Analysis of lung function by spirometry. Forced vital capacity (FVC); forced expiratory flow in the first second (FEV1); FEV1/FVC.

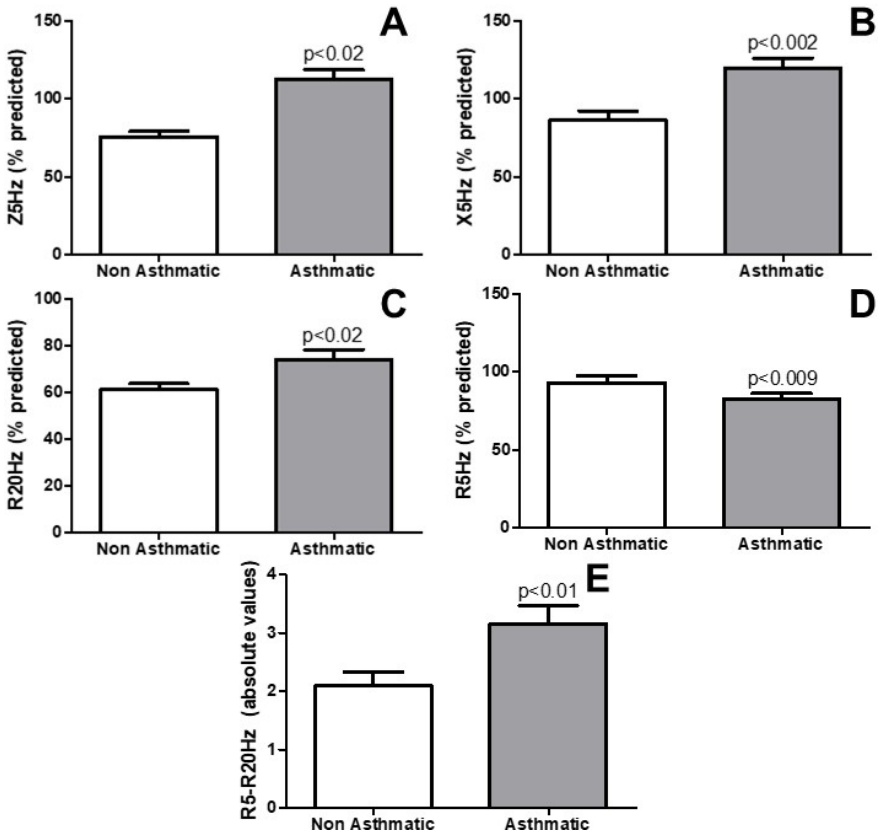
Figure 2 . Analysis of lung mechanics by impulse oscillometry system (IOS). Total respiratory impedance (Z5Hz), pulmonary reactance (X5Hz), resistance of proximal airways (R20Hz), total resistance of respiratory system (R5Hz), resistance of distal airways (R5-R20Hz).

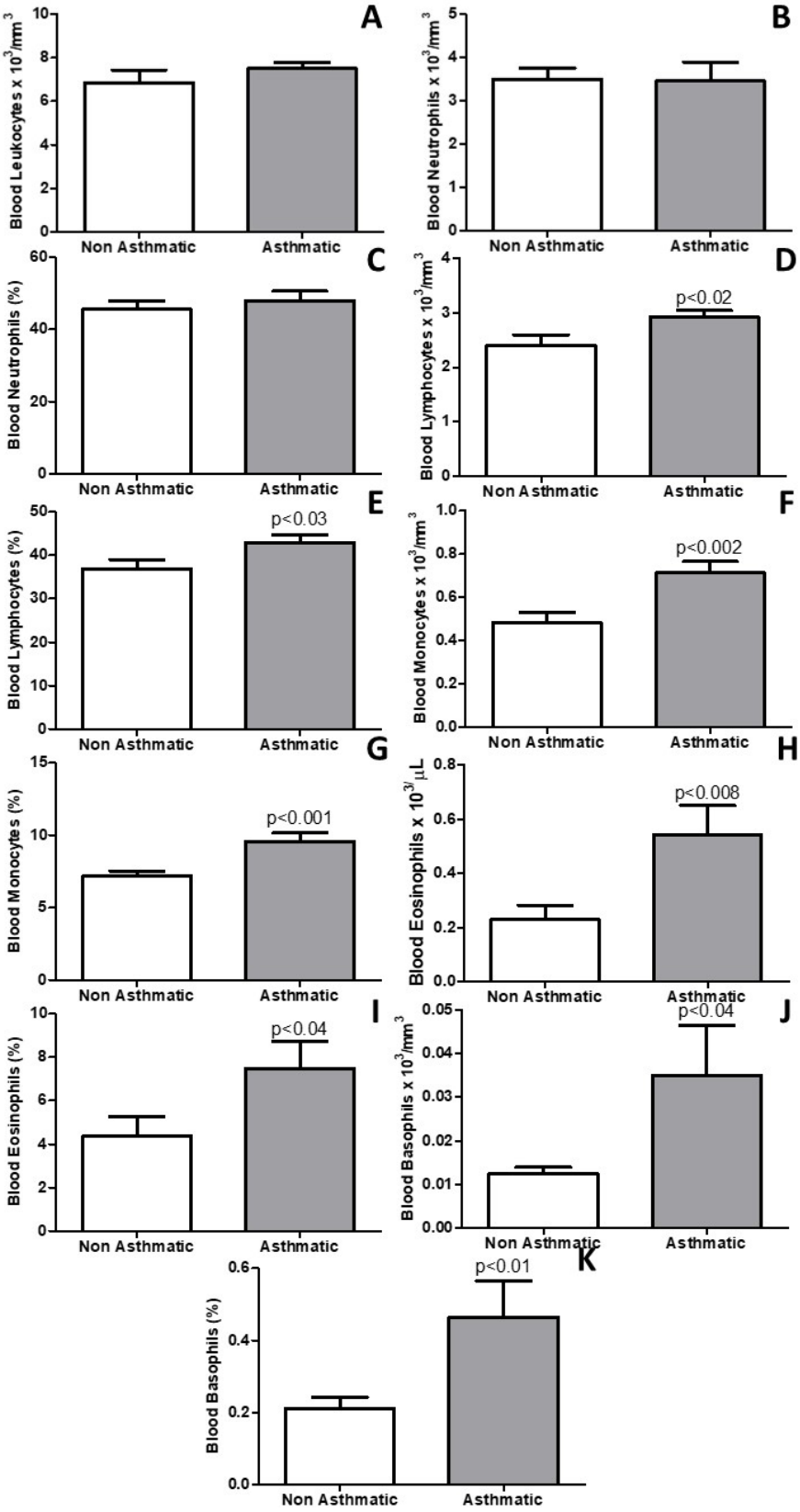
Figure 3 . Whole blood analysis, including total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils and basophils.

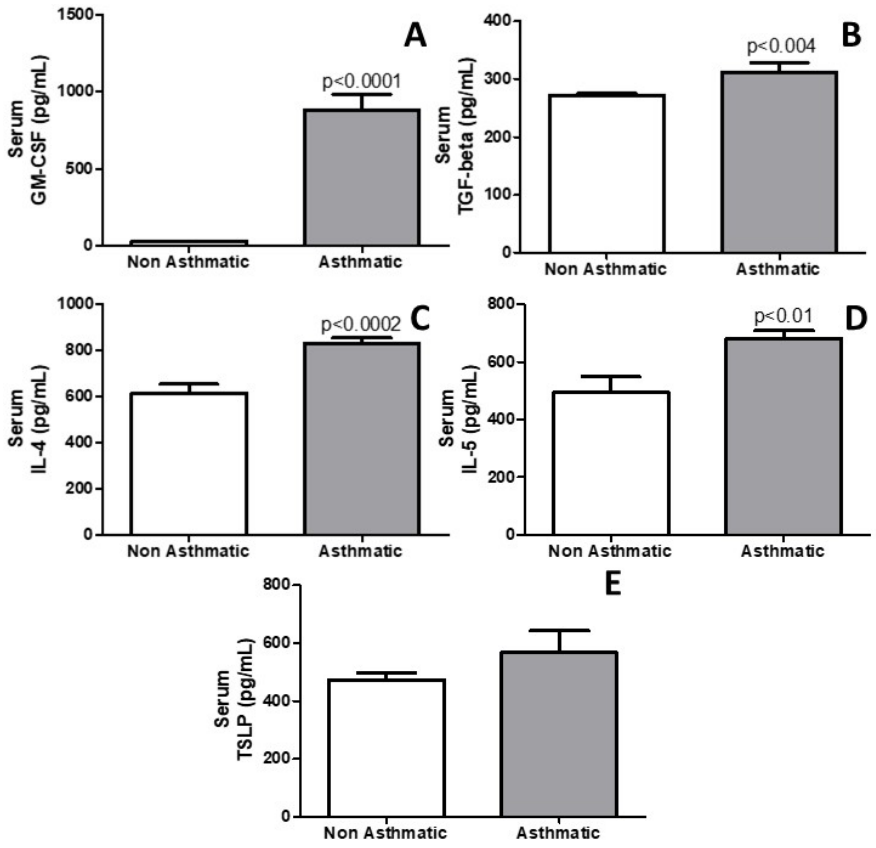
Figure 4 . Analysis of serum humoral mediators. Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF); transforming growth factor beta (TGF-beta); interleukin 4 (IL-4), interleukin 5 (IL-5), Thymic stromal lymphopoietin (TSLP).

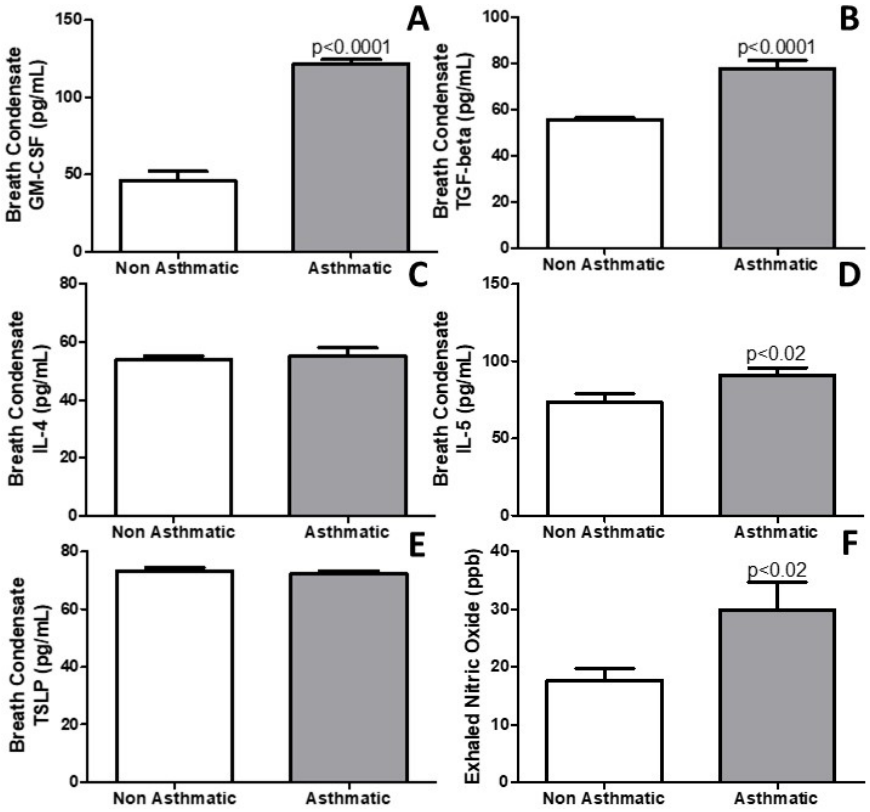
Figure 5 . Analysis of pulmonary humoral mediators in the breath condensate. Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF); transforming growth factor beta (TGF-beta); interleukin 4 (IL-4), interleukin 5 (IL-5), Thymic stromal lymphopoietin (TSLP) and exhaled nitric oxide (eNO).











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