Association of selected inflammatory biomarkers with cough reflex sensitivity in asthmatic children

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Abstract
Bronchial asthma is the most common chronic respiratory disease of childhood. Cough is one of its defining symptoms. This study investigated the associations between selected inflammatory biomarkers and cough reflex sensitivity after capsaicin inhalation in children with mild and moderate well-controlled type 2 endotype asthma compared with non-asthmatic probands. Sensitivity to the cough reflex was measured by recording the cough response after capsaicin inhalation. The sandwich ELISA method was used to measure serum concentrations of the investigated potential inflammatory biomarkers (interleukin 13, interleukin 1β, eosinophil-derived neurotoxin). The acquired data were statistically evaluated according to descriptive analyses for summarization and comparison between cough reflex sensitivity parameters and individual biomarker values in the observed and control groups modeled by a simple linear regression model. Statistical significance was defined as $p<0.05$. We showed a statistically significant association (p-value 0.03) between cough reflex sensitivity - C2 value (capsaicin concentration required for two cough responses) and interleukin -1β serum concentrations in the asthma group compared with the control group of non-asthmatic children. Our results support the possibility of interleukin-1β as a potential additive inflammatory biomarker used in clinical practice in children with asthma because of its correlation with the activity of the afferent nerve endings in the airways.

Keywords: asthma, biomarkers, children, cough, cough hypersensitivity, interleukin 1 beta

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Introduction

Cough is the most commonly reported symptom of respiratory diseases and one of the dominant components of the clinical manifestation of bronchial asthma in children. Accordingly, cough is a complex respiratory phenomenon mediated by the activation of the arc of the cough reflex. Bronchial asthma is the most common chronic inflammatory respiratory disease in the pediatric population with a constantly increasing incidence and prevalence. The combination of genetic predisposition and various endogenous and exogenous

ethiopathogenetic determinants leads to the development of chronic allergic or non-allergic airway inflammation, resulting in the development of bronchial hyperresponsiveness to specific and nonspecific factors [1]. Asthma is a disease of many faces. The concept of asthma is now considered a clinical entity representing several diseases with defined pathological pathways (endotypes) with variable clinical presentation (phenotypes). Measurable and reproducible indicators that link the different endotypes of asthma with phenotypes represent useful asthma biomarkers [2]. From a pathophysiological perspective, the chronic airway inflammation is a defining feature of asthma. Clinically useful noninvasive biomarkers can be used for measuring the degree of immunologically defined airway inflammation. Chronic asthma inflammation exerts comprehensive multimodal effects on the potential increase in afferent neuronal sensitization and response to cough stimuli. Various inflammatory mediators, cytokines and adhesion molecules are thought to be involved in modulating the peripheral sensitization of the cough reflex [3]. The interaction between these interrelated factors in the microenvironment affected by inflammation increases the neuroplasticity of the cough reflex. These circumstances play an important role in the onset of cough hypersensitivity syndrome. Under these conditions, the cough reflex in asthmatics can be initiated by subthreshold stimuli that do not trigger a cough response in the general population [4]. Cough reflex sensitivity even in children with well controlled mild or moderate asthma could be increased compared to healthy controls [5]. Therefore, some asthma biomarkers could serve as an additive tool for assessing control of disease. The correlation of asthma biomarkers and the grade of cough reflex reactivity could also be used for this purpose.

Aim of the study

The aim of our study was to determine the severity of chronic airway inflammation using well-defined inflammatory biomarkers in correlation with the reactivity of the afferent nerve endings in the airways in a cohort of children with bronchial asthma.

Methods

To assess the reactivity of the afferent nerve endings, we used the cough reflex sensitivity (CRS) test with capsaicin inhalation. We correlated the selected biomarkers of airway hypersensitivity in children with bronchial asthma with the reactivity of airway afferent nerve endings (CRS). Selected biomarkers according to clinical usefulness are generally defined in **Table 1**.

Study design, setting and participants

We enrolled 25 asthmatic children and 15 healthy controls in the study. This study was conducted at the National Institute of Pediatric Tuberculosis and Respiratory Diseases, Dolny Smokovec, Slovakia, between 2018 and 2019. The inclusion criterion for the tested subjects with asthma was diagnosed T2 endotype of mild or moderate and well-controlled asthma. Asthma severity was assessed retrospectively from the level of treatment required to control symptoms and exacerbations in the last year according to GINA guidelines [6]. Mild asthma is asthma that is well controlled with step 1 or step 2 treatment and moderate asthma requires manageable control with step 3 treatment. The patients' asthma control status has also been evaluated in accordance with GINA guidelines [6]. Patients with asthma were verified for the study by board-certified pediatric pneumologist based on either of the following criteria: more than 2 episodes of wheeze within the previous year, the usage of reliever (bronchodilator) more

than 3 times within the previous year or hospital admission due to respiratory insufficiency and wheeze in the previous year. Each child in the tested group of asthmatics had a history of spirometry-proven reversal of bronchial hyperreactivity and atopy with in vitro or in vivo diagnosed sensitization to aeroallergens. Asthmatics were free of worsening disease for at least one month. Healthy controls without any medications had undergone necessary evaluations, which had ruled out asthma or other respiratory diseases. Children with an acute infection in the past 14 days, a history of other chronic respiratory or non-respiratory diseases (such as cardiovascular, perinatal abnormality, severe primary or secondary immunodeficiency, malignancy), and obesity (body mass index > 97 percentile) were excluded.

After written informed consent was obtained from the legal guardians, all participants underwent obtaining of detailed medical history and physical examination. Blood samples were collected for determining biomarkers according to the manufacturer's protocols. All anti-asthmatic drugs were discontinued at least 48 h before performing lung function tests. On the third day after admission to the department, the spirometry was performed before and after the CRS examination with a baseline level of FEV1 greater than 80%.

The study was approved by the institutional Ethics Committee of the National Institute of Pediatric Tuberculosis and Respiratory Diseases, Dolny Smokovec, as a prospective clinical control study (ID01052018). Human investigations were carried out following the rules of the Declaration of Helsinki of 1975, as revised in 2013.

Pulmonary function tests

Pulmonary function tests were performed using a spirometry device Geratherm Spirostik (*Geratherm Respiratory GmbH, Germany*) according to the recommendation of the European Respiratory Society [7]. Main dynamic ventilatory parameters (forced expiratory volume in the first second - FEV1, forced vital capacity - FVC, and their ratio FEV1/FVC% were recorded. Participants also underwent a salbutamol bronchodilatation test to confirm or exclude bronchial hyperresponsiveness.

Assessment of cough reflex sensitivity

The reactivity of the afferent nerve endings was measured by recording the cough response after capsaicin inhalation, following the guidelines of the European Respiratory Society with pediatric modification by Varechova [8,9]. Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is a plant alkaloid responsible for the spicy taste of peppers. Each tested subject randomly inhaled saline (0.9% sodium chloride solution) between 12 inhalations of protocolary gradually doubling capsaicin aerosol (0.61 – 1250 µmol/l) using the one-breath method. Each inhaled solution was administered for 60 seconds intervals with 400 msec long-lasting inhalation. The monitored parameters represent capsaicin concentrations that induce 2 coughs (C2 value) and 5 coughs (C5 value) recorded within 30 seconds after capsaicin administration. The test was ended after reaching a capsaicin concentration that caused five or more coughs. We used a compressor air nebulizer (*model 646; DeVilbiss Health Care, Inc., Somerset, PA, USA*) and a spirometer with automatic timing and capsaicin dosing (*KoKo DigiDoser-Spirometer; nSpire health Inc., Louisville, CO, USA*) with an additional control valve (*RIFR; nSpire health Inc., Louisville, CO, USA*) to ensure a constant inspiratory flow rate.

Measurement of serum biomarkers

Sandwich ELISA (enzyme-linked immunosorbent assay) is the plate-based assay technique designed for detecting and quantifying soluble substances such as proteins. We used it to measure serum concentrations of IL-13 (*Interleukin 13 ELISA kit, Wuhan Fine Biotech Co., Ltd., China*), IL-1β (*Human IL-1 beta ELISA kit, Wuhan Fine Biotech Co., Ltd., China*) and EDN (*RNASE2 ELISA kit, Abbexa Ltd., Cambridge, UK*).

Statistical analysis

The data obtained were analyzed with R statistical software (*R Foundation for Statistical Computing, Vienna, Austria; URL https://www.R-project.org/, version 4.0.3*). Median, lower, and upper quartile statistical parameters were used to summarize the data gained. We used a box plot overlaid with a swarm plot to examine the differences in data between patients with asthma and controls. The null hypothesis for the equality of population medians in asthma and control groups was evaluated with the Wilcoxon test. Statistical associations between CRS parameters and individual values of biomarkers in the observed and control groups were visualized by cross-plot and modeled by a simple linear regression model with an interaction term. Regression was performed on a log-log scale and model parameters were estimated using the ordinary least squares method. Multivariate logistic regression analysis was used to quantify the association between predictors (age, biomarkers, and CRS parameters) and group (asthma, control) for the response variable. Findings with a significance level $p < 0.05$ were considered statistically significant.

Results

Descriptive statistics and clinical features of children with asthma

The tested group consisted of 25 asthmatic children (11 boys and 14 girls, mean age 9 ± 1 year) and 15 healthy controls (6 boys and 9 girls, mean age 8 ± 1 year). The demographics and clinical characteristics of the study participants are shown in **Table 2**. There were no significant differences between children with asthma and the control group in terms of age, gender, and body mass index.

Out of 25 children with asthma, 19 (76 %) had mild and 6 (24 %) had moderate asthma. Thirteen children (52%) with asthma have been receiving regular asthma controller treatment with inhaled corticosteroids. As expected, children with asthma had significantly higher levels of total IgE and eosinophil count. No one in the control group was sensitized to any aeroallergens. Spirometry parameters were comparable according to descriptive analysis in both groups. Eleven children with asthma had a positive salbutamol bronchodilator test before performing the assessment of CRS.

Descriptive statistics of biomarker levels and acquired parameters of CRS

All cooperating participants with the consent of their guardians were prospectively recruited into the study once the inclusion criteria had been met. Serum was collected 2 days before performing CRS for ELISA testing of biomarkers. Capsaicin concentrations causing two (C2) and five (C5) cough responses were obtained. The results of descriptive statistical analyses (median/p50, upper/p75, and lower/p25 quartiles) of the outcomes are shown in **Table 3.** No significant differences in serum levels of IL-13, IL-1β and EDN were found between the study

groups. Also, the obtained CRS parameters (C2, C5) did not statistically differ between the two groups.

Linear regression analyses for C2 parameter of CRS and serum levels of IL-1β

Simple linear regression of logarithmic ranked data demonstrated a statistically significant (p-value 0.03) positive association (slope 1.59) between log (CRS_C2) and log (IL-1β) values in the asthma group (**Table 4**). The association between log (IL-1β) and log (CRS_ C2) was negative (slope -0.09) and statistically insignificant (p-value 0.06) in the control group. Statistical associations between CRS_C2 and log (IL-1β) values are shown using a cross-plot (**Figure 1**).

Logistic regression of group on age, IL-13, IL-1β, EDN, SPD, CRS_C2, CRS_C5

None of the predictors was statistically significantly associated with the asthma/control group variable in the multivariate regression model. Model selection by the Akaike Information Criterion identified IL-13, IL-1β, and CRS_C2 as the important predictors. However, none of them was statistically significant. Tjur´s R^2 was 0.292.

Discussion

Our study pointed to a mutual relationship between the activity of afferent nerve endings in the airways responsible for the origin and modulation of cough and selected inflammatory biomarkers in children with bronchial asthma. Bronchial asthma is predisposed as a suitable candidate for personalized and evidence-based precision medicine due to the heterogeneity of the disease with several defined endotypes and phenotypes. Novel non-invasive biomarkers represent a suitable tool for achieving these therapeutic and diagnostic goals. The identification of clinically applicable biomarkers could help to improve and refine the diagnosis of asthma (asthma endotyping) and to improve the estimation and monitoring of the therapeutic response of treated patients (asthma phenotyping). Cough is one of the most common and one of the defining core symptoms of asthma. The intensity and frequency of coughing in chronic airway inflammation condition affect the quality of life of an asthma-treated patient. Thus, successful therapeutic management of chronic cough is one of the conditions for achieving disease control. Therefore, subjective evaluation of cough is an important part of asthma management and is also included in various questionnaires to monitor disease activity. The question of the relationship between various disease biomarkers and the degree of reactivity of the cough response is theoretically coming into focus. This indirect objectification could contribute to a better diagnosis and therapy of asthmatic children after statistical confirmation of the hypothesized associations.

In our study, we demonstrated the potential of IL-1β as an inflammatory biomarker of asthma in children due to the confirmed correlation between IL-lβ and the reactivity of airway afferent nerve endings in clinical study. We did not confirm an association of CRS with other biomarkers investigated (IL-13, EDN) in asthmatics compared to controls.

Interleukin-1 is one of the final products of inflammasome activation. The inflammasome consists of many functionally linked structural intracellular molecules located in myeloid cells. Interleukin-1 belongs to the first line of the innate immune response to foreign antigen determinants [10]. Once activated, the components of interleukin-1 trigger an inflammatory cascade. The products of activation modulate the body's inflammatory response

[11]. Two related forms of interleukin-1 exist, including IL-1α and IL-lβ with similar structures and functions but with different affinity for coupled receptors. Specifically, IL-1β enhances the activation of B lymphocytes, Th-2 lymphocytes and IgE immunoglobulin production [12].

Based on the published expert studies, IL-1β belongs to a wide group of potential asthma biomarkers. Natural lymphoid cells type 2 (ILC-2) as a part of innate immunity are also critical in the pathogenesis of the Type 2 asthma endotype. These cells represent the equivalent to CD4+ lymphocytes with essential functions in the defense of the body against pathogens; however, ILC-2 cause remodeling of the airway epithelium after damage [13]. Besides, after activation and stimulation by epithelial cytokines - alarmins (IL-25, IL-33, TSLP), ILC-2 produce a spectrum of variable cytokines IL-5, IL-13, and IL-9 due to the presence of exogenous noxes (pollutants, allergens, microorganisms) [14]. Moreover, ILC-2 are an important source of IL-13, a central cytokine of T2 inflammation with pluripotent activity responsible for mucus overproduction, susceptibility to bronchoconstriction, and airway remodeling [15]. Also, IL-1β acts as an essential activator of ILC-2 responsible for proliferation, cytokine production by ILC-2, and regulator of epithelial cytokine receptor expression in the context of ILC-2 in the pathogenesis of T2-type inflammation [16]. Importantly, recurrent respiratory syncytial virus (RSV) infections in predisposed infants increase the risk of developing asthma. Severe RSV infection in the bronchial epithelium generates excessive immune responses modulated by Th-2 lymphocyte clones, which persist even after the disease resolution [17]. IL-1β levels are elevated during RSV infection suggesting the involvement of IL-1β in the disease pathogenesis [18]. Interestingly, the inhibition of IL-1β and uric acid metabolites reduced the infiltration of ILC-2 into the lung in a neonatal experimental model of RSV bronchiolitis, which could contribute to stopping the development of T2 inflammation [17]. The pathological IL-1β-induced signaling pathway determined both Th-2-mediated and neutrophil-mediated airway infiltration in an experimental murine model of virus-triggered asthma exacerbation [19].

Other studies have confirmed the multifactorial role of IL-1β in the etiopathogenesis of asthma. Bronchial hyperresponsiveness in the pathophysiological functional presentation underscores the basic symptoms of asthma. IL-1β is also a key modulator of bronchial hyperresponsiveness by affecting toll-like and muscarinic signaling pathways associated with airway epithelial cell receptors [20]. Additionally, in the field of asthma genomics, several interleukin-1 signaling pathway gene polymorphisms (IL-1RN, IL-1RAP, IRAK3, and PELI1) have been confirmed to be related to childhood asthma pathogenesis [12]. The authors of another prospective comparative study demonstrated an association between some IL-1β gene polymorphisms (rs1143634, rs1143633, rs1143643) and a higher risk of occurrence of asthma in children [21]. There is also association between polymorphism of interleukin-1 beta and interleukin-1 receptor antagonist gene and asthma risk [22] and a new locus associated with time to asthma onset at 16q12 was identified [23].

Clinical studies also confirm the important role of IL-1β in asthma pathogenesis in the pediatric population [17,18,21,24,25]. One of the strongest potential predisposing factors affecting the development of the allergic endotype of asthma in school-aged children is recurrent viral infections with bronchoconstriction, particularly of RSV and rhinovirus etiology. Furthermore, in this field, the importance of IL-1β in the pathogenesis of asthma was validated. Infant probands from the referred clinical study with acute RSV infection had an increased level of IL-1β in the tracheal aspirates with higher predispositions to acquire asthma at school age

[17]. Results of recent clinical study demonstrated that children with asthma have significantly higher IL-1β levels compared to the control group [24]. The neonatal airway immune profile in a prospective observational clinical study showed enhanced IL-1β levels with the development of elevated specific IgE to inhaled allergens, a positive skin prick test, and allergic rhinitis at 6 years of age [25]. Consequently, IL-1β hypothetically links two sequentially emerging clinical entities that lead to an increased susceptibility to chronic cough under conditions of bronchial hypersensitivity.

This is the first clinical study to deal with associations of changes in cough reflex sensitivity with selected biomarkers of bronchial asthma according to the available databases of medical research (EBSCO, PUBMED, SCOPUS). In our previous research we confirmed, that FENO values do not correlate with cough reactivity in childhood asthmatics or healthy controls [26]. Based on our results, we decided to implement our intention for a new concept of the clinical entity of cough hypersensitivity syndrome. One of the main mechanisms of the increased tendency to cough is assumed to be neuronal dysregulation of sensory fibers and the central processing of tussigenic stimuli. Similarly, the microenvironment of asthmatic inflammation is a factor that creates the conditions for cough hypersensitivity [27,28]. Considering the importance of cough hypersensitivity, it is crucial to reveal the individual causative links between deteriorative factors of asthmatic cough with clinically reproducible and applicable tools represented by non-invasive biomarkers.

At this time, the main limitation of the presented study is the small number of probands in both groups, which may have partially affected the lower performance of the statistical analysis. The small number of enrolled probands was mainly due to the onset of the COVID-19 pandemic. Therefore, further, and larger investigations are necessary to confirm our findings.

Conclusion

The results suggest the potential of IL-1β as an inflammatory biomarker of asthma in children due to the confirmed correlation between IL-lβ and the reactivity of airway afferent nerve endings (cough reflex sensitivity). Complementary IL-1β testing could improve the treatment of asthmatic children by focusing on the control of chronic cough.

Author contribution

P.K. and P.F. performed cough reflex sensitivity testing, lung function tests, and their interpretation. M.G. performed statistical analysis. P.K., J.F., P.F. determined the diagnosis of asthma. K.I. measured serum concentrations of biomarkers by ELISA method. R.P. and T.H. supervised the work manuscript. All authors proofread the manuscript and met the criteria for authorship.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Table 1 *Main clinical features of selected inflammatory biomarkers*

Biomarker	Full name	Clinical and etiopathogenic highlights
IL-13	**Interleukin 13**	• central effector cytokine of the T2-mediated inflammatory process [29] • the activity of IL-13 determines the proliferation and differentiation of eosinophils and mast cells, the activation of fibroblasts and the secretion of airway mucus • strongly involved in the process of bronchial hyperresponsiveness and airway remodeling
IL-1β	**Interleukin 1β**	• one of the major pro-inflammatory cytokines produced by the activation of the inflammasome • can activate eosinophils and is involved in bronchoconstriction induced by muscarinic bronchial smooth muscle receptors [20]
EDN	**Eosinophil-derived neurotoxin**	• one of the secretory granule mediators that is effused in the extracellular space after eosinophil activation • the amount of this biomarker secreted by eosinophils indicates the functional activity of these cells [30]

Table 2 *Demographics and clinical characteristics of study patients*

	Asthmatics	Controls
No.	25	15
Age, y (range)	9 (8 – 12)	8 (8 – 11)
Male-to-female ratio	11:14	6:9
Weight, kg (range)	40.76 (22 – 68)	38.2 (26 – 49)
BMI, kg/m2 (range)	19.8 (13,5 – 26,1)	19.5 (14,4 – 25,4)
Height, cm (range)	141.80 (125 – 162)	140.2 (129 – 153)
Controller		
None	8	0
LTRA alone	4	0
ICS+LTRA	7	0
ICS/LABA	1	0
ICS/LABA+LTRA	5	0
Asthma severity (GINA)		
Mild/moderate/severe	19/6	0
Sensibilization on aeroallergens		
Skin prick test	21	0
Specific IgE	7	0
Total IgE, IU/l (range)	300.20 (4 – 3270)	89.33 (5,22 – 391)
Eosinophil count (per/µl)	778.44 (67 – 1950)	170.2 (62 – 456)
Passive smoking exposure	10	9
Spirometry		
FEV 1, % (range)	103.42 (82 – 121)	110.93 (91 – 134)
FVC, % (range)	101.34 (87 – 130)	100.60 (86 – 119)
Positive (salbutamol) bronchodilation test	11	0

BMI – Body Mass Index, LTRA - Leukotriene Receptor Antagonists, ICS – Inhaled Corticosteroids, LABA - Long-Acting Beta Agonists, GINA – Global Initiative for Asthma, IgE – immunoglobulin E, FVC – forced vital capacity, FEV1 – forced expiratory volume at the end of the first second of forced expiration

Table 3 *Median, lower, and upper quartile estimated for the asthma and control group with p-value from Wilcoxon test*

parameter	**unit**	**asthma group (n=25)**			**control group (n=15)**			**p-value**
		median	lower quartile	upper quartile	median	lower quartile	upper quartile	
age	years	10.00	9.00	11.00	11.00	10.00	11.50	0.3
IL-13	pg/ml	16.00	16.00	63.00	16.00	16.00	16.00	0.15
IL-1β	ng/ml	1.80	1.50	2.80	2.00	1.60	3.45	0.39
EDN	ng/ml	100.00	71.00	414.00	75.00	69.00	776.00	0.71
CRS_C2	µmol/l	10.00	3.00	39.00	5.00	2.00	5.00	0.048
CRS_C5	µmol/l	78.00	39.00	547.00	39.00	20.00	234.00	0.37

IL – interleukin, EDN - Eosinophil-derived neurotoxin, CRS_C2 – cough reflex sensitivity C2 value, CRS_C5 – cough reflex sensitivity C5 value

Table 4 *Parameters of the estimated log (CRS_C2) regression model in the log (IL-1β) group*

parameter	**estimate**	**std. error**	**t-value**	**p-value**
intercept	1.37	0.54	2.56	0.02
log IL-1β	1.59	0.70	2.29	0.03
group control	0.16	0.80	0.20	0.84
log IL-1β: group control	-1.68	0.85	-1.97	0.06

IL-1β – interleukin 1β, CRS_C2 – cough reflex sensitivity C2 value

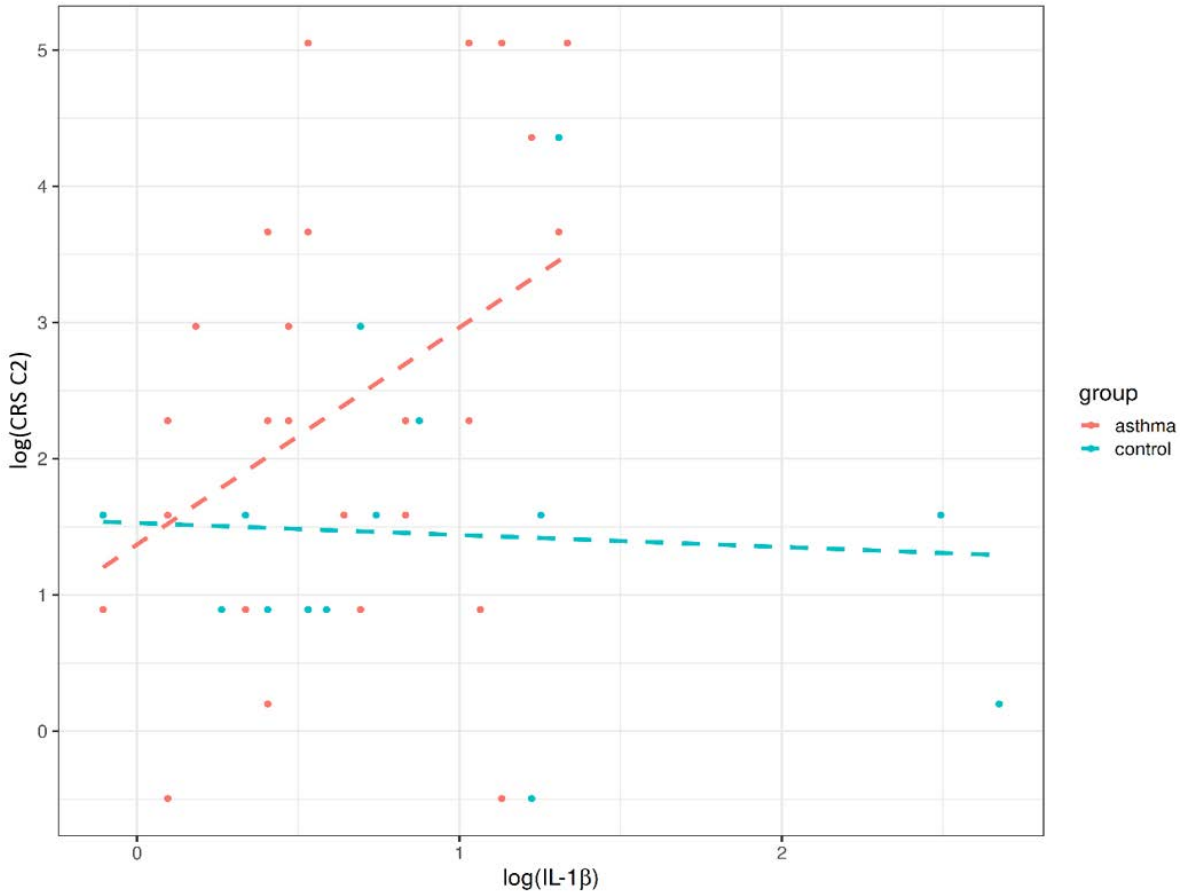

Figure 1 *Cross-plot of acquired data by statistical analysis showing log (CRS_C2) versus log (IL-1β) in asthma (red dots) and control (cyan dots) groups with fitted lines obtained from a linear regression model.*