

## A study of cytokine profile and serum IgE level and their association with montelukast therapy in childhood asthma

Ali Mansoor Al-Ameri<sup>\*</sup>, Jaafar Kadhim Naama<sup>\*\*</sup>, Munther Hussain Al-Kadhimi<sup>\*\*\*</sup>

<sup>\*</sup> Lecturer, Medical Microbiology Dept., College of Medicine, Karbala University

<sup>\*\*</sup> Assist. Prof., Medical Microbiology Dept., College of Medicine, Kufa University.

<sup>\*\*\*</sup> Assist. Prof., Institute of liver studies, King's College hospital/ London.

### Abstract

**A**sthma is the most common allergic disease giving rise to the morbidity or school absence in children and has an increasing incidence worldwide. Montelukast is a well known leukotriene receptor antagonist used to treat children with asthma. Several cytokines in addition to IgE are known to affect the course of the disease and response to treatment in this regard.

This is a prospective placebo-controlled cohort study done in Kerbala Pediatric Teaching Hospital during April, 2011 through February, 2013. It aims to test the effect of cytokine profile and serum IgE level (measured by ELISA) on asthma development and their influence on 1-month course of montelukast therapy compared to a placebo group (30 patients each).

Of the 60 patients, 30 were given montelukast as add-on therapy and the other 30 were regarded as placebo group. Serum level of IL2, 4, 10, 17 and IFN-gamma were determined pre- and post- treatment course in all patients and compared with 30 healthy controls. Response was assessed according to answer to a pre- formed questionnaire formula answered by the parents regarding frequency and severity of wheezy attacks.

Results showed a significantly higher level of serum IL4 in asthmatic children than in healthy control. More importantly, its level was shown to be directly related with poorer clinical response to montelukast, p value < 0.05. In addition, montelukast was shown to significantly decrease serum IL4 level in the treatment group (30 asthmatics) compared to the placebo group, p value < 0.05.

Furthermore, our data revealed that the serum level of IL10 was significantly lower in asthmatics compared to the control healthy children and that the higher the level of IL10, the better the clinical response to montelukast ( $r=0.73$ ). While the role of the other cytokines tested in this study, IL 2, IL 17 and IFN-gamma was non-significant regarding association with disease or influence on response to montelukast.

Data regarding serum IgE level in the recruited 60 asthmatic children revealed that there is a significantly increased level compared to the healthy control group, p value < 0.05. Additionally its serum level negatively correlates with clinical response to montelukast,  $r=0.58$ .

In conclusion, serum level of IgE, IL4 and IL10 are important markers associated with childhood asthma development and influencing response to montelukast. Secondly, it was shown that montelukast add-on therapy has significantly better biochemical and clinical response in childhood asthma than conventional asthma controllers alone.

### الخلاصة

يعد الربو من أكثر امراض الحساسية المفرطة شيوعا مسببا الضرر الجسدي والتغيب عن المدرسة لدى الاطفال وان انتشاره يتزايد في انحاء العالم شتى. ان عقار المونتيلوكاست هو مثبط معروف لمستقبلات اللوكوترايين حيث يستعمل

كعلاج للربو عند الاطفال. هناك عدة انواع من المحركات الخلوية والكلوبيولين المناعي نوع E تعرف بتأثيرها على طبيعة المرض ومدى الاستجابة للعلاج.

اجريت هذه الدراسة التجريبية في مستشفى الاطفال التعليمي بمحافظة كربلاء اثناء الفترة من نيسان 2011 الى شباط 2013. حيث تهدف الى اختبار فاعلية مستوى المحركات الخلوية والكلوبيولين المناعي نوع E (والتي يتم قياسها بتقنية الادمصاص المناعي المرتبط بالانزيم) على طبيعة المرض ومدى الاستجابة لعلاج المونتيلوكاست لمدة شهر واحد مقارنة مع مجموعة السيطرة (30 مريض لكل مجموعة).

تم اعطاء المونتيلوكاست كعلاج اضافي لثلاثين مريضا فيما اعتبر الثلاثين الاخرين كمجموعة سيطرة. تم قياس المستوى المصلي للسايوتوكينات IL2,4,10,17 وIFN-g قبل وبعد فترة العلاج لجميع المرضى فضلا عن 30 طفلا كمجموعة سيطرة اصحاء. تم استقصاء مدى الاستجابة اعتمادا على الاجابة عن لائحة اسئلة معدة مسبقا من قبل الاباء تتضمن عدد وشدة نوبات ضيق التنفس.

بينت النتائج ارتفاعا معنويا في المستوى المصلي للسايوتوكين IL4 لدى الاطفال المصابين بالربو مقارنة بمجموعة السيطرة. والاهم من ذلك ان مستواه يتناسب طرديا مع سوء الاستجابة السريرية للمونتيلوكاست. بالإضافة الى ذلك تبين ان المونتيلوكاست سبب نقصانا معنويا في المستوى المصلي للسايوتوكين IL4 لدى مجموعة المعالجة (30 مصابا بالربو) مقارنة بمجموعة السيطرة.

علاوة على ذلك بينت النتائج ان المستوى المصلي للسايوتوكين IL10 قد انخفض انخفاضاً معنوياً لدى الاطفال المصابين بالربو مقارنة بمجموعة السيطرة. وانه كلما ارتفع مستوى IL10 تكون الاستجابة للمونتيلوكاست افضل. فيما كان دور السايوتوكينات الاخرى التي تم استقصاؤها في هذه الدراسة (IFN) (IL 2, IL 17) غير معنوي احصائياً من حيث ارتباطها بالاصابة بالمرض او الاستجابة للمونتيلوكاست.

اما بخصوص المستوى المصلي للكلوبيولين المناعي نوع E لدى 60 طفلا من المصابين بالربو فتشير البيانات الى ان هناك ارتفاعاً معنوياً لديهم مقارنة بمجموعة السيطرة. بالإضافة الى ان مستواه المصلي يتناسب عكسياً مع مدى الاستجابة السريرية للمونتيلوكاست.

الاستنتاج: ان المستوى المصلي للكلوبيولين المناعي نوع E و السايوتوكينات IL4 و IL10 يعد مؤشرا مهما من حيث ارتباطه بالاصابة بالربو لدى الاطفال ومن حيث تأثيره على الاستجابة للمونتيلوكاست. من ناحية اخرى ان اضافة علاج المونتيلوكاست يؤثر تأثيراً ايجابياً معنوياً على مدى الاستجابة السريرية والكيميائية لدى الاطفال المصابين بالربو مقارنة بحالة استخدام علاجات الربو الدارجة لوحدها.

## Introduction

### Background

Asthma is the most common chronic disease of childhood and the most frequent reason for paediatric hospital admission, and its incidence is on the rise<sup>[1]</sup>. In 2006, it was reported that 3 children out of classroom of 30 are likely to have asthma worldwide<sup>[2]</sup>.

Asthma phenotypes in children could be summarized as; transient wheeze, persistent wheeze and late-onset wheeze with characteristic manifestations.<sup>[3]</sup>

Although airway inflammation is of crucial pathogenetic importance in asthma, the allergic immune reaction and the associated inflammation involve many cells which release mediators contributing to the pathogenesis of the disorders. It is well known that IgE plays a pivotal role in immunopathogenesis of asthma. Cross-linking of IgE-allergen complex with Fc εRI is the key step in immediate-type and

inflammatory allergic reactions including asthma<sup>[4]</sup>.

Determination of serum cytokine levels in asthmatic patients could have potential utility in diagnosis of asthma and certain phenotypes, in prediction of attacks, and treatment choice. Th2 cells secrete a panel of cytokines with several overlapping functions including Interleukin-4 (IL-4), IL-5, IL-13, and granulocyte-macrophage colony stimulating factor (GM-CSF). By mediating differentiation of the Th2 subpopulation and eosinophils, as well as modulating B-cell proliferation and IgE switching, the Th2 cytokines are thought to play a prominent role in asthma<sup>[5]</sup>.

The sentinel Th1 cytokine, interferon gamma (IFN γ) and IL12 reciprocally stimulate their production and function during cell-mediated immunity and development of naïve T lymphocytes into Th1 cells. Evidence suggests a contributory role of Th1 cells and their cytokines in asthmatic inflammation and airway hyperresponsiveness<sup>[6,7]</sup>. Although

the involvement of IL-17A in asthma is supported by several recent studies, its causative role is still uncertain.<sup>[8]</sup>

Leukotrienes (LT) especially LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> are potent pro-inflammatory agents responsible in part for bronchoconstriction during an asthmatic attack.<sup>[9]</sup>

Montelukast is a LT-receptor antagonist. It is widely used to treat asthma as an adjunct with other asthma therapies.<sup>[10,11]</sup> In the current study, the effect of montelukast on cytokine profile and IgE level was investigated as well as the effect of the latter markers on response to montelukast therapy.

Although there are numerous *in vitro*, as well as *in vivo* data from animal studies<sup>[12-14]</sup>, to date no information of cohort clinical studies exist that examines a correlation between circulating cytokine levels and childhood asthma phenotypes and their correlation with response to montelukast<sup>[15]</sup> Thus, the present study aimed at investigating serum IgE and some cytokine levels of healthy and asthmatic children and to test their association with asthma development and with response to montelukast therapy.

### Aim of the Present Study

- To determine the predictive value of serum IgE level and some cytokines (IL 2, 4, 10, 17 and IFN $\gamma$ ) in childhood asthma.
- To predict the effect of montelukast therapy on the mentioned cytokines and IgE level.
- To address the effect of cytokine profile on response to montelukast, if any. Meanwhile, to test whether IL4 may constitute useful target for pharmacotherapeutic intervention especially in montelukast-non responders.

### Patients, materials and methods

#### Study design

This is a placebo-controlled cohort interventional study. It was done during January, 2011 through February, 2013 when a total of 60 doctor-diagnosed asthmatic children (43 boys and 17 girls; mean age was 6 years, range 2–15 years) and 30 healthy control children, were recruited from Kerbala Pediatric Teaching Hospital.

Informed consent was obtained for all the participants and/or their parents. All subjects were tested for total serum IgE and IL2, 4, 10, 17 and IFN  $\gamma$  levels and responded to a pre-formed questionnaire to stage the disease in term of attack frequency, severity (effect on sleep or activity), respond to current on/need therapy and exacerbation after colds and on exposure to smoking. Clinical asthma, characterized by increased airway responsiveness, reversible airway obstruction, and airway inflammation, was defined according to podiatrician's diagnosis.

Here in, serum IgE and the serum cytokines profile (represented by IL2, 4, 10, 17 and IFN  $\gamma$ ) were demonstrated by enzyme-linked immunosorbant assay (ELISA) in sera from 60 asthmatic children and 20 controls. For 30 asthmatic children a short course of montelukast therapy, a leukotriene antagonist, (4-5 mg once daily) was given orally for one month and followed by post treatment measurement of cytokine profile and response to treatment compared to data from placebo group (30 children).

This was done in attempt to predict to which degree these molecules are involved in the disease and their association with response to montelukast therapy. The study also addresses the potential therapeutic utility of drugs targeting IL-4 for montelukast-non responder asthmatics.

#### Patient selection

Children with doctor-diagnosed moderate-severe asthma, (60 patients) who haven't taken previous montelukast course or regular steroid therapy during the last

six months, were recruited for this placebo-controlled prospective cohort. They were tested for serum levels of IgE, IL2, 4, 10, 17 and IFN-gamma. Data were compared with those from 30 control healthy children.

Thereafter, the asthmatic children were divided into two groups; montelukast group (30) who were given short course of montelukast (4mg or 5mg) once daily together with the common on-need asthma controllers for 1 month during which period a complete diary was recorded by the parents regarding frequency and severity of wheezy attacks.

Post treatment levels of the mentioned markers were demonstrated and data were compared with those from the placebo group (30 asthmatic children) who were given the conventional on-need asthma therapy only.

#### **Inclusion criteria**

Inclusion criteria for patients include those children aged 2-15 years with doctor-diagnosed moderate-severe asthma. The clinical-based diagnostic criteria include the followings: Major criteria: parent asthma, eczema and inhalant allergen sensitization

Minor criteria: allergic rhinitis, wheezing apart from colds, eosinophils  $\geq 4\%$  and food allergen sensitization<sup>[16]</sup>.

#### **Exclusion criteria**

Exclusion criteria involve children with cardiac diseases or respiratory illness due to other causes a foreign body inhalation, cystic fibrosis<sup>[3]</sup> or those who have recently received a full course of steroid, montelukast or other immunomodulator(s).

#### **Blood sample processing**

After an informed consent was taken from the participants and/or their parents, 3-ml venous blood samples were taken from all selected children to measure cytokine profile and serum IgE level.

Blood sample, was centrifuged after being clotted to collect serum which was stored as aliquots at  $-20^{\circ}\text{C}$  until use for ELISA determination of serum cytokine profile and IgE level.

#### **Cytokine profile assay by ELISA:**

Serum levels of interleukins (IL) 2, 4, 10, 17 and interferon-gamma (IFN $\gamma$ ) were determined by classic sandwich-ELISA. This was done using ELISA minikits (Peprotech) according to the instructions enclosed with the kits. There was additional step of plate coating with the provided primary antibody prior to the test

#### **Total serum IgE level measurement:**

Total serum IgE level was measured by sandwich-ELISA technique, (eBioscience, Human IgE ELISA Ready-SET-Go, Cat. No. 88-50610-22). Quantitative analysis was performed for total serum IgE levels according to instructions enclosed with the kit.

#### **Medical intervention; Montelukast therapy:**

One-month course of chewable montelukast tabs (Zentiva) in dose of 4mg for children up to 6 years old or 5mg for those above 6 once daily at night was given to the 30-asthmatic group as add-on therapy (i.e.) together with the use of classical "on-need" treatment for asthma attacks. Other group of 30 asthmatic children were regarded as placebo group, receiving the on-need treatment without montelukast.

#### **Follow up, assessing level of response:**

During the treatment period, parents were requested to bring their child to the research clinic 2-weekly as well as for acute respiratory tract symptoms, including wheezy episodes (as predefined from diaries).

At each visit the children were examined for objective wheeze which was diagnosed by a pediatrician at the research clinic. Meanwhile, a follow up including response and compliance to montelukast treatment was ensured.

The parents were taught to record symptoms, with emphasis on their frequency and severity together with use of "on-need" treatment. Diary cards at the 2-weekly visits together with examination findings were collected and reviewed accordingly to mount the overall level of

response to montelukast at the end of the treatment course. Response to montelukast was assessed according to the following bases<sup>[17]</sup>:

- Well controlled (Good responders)
  - Symptoms  $\leq 1$  day per week
  - Nighttime awakenings  $\leq 2$  times per month
  - Short-acting beta-agonist use (rescue)  $< 2$  days per week
  - No limitation in normal activity
- Not well controlled (Fair responders)
  - Symptoms  $> 1$  day per week
  - Nighttime awakenings 1-2 times per week
  - Short-acting beta-agonist use (rescue)  $> 2$  days per week
  - Some limitation in normal activity
- Very poorly controlled (Poor responders)
  - Symptoms throughout the week
  - Nighttime awakenings  $\geq 3$  times per week
  - Short-acting beta-agonist use (rescue) several times per day
  - Extreme limitation in normal activity

#### Post treatment evaluation:

Post treatment specimens' collection and sample processing was done as mentioned in the pretreatment stage for determination of serum cytokine profile and serum IgE level estimation compared to the response score mentioned above.

#### Statistical analyses

We used logistic regression with generalised estimating equation to assess differences in the cytokine profile and serum IgE level before and after treatment

with montelukast using paired t-test. Also comparing the data of serum cytokines and IgE levels in samples from children with asthma and the healthy controls using Pearson's correlation coefficient and student t-test.

All the above statistical analyses were performed by Microsoft excel and the online Graph Pad software. The difference was considered significant if  $p < 0.05$ .

## Results

### Cytokine profile and asthma

Data from recruited 60 asthmatic children, has shown a significantly higher level of serum IL4 than that in healthy control (20 children) [394.68 versus 111.95 pg/ml]. Meanwhile, results revealed that the serum level of IL10 was significantly lower in asthmatics compared to the control healthy children (107.61 versus 390.6 pg/ml).

Data concerning other cytokines tested in this study, IL 2, IL 17 and IFN-gamma were shown to be non significant both regarding association with asthma and influence on response to montelukast. (see below).

### Clinical response to montelukast

Regarding the clinical response to montelukast, data from the supplied diary formula revealed significantly different levels of response in both treatment group and placebo group as shown in figure 1.

Table 1: Serum cytokine levels measured by ELISA from 60 asthmatic children compared to the control group.

cytokine	mean serum level, pg/ml		Significance
	control healthy	Asthmatics	
IL2	274.9 $\pm$ 29.53	276.55 $\pm$ 75.76	No
IL4	111.95 $\pm$ 22.72	394.6833 $\pm$ 55.27	Yes
IL10	390.6 $\pm$ 57.15	107.6167 $\pm$ 19.45	Yes
IL17	278.95 $\pm$ 52.59	262.3167 $\pm$ 42.20	No
IFNg	269.35 $\pm$ 62.10	280.1167 $\pm$ 59.01	No

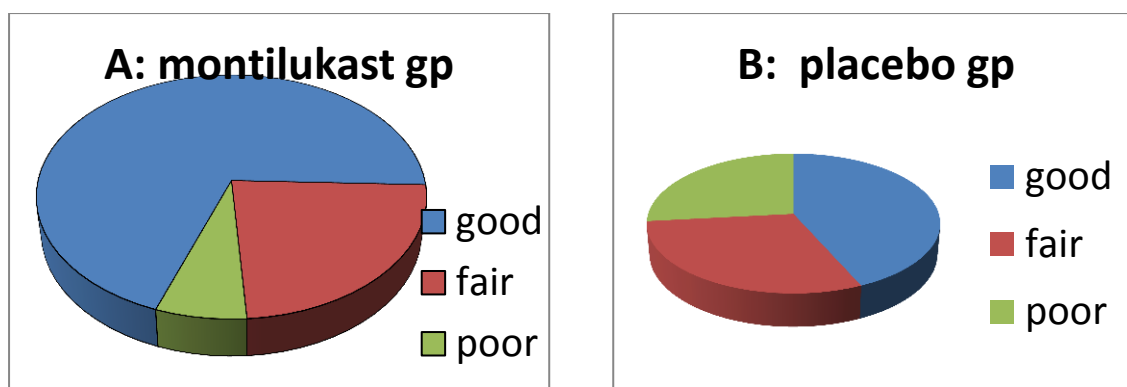


Figure 1: Representative charts of different levels of clinical response to one-month montelukast course "A" compared to the placebo group "B" of asthmatic children.

### Effect of montelukast on serum cytokine profile

In the treatment group, results showed that montelukast has caused significant reduction in serum levels of IgE and IL4 and elevation of IL10, while confers non

significant impact on other cytokines. In addition, serum levels of IgE and IL4 and IL10 have significant association with level of clinical response to montelukast determined by the diary formula supplied to the parents.

Table 2: Effect of montelukast therapy on serum cytokine levels (measured by ELISA) in treatment group compared to the placebo group of asthmatic children.

Cytokine	mean differences of pre- and post-treatment serum level pg/ml		Significance
	montelukast group	placebo group	
IL2	26.56±2.74	19.78±2.97	No
IL4	104.36±5.47	45.23±4.86	Yes
IL10	98.88±6.55	35.57±3.05	Yes
IL17	30.32±3.64	18.89±2.96	No
IFNg	41.95±3.38	16.23±5.09	No

### Cytokine influence on response to montelukast

A strong and inverse relationship ( $r = 0.78$ ) between serum IL-4 levels and clinical response to montelukast has been demonstrated. *i.e.* its level was directly related with poorer response to montelukast in the studied treatment group. In addition, our data revealed that the serum level of IL10 is positively related with better clinical response to montelukast as shown in figure 2 "A" and "B".

### Serum IgE level association with childhood asthma

Data from the current study regarding serum IgE level in the recruited 160 doctor-diagnosed asthmatics revealed that

there is a significant association with childhood asthma, mean  $99.81 \text{ IU} \pm 63.3$  in asthmatic children versus  $37.36 \text{ IU} \pm 13.47$ ;  $P\text{value} < 0.0001$ . (Figure 3).

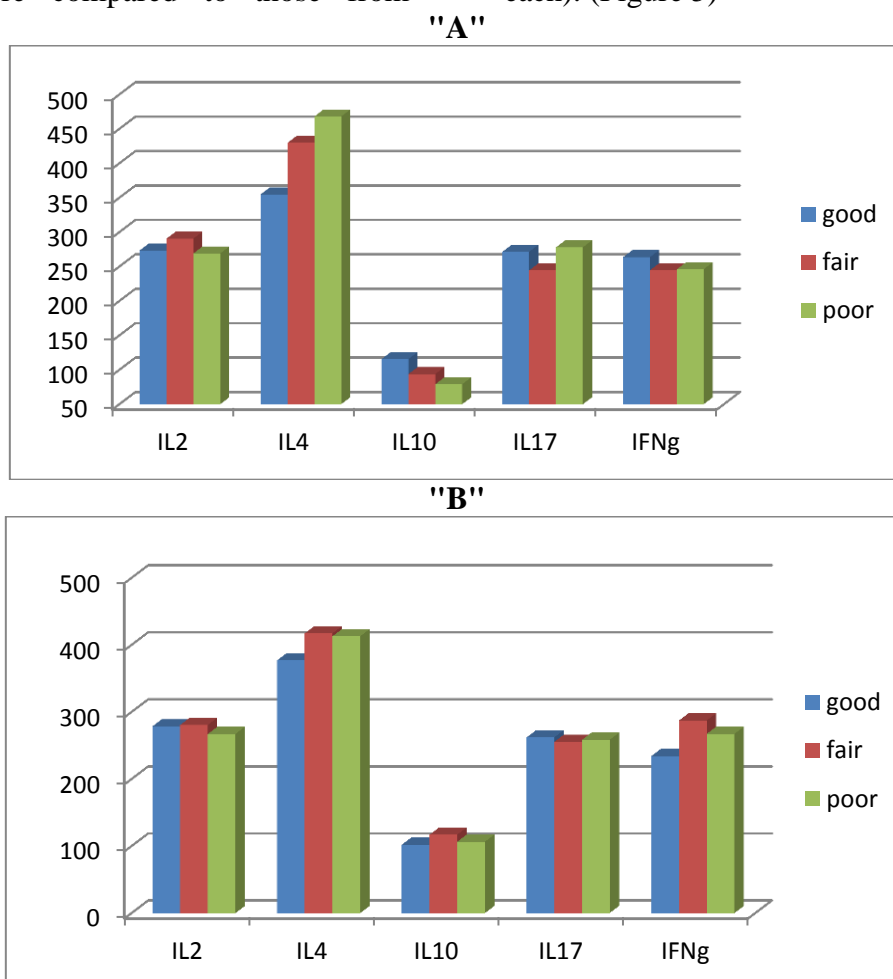
### Effect of montelukast on serum IgE level

Our results concerning influence of montelukast therapy on serum IgE level in the treatment group (30 patients) revealed that there is a significant decrease of its level compared to data from the placebo group.  $p$  value from the two-tailed paired  $t$ -test  $< 0.05$ . (Figure 4).

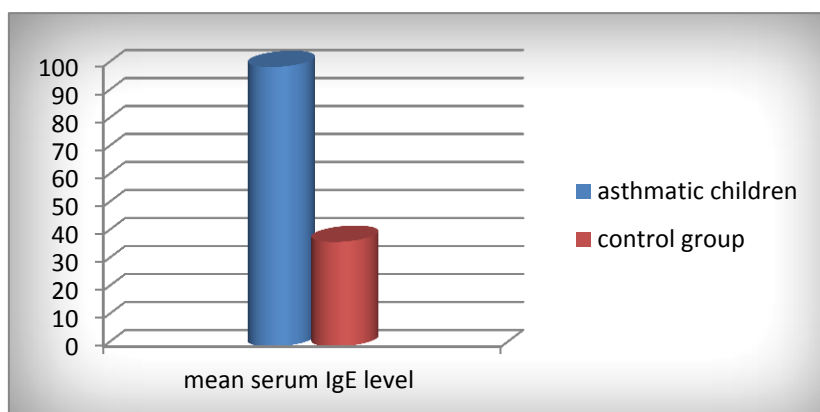
### Effect of serum IgE level on response to montelukast

It has been shown, here, that pretreatment serum IgE level has a statistically significant impact on clinical response to

montelukast in the treatment group and placebo group (30 asthmatic patients each). (Figure 5)



**Figure 2:** Influence of serum cytokine levels (measured by ELISA) on response to montelukast "A" from treatment group compared to "B" placebo group.



**Figure 3:** Representative chart of association of serum IgE level with childhood asthma

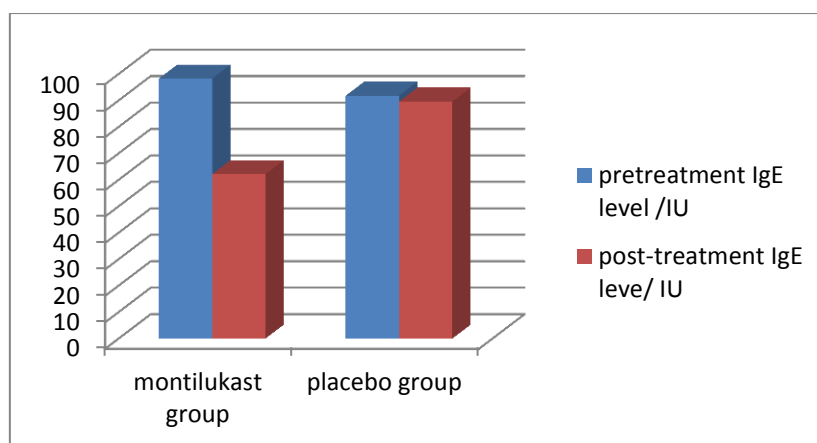


Figure 4: Representative chart of influence of montelukast on serum IgE level in the treatment group of asthmatic children compared to placebo group.

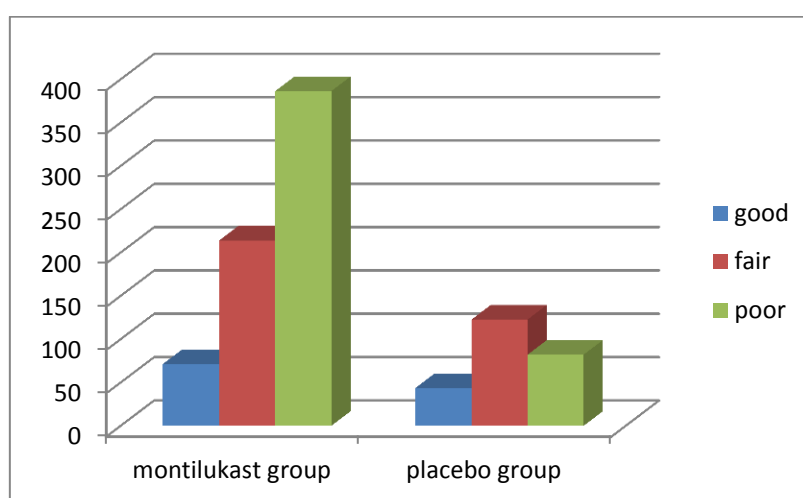


Figure 5: Representative chart of influence of pretreatment serum IgE level on clinical response to montelukast in the treatment group and placebo group of asthmatic children.

## Discussion

### Cytokine profile and asthma

Data from the current study has shown a significantly higher level of serum IL4 in asthmatic children than in healthy control. This is parallel to what was reported by a previous study. Some studies suggest that it plays a crucial role in the development of asthma through its role in the induction of IgE synthesis by B cells and differentiation to a Th2 phenotype<sup>[18]</sup>. Conversely, our data revealed that the serum level of IL10 was significantly lower in asthmatics reflecting the poor regulatory T helper cell function in this group compared to the control healthy children. This finding was reinforced by previous data implicating that impaired

IL10 production has been associated with Th2 dominated immune response reported in asthmatic patients<sup>[19]</sup>

### Clinical response to montelukast

Results from our placebo-controlled cohort study revealed statistically significant better clinical response to montelukast as add-on therapy in the treatment group than that with the conventional asthma controller therapy used in the placebo group. Our findings are consistent with what was observed by previous trials in this regard. It was found that 6 weeks of montelukast add-on to usual anti-asthmatic therapy was able to reduce asthma morbidity compared with placebo<sup>[20]</sup>. Similarly, data from a recent cohort study has shown that oral leukotriene receptor antagonists, including montelukast, reduce

the incidence of asthma exacerbations compared to conventional asthma controllers therapy<sup>[21]</sup>.

#### **Effect of montelukast on serum cytokine profile**

Regarding the effect of montelukast course on cytokine profile, no data were significant except that montelukast was shown to significantly decrease serum IL4 level in the treatment group (30 asthmatics) compared to the placebo group, p value <0.05. Such finding is consistent with previous placebo-controlled cohorts in school-aged children revealing significant reduction in blood level of eosinophils, IL4, intercellular adhesion molecule (ICAM)-1 and eosinophils-chemotactic protein (ECP) in response to montelukast<sup>[22,23]</sup>.

In addition, owing to this central role of IL4, attempts to target it by antibodies in order to mount new asthma controller therapy is giving promising results although still under research. Meanwhile, Preclinical efficacy and safety of an IL4 antagonist pascolizumab a humanised antiinterleukin-4 antibody with therapeutic potential in asthma was tested in two phase 2a studies<sup>[24-26]</sup>

#### **Cytokine influence on montelukast therapy**

More importantly, serum IL4 level was shown to be directly related with poorer clinical response to montelukast (p value < 0.05). Our finding is consistent with previous investigators' observation in this regard<sup>[24]</sup>, while disagrees with data from others which negated such association between IL4 and clinical response to montelukast<sup>[19]</sup>.

In addition, our data revealed that the higher the level of IL10, the better the clinical response to montelukast. This further ensure the fact that IL10 plays a pivotal regulatory role in airway inflammation<sup>[19]</sup> and its deficiency causes resistance to asthma controller therapy including montelukast.

The role of the other cytokines tested in this study, IL 2, IL 17 and IFN-gamma

was much less significant than IL4 and 10. Their serum levels were neither significantly related to asthma nor affecting the clinical response to montelukast. Again our results agree with some previous data which found a similar non-significant link between such cytokines and childhood asthma development<sup>[19]</sup>.

On the other hand, some reports have stated that these markers are strongly affecting immunopathogenesis of asthma and, thus, are inconsistent with data from the current study<sup>[8,27,28]</sup>.

For instance, it was reported that IL-17 may play a role in the development of various allergic diseases that have been classically considered to be Th2-mediated disorders<sup>[29]</sup>. However those studies investigated such relation in adult participants which might explain, in part, this disagreement<sup>[19]</sup>.

Nevertheless, a genetic study of IFN gamma done in children showed significant protective effect of lower expression of this cytokine against genetic risk for allergic airway inflammation later on<sup>[30]</sup>. On the other hand, other researchers reported significant negative correlation between IFN gamma and IL17 levels and development of childhood asthma. The latter finding could be explained by the fact that these cytokines simply down regulate Th2 arm and thus protect against asthma development<sup>[31,32]</sup>.

#### **Serum IgE level association with childhood asthma**

There is no doubt about the central role of IgE in the immunopathogenesis of asthma<sup>[33-35]</sup>. Data from the current study regarding serum IgE level in the recruited 160 asthmatic children revealed that there is a significantly increased level compared to the healthy control group. However, we can't rely on this association to mount an asthma diagnosis, a condition requiring larger scale survey study with much larger sample size.

Similarly, recent data suggested limited linkage for the serum IgE level with

asthma diagnosis, showing that it is elevated only approximately half the time in patients with allergic disease and that obtaining an IgE level is not indicated in most patients with asthma, although levels greater than 1000 ng/mL (1 IU= 2.4 ng) may suggest an alternate diagnosis, such as allergic bronchopulmonary aspergillosis (confounding illness such as atopic dermatitis may also result in high IgE levels)<sup>[36]</sup>.

### Effect of montelukast on serum IgE level

The current study has shown a significant lowering effect of montelukast therapy on serum IgE level (p value < 0.05). This is consistent with data from previous cohort studies in this field which reported significant reduction of serum IgE level in response to montelukast compared to the placebo group<sup>[22,23]</sup>. These data indicate that this immunoglobuline could be implicated in planning treatment options and its serum level is a good indicator for the degree of clinical; response to montelukast add-on therapy.

### Effect of serum IgE level on response to montelukast

Data from the current study has shown that the existence of elevated pretreatment serum IgE level is potentially of great importance and may explain in part the unresponsiveness to the given montelukast therapy. The latter finding could be explained by the overt central immunopathogenetic role played by IgE in asthma development<sup>[35]</sup>.

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