

Clinical characteristics, cytokine profiles and plasma IgE in adults with asthma

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ABSTRACT

Asthma is a disease with complicated network of inflammatory responses of cytokines and ImmunoglobulinE (IgE). The aim of this study was to explore the clinical characteristics, cytokine profile and plasma IgE in the Malaysian population. This is a cross-sectional study involving physician-diagnosed asthma patients ($n = 287$) recruited from the Chest Clinic, University of Malaya Medical Centre (UMMC). Blood (8 mL) was taken after consent was obtained. The peripheral blood leucocytes (PBL) were cultured in presence of a mitogen for 72 h to quantify cytokines [Interleukin-5(IL-5), Interleukin-9 (IL-9), Interleukin-12 *Beta* (IL-12 β) and granulocyte-macrophage colony-stimulating factor (GM-CSF)] and plasma was used to quantify IgE levels with commercial ELISA kits. Results were compared against the same biomarkers in healthy subjects ($n = 203$). In addition, the amount of the biomarkers in the asthma patients were compared with their disease severity and clinical characteristics. Statistical tests in the SPSS software (Mann-Whitney *U* test and the Kruskal Wallis) were used to compare cytokine production and plasma IgE levels. The mean plasma IgE level was markedly higher ($p < 0.0001$) in asthmatics compared to controls. There were higher levels of IL-5, IL-9, IL-12 β and GM-CSF ($p < 0.0001$) produced by cultured PBL from asthma patients compared to controls. However, our results did not expose a significant association between these cytokine levels and severity and clinical symptoms of asthma. However, there was a marked association between asthma severity and blood lymphocyte count [$\chi^2(2) = 6.745$, $p < 0.05$]. These findings support the roles played by cytokines and IgE in the airway inflammation in asthma. The findings of this study provide new information about inflammatory cytokines in Malaysian asthma patients.

1. Introduction

Asthma, a chronic airway inflammatory disease, is the leading respiratory disease in the country (World Health Organization, 2018). According to the latest data by the World Health Organization (WHO) in 2018, the number of deaths due to asthma in Malaysia was 1,069, accounting for 0.76 % of the total number of deaths (World Health Organization, 2018). According to the Fourth National Health and

Morbidity Survey (NHMS IV) 2011, the prevalence of asthma in Malaysia is 6.4 % (Malaysian Ministry of Health, 2011). However, there are relatively few studies on asthma in Malaysia. The prevalence, morbidity and mortality rates of asthma appear to be increasing, especially in industrialized countries (Boulet, 2018; Pawankar, 2014). It has been proposed that asthma might be preventable if people at risk are identified early, so that they could take the correct precautions before the onset of symptomatic disease (Pawankar, 2014). There are many

Abbreviations: Af, *Aspergillus fumigates*; AHR, Airway hyper-responsiveness; APC, Antigen-presenting cells; BAL, Bronchoalveolar lavage; Con A, Concanavalin A; COPD, chronic obstructive pulmonary disease; ELISA, enzyme-linked immunosorbent assay; GINA, Global Initiative for Asthma; GM-CSF, granulocyte-macrophage colony stimulating factor; ICOS, Inducible T-cell COStimulator; ICS, inhaled corticosteroids; IgE, Immunoglobulin; IL, Interleukin; LABA, long-acting β_2 agonist; LAK, lymphokine-activated killer; LT, leukotrienes; PCA, Principle components analysis; SABA, short-acting β_2 agonist; SNP, Single Nucleotide Polymorphism; SPSS, Statistical Package for the social sciences; TCR, T-cell receptor (TCR); Th, T helper; WHO, World Health Organization.

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studies, which have reported on the roles of T-helper-2 (Th2) cytokines and IgE on the pathogenesis of asthma (Beeh et al., 2000; Khadadah et al., 2000; Sears et al., 1991). However, much remains to be discovered in order to understand the exact mechanisms that can explain the pathogenesis of asthma.

Spirometry, is a principal tool for primary care physicians to detect and manage respiratory problems (Johnson and Theurer, 2014). It includes a computer, PFT software, pneumotach, printer, disposable mouthpiece, disposable nosepiece, and a 3-L syringe for calibration (Johnson and Theurer, 2014). Forced expiratory volume in the first second (FEV1) can be used to determine the severity of airflow limitation in asthma and chronic obstructive pulmonary disease (COPD) (Gallucci et al., 2019). This severity is derived by expressing FEV1 as a percentage of the predicted value in a patient (Devasahayam et al., 2019; Pahal et al., 2019). FEV1 values greater than 80 % of the predicted average value are considered to be normal (Shapira et al., 2019). Forced vital capacity (FVC) is the maximum amount of air that can be exhaled when blowing out as fast as possible (Gallucci et al., 2019). The ratio of FEV1 to FVC (FEV1/FVC, also known as FEV1%) can help differentiate obstructive and restrictive lung diseases (Devasahayam et al., 2019).

Most cytokines are implicated in the chronic inflammatory processes of asthma which eventually contribute to the release of mediators such as histamine and leukotrienes (LT), airway remodelling, bronchial hyper-responsiveness (BHR) and bronchoconstriction (Lambrecht et al., 2019). The CD4⁺ T-lymphocytes from the airways of asthmatics express a panel of cytokines associated with Th2 inflammations (Renaud, 2001). For instance, interleukin-5 (IL-5) produced by Th2 cells is a major cytokine that is responsible for the development of eosinophilia *in vivo* (Wilson et al., 2011). Increased expression of IL-5 mRNA was reported in CD4⁺ T cells isolated from airways of asthmatics (Truyen et al., 2006). Similar elevated levels of IL-5 levels were also reported in BAL (Hosoki et al., 2015) and induced sputum following allergen challenge (Liu et al., 2000) in asthmatic patients.

Interleukin-9 (IL-9) is a cytokine with pleiotropic actions on allergic mediators such as mast cells (Chakraborty et al., 2019) eosinophils (Sitkauskienė et al., 2005), B-cells and epithelial cells (Steenwinkel et al., 2007). This cytokine is reported to induce mucus production in asthma patients (Louahed et al., 2000). Lungs from IL-9 transgenic mice showed selective expression of IL-9 and large infiltration by eosinophils and lymphocytes with increased numbers of mast cells within the airway epithelium (Temann et al., 1998). Researchers have proposed that IL-9 (Renaud, 2001) and its' receptor, IL-9 receptor (IL-9R) (Grasso et al., 1998) are good targets for therapeutic interventions in asthma as IL-9 was found to regulate bronchial hyper-responsiveness (BHR) in mice (Saeki et al., 2016).

Interleukin-12 (IL-12) is a cytokine that plays a key role in T-helper-1/2 (Th1/Th2) differentiation during primary antigen presentation (Meys et al., 2006; Risoan et al., 1999), which is produced mainly by antigen-presenting cells (APC) such as dendritic cells (DC) and macrophages (Hofstra et al., 1998). This cytokine promotes differentiation of CD4⁺ Th0 cells to CD4⁺ Th1 cells, which produce cytokines like IL-2, tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) (Wills-Karp et al., 1999). In addition, IL-12 is reported to have direct and indirect inhibitory effects on Th2-mediated allergic airway inflammation (Tournoy et al., 2001) as it was shown that IL-12 can inhibit BHR and pulmonary inflammation after a single antigenic challenge (Gavett et al., 1995).

Granulocyte-macrophage colony stimulating factor (GM-CSF), also known as the colony stimulating factor 2 (CSF2), is produced by many cells in the airway such as macrophages, T lymphocytes, mast cells, eosinophils, fibroblasts, endothelial cells and epithelial cells (Saha et al., 2009). Increased expression of GM-CSF was also observed in the epithelium of bronchial biopsy specimens from asthmatics (Sousa et al., 1993), T-lymphocytes and eosinophils after endo-bronchial challenge with allergen (Broide and Firestein, 1991). However, phase II interventional clinical trials involving severe asthma patients using human

anti-GM-CSF antibody (KB003) showed little treatment success (Molfino et al., 2016).

Immunoglobulin E (IgE) is a class of antibody that is associated with airway inflammation and allergic reactions (Crespo-Lessmann et al., 2020). Asthma patients often have elevated plasma levels of IgE (Giuffrida et al., 2014). Several studies have reported a link between serum IgE levels and markers at chromosome 5q31–33 in the Amish (Marsh et al., 1994), Dutch (Meyers et al., 1994) and UK (Doull et al., 1996) populations. It is also possible that the levels of IgE may play a role in modulating the severity of asthma. Previous studies reported an association between high IgE levels and increased asthma severity with airway hyperresponsiveness (Borish et al., 2005; Siroux et al., 2003; Sunyer et al., 1995).

Several human genetic studies point to a limited set of cytokines as key targets for the treatment of asthma, mainly IL-4, IL-5, IL-9 and IL-13. Thus far, the combination of these four cytokines (IL-5, IL-9, IL-12β and GM-CSF) has not been studied in Malaysian asthmatics. Hence, the aim of this study was to investigate the relationship between these cytokines (IL-5, IL-9, IL-12β and GM-CSF) and plasma IgE levels and the clinical characteristics of asthma in the Malaysian population. The findings from this study may contribute to a better understanding of the mechanisms of asthma airway inflammation and a better management of the disease in the country.

2. Material and methods

2.1. Ethics approval

The study was approved by the University of Malaya Medical Centre (UMMC) Ethics Committee (Reference number: 20159–1652). Informed written informed consent was obtained from all study subjects who consented to participate in this study.

2.2. Subjects

Two hundred and eighty-seven patients (115 Malays, 72 Chinese and 100 Indians) with no asthma exacerbation requiring systemic corticosteroids for the last one year were recruited for this study from the Chest Clinic, University of Malaya Medical Centre (UMMC). The asthma patients were stratified into mild ($n = 109$), moderate ($n = 130$) and severe ($n = 48$) asthmatics. The diagnosis of asthma was based on guidelines by the Global Initiative for Asthma (GINA) 2016 and patients with all levels of asthma severity were recruited. Following diagnosis, severity of chronic persistent asthma was categorized into mild, moderate and severe according the controller medications recommended in the GINA treatment steps required to control the asthma (GINA guidelines 2016). Patients with mild asthma required GINA step 2 treatment which included low dose inhaled corticosteroids (ICS) for asthma control. Moderate asthma was asthma which was well controlled with GINA step 3 treatment which consisted of low dose ICS combined with long-acting β₂ agonist (LABA) or medium dose ICS alone. Severe asthma required GINA step 3 or step 4 treatment which consisted of moderate or high dose ICS combined with LABA plus a leukotriene receptor antagonist and/or tiotropium bromide (a long-acting muscarinic antagonist) and/or theophylline and/or oral corticosteroid and/or omalizumab (an-anti-IgE) or remained uncontrolled despite these treatments. In all grades of asthma severity, short-acting β₂ agonist (SABA) could be used as needed.

The level of the patient's asthma was categorized into well controlled ($n = 202$), partly controlled ($n = 54$), and uncontrolled ($n = 31$). Patients with well controlled asthma were those with daytime symptoms less than 2 times a week, no night time waking due to asthma, reliever needed less than twice a week and no limitation in their daily activities due to asthma (GINA guidelines 2016). Patients with uncontrolled asthma had three or four of the following features, daytime symptoms more than twice a week, night waking due to asthma, needed reliever

more than twice/week and experienced limitations in their daily activities due to asthma. Patients with partly controlled asthma had one or two of the symptoms of uncontrolled asthma.

Two hundred three ($n = 203$) volunteers (85 Malays, 59 Chinese and 59 Indians) were enrolled in this study as control healthy subjects. All volunteers for the study were asked to complete a questionnaire to collect information on demographic characteristics, individual history of respiratory diseases or any other major illnesses. None of the healthy subjects reported any history of acute or chronic medical conditions. Individuals with recent upper respiratory tract infections were also excluded. Age criteria for asthma patients and normal subjects ranged from 18 to 72 years old. All the subjects were recruited in the period from October 2016 to November 2020.

2.3. Isolation of peripheral blood lymphocytes

A blood sample (8 mL) was collected from each eligible subject (asthma patients) during routine follow-up at the Chest Clinic, UMMC. Blood samples were taken when patients were not on systemic corticosteroids. The same amount of blood was collected from eligible healthy volunteers. For each subject, 3-mL's of blood were collected in EDTA blood collection tubes while 5 mL's were collected in heparinized blood collection tube. Blood collected in the EDTA tubes was sent to the Clinical Diagnostic Laboratory, UMMC for complete blood count (CBC) and differential white blood cell count (WBC). This was performed to ascertain the levels of total WBC as well as the eosinophils and lymphocytes in each blood sample.

Blood collected in the heparinized tube was centrifuged (1500 rpm for 10 min) to separate plasma from the cellular component. Then, 2 mL's of plasma were stored at -20°C for IgE analysis later. The heparinized tube containing the remaining blood cells mixed thoroughly with sterile G-Dex II RBC lysis buffer by inverting the tube five times. The samples were then centrifuged at $2000 \times g$ for 10 min to isolate the leucocytes. The supernatant containing the lysed erythrocytes was discarded leaving the pellet containing the WBC which was submitted for lymphocyte culture.

2.4. Lymphocyte culture

The number of lymphocytes in each sample was calculated from the results of the CBC and differential count performed by the Clinical Diagnostic Laboratory, UMMC. Cell count was adjusted to 2.0×10^6 cells/mL using RPMI 1640 medium supplemented with L-glutamine, 10 % heat-inactivated fetal bovine serum (FBS), 0.1 mg/mL streptomycin and 100 units/mL penicillin-streptomycin. Concanavalin A (Con A) was added to give a final concentration 25 $\mu\text{g/mL}$ and this suspension was transferred into a T25 tissue culture flask (Corning, USA) and incubated at 37°C in a humidified 5% CO_2 incubator. After 72 h, the cell culture supernatants were harvested and stored at -20°C for subsequent quantification of IL-5, IL-9, GM-CSF, IL-12 β by enzyme-linked immunosorbent assay (ELISA).

2.5. Quantification of IL-5, IL-9, GM-CSF, IL-12 β from cultured lymphocytes and plasma IgE by ELISA

The amount of IL-5, IL-9, and GM-CSF produced by the cultured lymphocytes (in the cell culture supernatants) and IgE from asthmatic patients and control subjects were quantified using appropriate commercial ELISA kit according to the protocol recommended by the manufacturer (eBioscience, USA) with a detection range of 1 pg/mL to 750 pg/mL and 7.8–1000 ng/mL, respectively. The amount of IL-12 β cell in the culture supernatants was quantified using a IL-12 β ELISA Kit from another company (Sino-Biological, Inc. (Beijing, China) with a detection range of 15.63 pg/mL to 1000 pg/mL. The absorbance produced following the ELISA was read using a multi-plate reader (TECAN, USA) the wavelength of 450 nm with 570 nm as the reference

wavelength. All samples (rest and standard) were run in duplicate and the amount of cytokines (IL-5, IL-9, IL-12 β and GM-CSF) and IgE were quantified from the standard curve generated for each biomarker.

2.6. Statistical analysis

Data collected were transferred into a Microsoft Excel spread sheet and then transformed into SPSS (Statistical Package for the Social Sciences, IBM, Version 22.0) for statistical analyses. Parametric continuous variables were reported as mean $\pm$ standard deviation (SD) while non-parametric continuous variables were reported as median and inter-quartile range (IQR). Normality was determined using the Kolmogorov-Smirnov and Shapiro-Wilks tests. Categorical variables were presented as frequency and percentages and differences between groups were compared using Chi-squared test.

For parametric continuous variables, differences between groups were compared using Student *t*-test. For non-parametric continuous variables, differences between two groups were analyzed by the Mann-Whitney *U* test while comparisons between three groups (for asthma severity and asthma control) were performed using the Kruskal-Wallis test (non-parametric ANOVA). Further adjustment on age and gender criteria was performed with multinomial logistic regression. All tests were presented as two-sided, and a *P* value < 0.05 was considered statistically significant. Data were summarized into graphs by using GraphPad Prism 7.01 software (GraphPad Software Inc).

3. Results

Table 1 describes the characteristics of the asthma patients and the healthy controls. Statistical analysis showed that the selected sample groups did not differ significantly. Chi-squared and Student *t*-test performed to compare the demographic differences between asthmatic and control subjects in terms of ethnicity and gender. The study population comprised of three major ethnic groups in West Malaysia, i.e. Malays, Chinese and Indians. The proportion of subjects from each ethnic group was balanced between asthma patients and healthy controls ($p > 0.05$). Asthma patients were older with mean age ($\pm$ SD) of 52.52 (± 14.15) years compared to that healthy controls, mean age ($\pm$ SD) of 35.90 (± 10.80) years. There was significantly higher proportion of females among the asthma patients compared to the healthy controls ($p < 0.0001$).

The majority of the patients were diagnosed with well controlled asthma (70.4 %) and moderate asthma (45.3 %). Only a minority of patients had uncontrolled asthma (10.8 %) and severe asthma (16.7 %). Very small percentages of patients had an FEV₁ < 60 % predicted (5.23 %), had asthma exacerbation in the last 12 months (5.23 %) and a history of endotracheal intubated for asthma exacerbation (3.48 %) (Table 1).

ICS were the backbone of treatment, but patients with an increase in asthma severity, inhaled LABA in fixed-dose combination with higher dose ICS, with or without leukotriene receptor antagonist and with or without tiotropium were used. The healthy volunteers ($n = 203$) did not smoke and did not have any history of allergy/atopy, cardiac or pulmonary diseases. The peak expiratory flow (PEF) measurement in healthy controls range between 350–550 L/min, with a mean ($\pm$ SD) of 436.41 (± 51.54) L/min (Table 1). The mean eosinophils and lymphocyte counts were generally higher in asthma patients compared to the controls (Table 1).

The mean concentration of IL-5 in asthma patients (58.89 ± 79.36 pg/mL) was higher than the healthy subjects (32.71 ± 32.93 pg/mL; $p < 0.0001$) (Fig. 1). Similarly, the mean IL-9, IL-12 β and GM-CSF levels were significantly higher in the asthma patients compared to the healthy controls [(572.62 $\pm$ 636.98 pg/mL) versus (174.68 $\pm$ 125.01 pg/mL), $p < 0.0001$; (233.08 $\pm$ 219.76 pg/mL) versus (98.68 $\pm$ 100.65 pg/mL), $p < 0.0001$; (158.42 $\pm$ 143.01 pg/mL) versus (76.35 $\pm$ 77.77 pg/mL, $p < 0.0001$) (Fig. 1).] The asthma patients have raised plasma IgE levels

Table 1

Comparison of demographic and clinical characteristics and peripheral blood counts in asthma patients versus healthy controls.

Characteristics		Asthma patients (n = 287)	Healthy controls (n = 203)	P-value
Age⁺ (years)	Mean $\pm$ SD	52.52 $\pm$ 14.15	35.90 $\pm$ 10.80	<0.0001
	(Range)	(18–72)	(19–71)	
Race⁺, n(%)	Malay	115 (40.1)	85 (41.9)	0.364
	Chinese	72 (25.1)	59 (29.1)	
	Indian	100 (34.8)	59 (29.1)	
Gender⁺, n(%)	Male	80 (27.9)	103 (50.7)	<0.0001
	Female	207 (72.1)	100 (49.3)	
Peak Flow rate (L/min)	Mean $\pm$ SD	NA	436.41 $\pm$ 51.54	NA
	(Range)		(350–550)	
Severity of asthma, n (%)	Mild	109 (38.0)	NA	NA
	Moderate	130 (45.3)		
	Severe	48 (16.7)		
FEV₁, <60% predicted n(%)	Yes	15 (5.23)	NA	NA
	No	272 (94.77)		
	Well controlled	202 (70.4)		
Asthma Control, n (%)	Partly Controlled	54 (18.8)	NA	NA
	Uncontrolled	31 (10.8)		
	Yes	15 (5.23)		
Exacerbations, during the last 12 months (%)	No	272 (94.77)	NA	NA
	Yes	10 (3.48)		
History of endotracheal intubated for asthma, n (%)	No	277 (96.52)	NA	NA
	ICS	85 (29.62)		
Controller medication(s) needed for asthma control	ICS + LABA	119 (41.46)	NA	NA
	ICS + montelukast	2 (0.01)		
	ICS + LABA + montelukast	76 (26.48)		
	ICS + LABA + theophylline	5 (1.74)		
Lymphocyte count (x 10⁶ cells/mL)⁺	Mean $\pm$ SD	2.625 $\pm$ 1.072	2.288 $\pm$ 0.694	0.003
	Range	(0.0–11.08)	(0.48–4.09)	
Eosinophil count (x 10⁶ cells/mL)⁺	Mean $\pm$ SD	0.393 $\pm$ 0.363	0.203 $\pm$ 0.182	<0.0001
	Range	(0.0–2.72)	(0.0–0.90)	

Statistical analyses were performed using the chi-squared*/ Student *t*-test⁺.

Values in parenthesis are range or percentages as indicated.

NA: not applicable.

ICS: inhaled corticosteroids; LABA: long-acting β_2 -agonist.

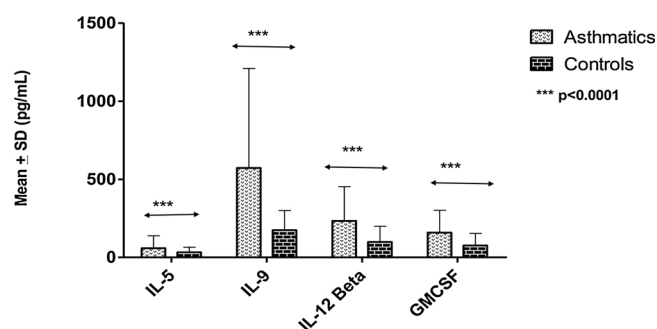


Fig. 1. Expression levels of IL-5, IL-9, IL-12 Beta and GMCSF in peripheral blood lymphocytes of asthmatics and control.

(818.73 $\pm$ 650.17 ng/mL) compared to the control group (356.44 $\pm$ 386.02 ng/mL; $p < 0.0001$) (Table 2) (Fig. 2).

We also looked for association between the severity and symptoms control with the cytokine profiles and plasma IgE levels (Table 3). There

Table 2

Comparison of Interleukin-5, Interleukin-9, Interleukin-12 β , granulocyte-monocyte colony stimulating factor and IgE in asthma patients versus healthy controls.

	Asthma Patients (n = 287)	Healthy controls (n = 203)	P-value*	Odds ratio (95 % CI) P-value**
IL-5				
Mean (pg/mL)	58.89 $\pm$ 79.36	32.71 $\pm$ 32.93		1.010 (1.004–1.016)
Range (pg/mL)	0–576.18	0–192.64	<0.0001	
Median (pg/mL)	33.77	18.72		0.002
Interquartile range	55.66	40.20		
IL-9				
Mean (pg/mL)	572.62 $\pm$ 636.98	174.68 $\pm$ 125.01		1.004 (1.003–1.005)
Range (pg/mL)	0–2761.06	0–669.29	<0.0001	
Median (pg/mL)	320.60	158.63		<0.0001
Interquartile range	550.88	170.46		
IL-12β				
Mean (pg/mL)	233.08 $\pm$ 219.76	98.68 $\pm$ 100.65		1.006 (1.004–1.008)
Range (pg/mL)	0.192–1290.27	0–647.35	<0.0001	
Median (pg/mL)	167.77	76.95		<0.0001
Interquartile range	250.72	93.66		
GM-CSF				
Mean (pg/mL)	158.42 $\pm$ 143.01	76.35 $\pm$ 77.77		1.007 (1.004–1.010)
Range (pg/mL)	0–819.47	0–482.70	<0.0001	
Median (pg/mL)	116.13	60.04		<0.0001
Interquartile range	127.47	76.85		
IgE				
Mean (ng/mL)	818.73 $\pm$ 650.17	356.44 $\pm$ 386.02		1.002 (1.001–1.002)
Range (ng/mL)	0–2804.31	18.40–1814.89	<0.0001	
Median (ng/mL)	611.03	187.24		<0.0001
Interquartile range	920.66	386.02		

P-value* for univariate analysis using Mann Whitney *U* test. Adjusted P value** with odds ratio (OR) after controlling for age and gender using multinomial logistic regression analysis.

CI: confidence interval; GM-CSF: granulocyte-monocyte colony stimulating factor; IL: interleukin; Ig: Immunoglobulin.

was no association between the levels of these cytokines and plasma IgE with the severity of asthma or the level of asthma control. However, significant results were noted on the lymphocytes counts in patients with different asthma severity (Table 3). A Kruskal Wallis test showed that there was statistically significant difference in asthma severity and lymphocytes blood count, $\chi^2(2) = 6.745$, p value < 0.05 with mean rank for severe 167.04, moderate for 146.83 and 130.48 for mild.

Pearson correlation test conducted to observe correlation for Interleukin-5, Interleukin-9, Interleukin-12 β , GM-CSF, eosinophils and lymphocytes against Plasma IgE (data not shown) for both healthy and patient samples. There was a positive correlation between eosinophil count and plasma IgE level among the asthmatics, which was

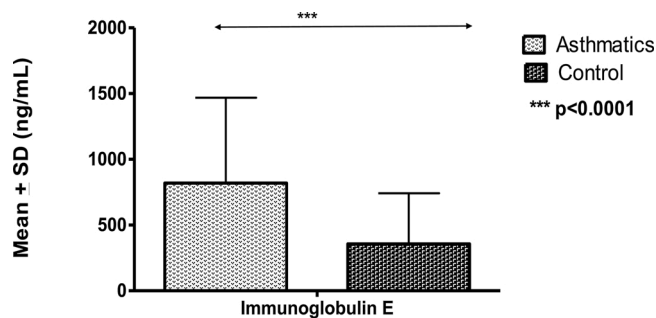


Fig. 2. Plasma Immunoglobulin E (IgE) levels in asthmatics and controls.

statistically significant ($p < 0.05$). Significant differences were noted when the blood counts were compared between asthma patients of different ethnicity and gender. The Kruskal Wallis test showed that there was statistically significant difference between peripheral blood lymphocyte counts between different ethnic groups, $\chi^2(2) = 39.088$, p value < 0.0001 with a mean rank of 172.98 for Indian, 150.18 for Malay and 93.88 for Chinese. The results indicate lymphocytes counts were higher among Indian asthma patients compared to Malay and Chinese patients. A similar statistically significant difference was found with eosinophils count with $\chi^2(2) = 6.803$, p value < 0.05 , with a mean rank of 152.27 for Indian, followed by 150.61 for Malay and 121.96 for Chinese. When compared between genders, only lymphocyte count showed a significant difference among the asthma patients, ($U = 6179.0$, $p < 0.001$) with a mean rank of 117.74 for males and 154.15 for females.

4. Discussion

Asthma is characterized by increased responsiveness of the bronchial tree to environmental triggers resulting in chronic inflammation of the airways (Bousquet et al., 2000). The incidence of asthma and wheezing diseases among children and young people has been increasing globally (Mattiuzzi and Lippi, 2020; To et al., 2012; Trivedi and Denton, 2019). Airway inflammation plays a central role in mechanisms of asthma (Cevhertas et al., 2020). The specific inflammation leads to the characteristic physiological abnormality of AHR. This makes the airway constricted in response to a variety of stimuli, leading to the distinguishing symptoms of wheeze, cough and dyspnea (Doshi et al.,

2002).

Contrary to common perception, asthma is a complex and heterogeneous disease with many phenotypes (Anderson, 2008). Haldar and co-workers (2008) used principal components analysis (PCA) and clinical contribution to select a subset of variables that were measured in medical environment such as age of asthma onset, gender, allergic status, body mass index (BMI), peak flow variability, induced sputum eosinophil counts, and symptom scores (Haldar et al., 2008). The classification of asthma phenotypes and the acknowledgement that these phenotypes are associated with differing response to therapies, has prompted the quest for biomarkers to define the various asthma endotypes and phenotypes (Bhakta and Woodruff, 2011). With the assumption of the critical role of inflammatory cytokines in asthma, our study has taken a molecular approach to distinguish subgroups of asthma, according to disease severity and asthma control, and the possible association with cytokines, plasma IgE levels and blood eosinophils. We summarized and categorized our findings according to demographic features, clinical characteristics, lung function and biomarkers of airway inflammation.

The pharmacological treatment of asthma involves two main types of medications, controllers and relievers. The most important controller medications to address the airway inflammation are ICS. ICS are used to treat bronchial inflammation to improve asthma symptoms, lung function, and quality of life as well as to prevent exacerbations, whereas inhaled bronchodilators such as SABA provide temporary relief of breathlessness through bronchodilation (Bostantzoglou et al., 2015). ICS was the backbone of treatment for all our patients with chronic persistent asthma in keeping with the GINA guidelines recommendation. With increasing asthma severity, higher dose ICS with or without inhaled LABA, with or without a leukotriene receptor antagonist and with or without inhaled tiotropium were prescribed.

Most of the previous studies had been conducted in patients with mild to moderate asthma and had shown airway inflammation to be related to eosinophil accumulation and Th2-type cytokine production (Bahrami Mahneh et al., 2015; Seys et al., 2017). Numerous scientists targeted key Th2 cytokines such as IL-4, IL-5 and IL-13 which have been studied and accepted to treat severe asthma patients, largely eosinophilic, allowing a reduction in corticosteroids doses (Busse et al., 2013; Fahy, 2015; Seys et al., 2017). In the present study, cytokine concentration levels and their relationship with asthma severity, asthma control and pulmonary function were evaluated to access for possible

Table 3

Comparisons of cytokines concentrations (Interleukin-5, Interleukin-9, Interleukin-12 β , GM-CSF, plasma IgE levels and peripheral blood counts (eosinophil and lymphocyte) in patients with different categories of asthma.

	IL-5 (pg/ mL)	IL-9 (pg/ mL)	IL-12 β (pg/ mL)	GM-CSF (pg/ mL)	IgE (ng/ mL)	Lymphocytes count (x 10 ⁶ cells / mL)	Eosinophils count (x 10 ⁶ cells / mL)
Asthma severity							
Mild	150.73	141.33	142.93	155.05	152.60	130.48	139.96
Moderate	138.08	144.93	145.62	133.71	142.67	146.83	141.35
Severe	144.74	147.55	142.06	146.79	128.07	167.04	160.35
χ^2	1.383	0.218	0.094	3.984	2.970	6.745	2.255
P value	0.501	0.897	0.654	0.136	0.226	0.034	0.324
Median	33.77	331.51	167.77	116.13	611.03	2.61	0.30
Interquartile range	55.66	608.25	250.71	27.47	920.66	1.14	0.32
Asthma control							
Well controlled	145.93	146.23	140.60	144.06	146.09	141.83	143.42
Partly controlled	139.01	142.77	153.78	138.87	141.04	143.45	153.11
Uncontrolled	140.13	131.63	149.10	152.52	135.55	159.11	131.89
χ^2	0.372	0.846	1.205	0.533	0.518	1.169	1.321
P value	0.830	0.655	0.548	0.766	0.772	0.557	0.517
Median	33.77	331.51	167.77	116.13	611.03	2.61	0.30
Interquartile range	55.66	608.25	250.72	127.47	920.66	1.14	0.32

Comparison of asthma severity and asthma control with the mean rank and median using Kruskal Wallis Test, $\chi^2 =$ chi squared.

GM-CSF: granulocyte-monocyte colony stimulating factor; Ig: immunoglobulin; IL: interleukin.

clinical applicability (Table 3).

From the results obtained, eosinophils and lymphocytes counts recorded P value <0.05 , showed significant difference among the asthmatics and control (Table 1). This may be due to the eosinophilic asthma phenotype seems to be more related in patients with adult-onset asthma than in those with childhood-onset asthma (Miranda et al., 2004). Furthermore, based on routine blood data, Zhan et al. demonstrated eosinophils and lymphocyte counts can serve as a biomarker for asthma diagnosis (Zhan et al., 2020). According to study conducted by Carroll et al., numbers of eosinophils were associated to the numbers of lymphocytes in the pathogenesis of asthma (Carroll et al., 1997). The lymphocytes are vital among all T-cells, and show increased activation compared to controls (Bentley et al., 1992; Bradley et al., 1991).

The total plasma IgE levels were significantly higher in our asthma patients than in healthy subjects. The association between higher cytokine levels among asthma patients remained significant (P value <0.05) after further adjustment on the age and gender factor in both asthma and control groups. Our data showed similar results with the comparative study of Cho SH et al. found that the levels of serum IL-5 to be higher in asthmatic patients than in healthy controls (Cho et al., 2005). In addition, a study conducted in Iran population revealed IL-9 serum level was significantly correlated with asthma susceptibility and absolute eosinophil count (Mahdavi et al., 2021). It has been proposed that IL-9 might be a good target for therapeutic interventions in asthma (Renauld, 2001) as it has been reported to regulate BHR in mice (Saeki et al., 2016). Some researchers have also proposed that the IL-9 receptor (IL-9R) to be a potential therapeutic target for asthma (Grasso et al., 1998).

Saha et al., reported that over-expression of GM-CSF in sputum and the bronchial mucosa is a specific benchmark of severe asthma (Saha et al., 2009). Studies using cultured bronchial epithelial cells from asthmatics showed that GM-CSF could be involved in chronic eosinophilia and airway remodelling (Soloperto et al., 1991). Increased expression of GM-CSF was also observed in the epithelium of bronchial biopsy specimens from asthmatics (Sousa et al., 1993), T-lymphocytes and eosinophils after endo-bronchial challenge with allergen (Broide and Firestein, 1991). Nevertheless, IL-12 is reported to have direct and indirect inhibitory effects on Th2-mediated allergic airway inflammation (Tournoy et al., 2001). After a single antigenic challenge, it was shown that IL-12 can inhibit AHR and pulmonary inflammation (Gavett et al., 1995). Consequently, administration of IL-12 inhibited expression of Th2 cytokine genes in mice (Gavett et al., 1995).

Severe asthma occurs in only 10 % of the asthma population but is associated with greater morbidity and mortality and disproportionate health care utilization and costs (M. Kenawy et al., 2017; Taylor et al., 2008). In this study, patients with severe asthma were compared with patients with mild and moderate asthma. We did not find significant differences in the levels of pro-inflammatory cytokines (IL-5, IL-9, IL-12 β , GM-CSF) and plasma IgE in patients with different asthma severity and symptoms control. This lack of difference might have been due to the fact that 70.4 % of the asthma patients recruited were having well controlled asthma. Mouthuy et al., showed that sputum IgE levels though indicative of increased airway eosinophilic inflammation, are not related to disease severity (Mouthuy et al., 2011). Our results are in concurrence with previous studies that shows serum cytokine levels are produced from the activity of Th1, Th2 cells and clinical symptoms are independent of one another (Corrigan and Kay, 1990; Tatenno et al., 2004; Ulambayar et al., 2019). Ulambayar et al., demonstrated age-related changes of epithelial cell-derived cytokines may affect clinical phenotypes and severity of elderly asthma. The study concluded decreased levels of cytokine levels may contribute to less Th2 phenotype (Ulambayar et al., 2019).

However, significant results noted on the lymphocytes counts and asthma severity (Table 3). This results indicate patients with severe asthma have increases in lymphocytes in the blood from moderate and mild asthmatics. Increased peripheral blood lymphocytes in severe

asthma may be explained by increased expression of interleukins but differences eosinophil counts did not appear to be associated in asthma severity in our patients. Our findings are in keeping with the results of previous study by Darwesh et al., who in an Iraq population, they showed that mean of neutrophils/lymphocytes ratio was significantly increased among patients with severe asthma (Darwesh et al., 2020). Our finding is consistent with the results of the study by Gungen and Aydemir, who in a Turkish population, found significantly higher lymphocytes in asthma patients compared to controls (Gungen and Aydemir, 2016). These findings indicate the neutrophil to lymphocyte ratio is useful biomarker in assessment of asthma severity.

Eosinophils and serum immunoglobulin E (IgE) are considered good markers of airway inflammation in asthma (Kumar et al., 2017). Our study shows that in asthma patients raised plasma IgE levels associated with the eosinophil blood count in keeping with the findings of previous studies (Manise et al., 2013; Naqvi et al., 2007) (Miranda et al., 2004; Moore et al., 2010). When we compared race and gender, no significant association was found with cytokine concentration and plasma IgE levels in our patients (data not shown). In contrast, studies have shown significant differences in the clinical heterogeneity and allergic characteristics of asthma in African-Americans, Whites, and Hispanics in the United States of America (Lester et al., 2001). Similarly, Nyenhuis et al., revealed race is associated with differences in airway inflammation among asthmatics (Nyenhuus et al., 2017). However, we found significantly higher lymphocyte and eosinophil in our Indian asthma patients compared to our Malay and Chinese patients. From the results, we also found that our female asthma patient had significantly higher lymphocyte counts than our male patients.

Measurement of these immune mediators has become increasingly vital as a mediator of understanding immune responses during the progress of disease (Biancotto et al., 2013). The current study demonstrated higher variations in cytokine levels among healthy individuals than were observed for samples obtained from asthma patients (Table 2). Since our healthy subjects had been screened to exclude those individuals with any acute or chronic illness, we did not observe high levels of cytokines production in the samples compared to the patients. The significant increase in cytokine levels suggest that early detection of disease due to the host response may be possible. We also showed the production of cytokines in healthy subjects showed negligible and non-significant differences when investigated in relation to the sex and gender. The knowledge of cytokine concentrations in healthy subjects is an important reference for further comparison with patients. Variability in their levels can reveal an occurrence of potential importance for the understanding of the cytokine pattern in asthmatics.

To the best of our knowledge, this is the first study to investigate the association between the selected cytokine profile and asthma severity and asthma control in the Malaysia population. All the participants with asthma included in the analysis were recruited in chest clinics as asthma cases, with an observant procedure set up to include true asthmatics. Cytokines were measured in peripheral blood lymphocytes, a less invasive method than sputum assays for further application in clinical practice. We acknowledged that we have only explored a small part of the cytokines network, but we have included key inflammatory and Th2 cytokines as well pro-inflammatory cytokine (GM-CSF), cytokines that were often neglected in previous studies in the Malaysia population (Mudhafar and Alsailawi, 2019; Radhakrishnan et al., 2013).

However, there are limitations in our study. Firstly, the number of severe asthma subgroup is relatively small in present study ($n = 48$, 16.7 %). Although cytokines level and plasma IgE level is not associated with severe asthma, further study is still necessary to explore whether it is similar in a larger population. Secondly, although allergen challenge has also positive effect on cytokine expression (Al-Sajee et al., 2018; Pelikan, 2013), we did not analyze the influence of allergy on the association between cytokine and plasma IgE compared with the asthma severity. Thirdly, the majority of the asthma patients who attend the chest clinic were aged above 40 years whereas the majority healthy subjects were

recruited are younger, age ranging from 19 to 39 years old. For this reason, results were statistically adjusted for age and gender with multinomial logistic regression. Furthermore, this study was a single-center, case control study, and the patients were from one region (Klang Valley); thus selection bias might exist.

Overall, we found significant differences in cytokine profiles between asthma patients and healthy subjects. It is noteworthy that biological markers play a role either in the development, the activity or the evolution of the disease. We also showed blood counts such as lymphocytes and eosinophils may play a vital role in the pathogenesis of asthma.

5. Conclusions

In summary, this study identified the main characteristics of cytokine patterns in healthy and asthmatic adults, and provided the reference values for these four cytokines in the normal Malaysian population for the first time. It is obvious from our study that the cytokine levels are significantly different between asthma patients and non-asthma healthy subjects. However, we were unable to demonstrate any difference in the levels of the cytokines studied and plasma IgE between patients with different asthma severity and patients with different levels of asthma control.

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Declaration of Competing Interest

The authors report no declarations of interest.

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