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Determine gene expression of IL-17 in Iraqi Child Asthmatic Patients

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Abstract: A wide variety of cytokines and chemokine's plays an vital roles in asthma severity , includes *IL-17* which participate in the pathophysiology of asthma. The objective of this study is to investigate an association of gene expression and level of serum concentration of *IL-17* in pediatric patients with asthma . Two groups were enrolled in this study: seventy five asthma patients (27 female and 48 male) and twenty five apparently healthy as a control group (10 female and 15 male).The age of the samples ranged from (1-10) years old. Recruited from admitting the Central Teaching Hospital Pediatrics and Alzahra'a Center for Asthma Allergy in Baghdad during the period extended from October /2019 to last February /2020. Subject information's were collected using a specific questionnaire form as a descriptive study; on the other hand, the present study was approved by the council of institute of genetic engineering and biotechnology for post graduate studies / University of Baghdad. The RNA was extracted from the samples of blood of asthma patients and apparently healthy subjects by using TransZol Up Plus RNA Kit (blood) .The acceptable purity of RNA in asthmatic patient is range between 1.84-1.99 and for apparently healthy group is range between 1.84-1.96.mRANA expression for *IL-17*gene were determined by real time PCR assay while serum samples were used to detect the concentration of *IL-17* using ELISA technique. Results showed that the male's percentage was higher than the females. With regard to age, highest percentage was found in the third age group of asthma patients (more than 6 year) 32 (43%) of the total numbering patients. Family history revealed that 62 (82.67%) of 75 patients have positive family history of asthma while 13(17.33 %) have no family history. The highest percentage of the asthma cases was found in winter season. According to sensitive of disease showed that 43 (57.33 %) of 75 patients consider mild disease while 32 (42.67 %) of patients have severe asthma. On the other hand ,for *IL-17* gene expression the Ct of asthma patient group revealed (22.14) and control group (22.92) and the $2^{-\Delta\Delta Ct}$ of asthma patient group (9.253) and control group (5.314) and the fold of gene expression was statistically significantly ($P\leq0.01$) in asthmatic group than healthy non asthmatic group that show in ratio (1.74: 1.00). *IL-17*concentration was estimated by ELISA, the results were statistically significant ($P\leq0.01$), the level of IL-17 in asthma patient group (440.48 ± 59.02pg/ml) while in Control group(88.03 ± 27.70pg/ml) as well as increase concentration of *IL-17* in severe asthma patients than mild form asthma 544.97 ± 85.40 and 319.67 ± 76.18 respectively .Resent study concluded that asthma is a complex disease and the inclusion of different immune cells results in different pathological processes.

Keywords: Asthma Childhood, *IL-17*, qRT-PCR.

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Introduction

Asthma is a major non communicable disease characterized by recurrent attacks of breathlessness and wheezing, which vary in severity and frequency from person to person (1). During an asthma attack, the lining of the bronchial tubes swell, causing the airways to narrow and reducing the flow of air

into and out of the lungs, recurrent asthma symptoms frequently cause sleeplessness, daytime fatigue, reduced activity levels and school absenteeism (2). Asthma has a relatively low fatality rate compared to other chronic diseases (3). Disease onset in children usually occurs before their fifth birthday. The majority of children with asthma are sensitive to household allergens and irritants, and they can benefit from a smoke-free, dust-free and pet-free environment (4,5). According to World Health Organization estimates that more than 339 million people had asthma globally in 2016,It is a common disease among children, Most asthma-related deaths occur in low- and lower-middle income countries (6).

Interleukin -17 is a protein consists of 150 amino acids with a molecular weight of 15 kDa, and its gene lies on human chromosome 6p12 (7). Is a family of cytokines consists of 6 proteins (*IL-17A* to *IL-17F*) and 5 receptors (*IL-17RA* to *IL-17RE*) which are structurally unrelated to any other known cytokine receptor (8). Recent studies have provided convincing evidence that *IL-17*, the predominant product of Th17 cells, plays an imperative role in regulating the expression of inflammatory mediators and the recruitment and function of inflammatory cells in various inflammatory diseases including asthma (9).

Interleukin (*IL)-17* is recognized to play a critical role in numerous immune and inflammatory responses by regulating the expression of various inflammatory mediators, which include cytokines, chemokines, and adhesion molecules, there is growing evidence that *IL-17* is involved in the pathogenesis of asthma. The *IL-17* orchestrates the neutrophilic influx into the airways and also enhances T-helper 2 (Th2) cell-mediated eosinophilic airway inflammation in asthma. Recent studies have demonstrated that not only inhibitor of *IL-17* in itself but also diverse regulators of *IL-17* expression reduce antigen-induced airway inflammation, bronchial hyper responsiveness and can stimulate the release of neutrophils and eosinophil which can induce airway hyper-reactivity, broncho-constriction, mucus secretion, and airway remodeling (10). As to our knowledge, this is the first study about the genetic and serological aspect of child asthma disease in the institute of genetic engineering and biotechnology so that: The present study aims to increase knowledge about the occurrence of Asthma in Iraqi pediatric to determine the concentration of *IL-17* in patient by ELISA technique and determining gene expression of *IL-17* in patient by Real time PCR.

Subjects and Methods

Study consist of two groups, Seventy Five patients (27 female and 48 male) and twenty five as apparently healthy subjects (control) and personal information such as: age, asthma season, gender, family history, sensitivity ,incidence intensity, onset of disease, other diseases, the samples were admitting the Central Teaching Hospital Pediatrics and Alzahra'a Center for Asthma and Allergy in Baghdad during the period from October /2019 to February /2020. The study design was approved by the Institute of Genetic Engineering and

Biotechnology for Postgraduate Studies/ University of Baghdad. Writing informed consents were obtained from all patients and apparently healthy control group; all patients were diagnosed according to clinical examination by a chest physician and selected according to the criteria of the global initiate of asthma.

Genomic RNA Extraction

The RNA was extracted from blood of asthma patients as well as from apparently healthy groups (control) by using TransZol Up Plus RNA Kit (blood) company of kit (Transgen) Then, RNA concentration and purity were measured by nanodrop.

cDNA synthesis for mRNA

Total RNA was reversely transcribed to complementary DNA (cDNA) using EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix. The procedure was carried out in a reaction volume of 20 µl according to the manufacturer's instructions. The total RNA volume to be reversely transcribed was (20µl) as show in table (1).

Table (1): Reaction volume and components of reverse transcription reaction used to prepare cDNA from total RNA.

Component	Volume
Total RNA/mRNA	4 µ1
Anchored Oligo(dT) Primer (0.5 µg/µl)	1 µ1
Random Primer (0.1 µg/µl)	1 µ1
GSP	2 pmol
2×ES Reaction Mix	10 µ1
EasyScript® RT/RI Enzyme Mix	1 µ1
gDNA Remover	1 µ1
RNase-free Water	to 20 µ1

Real time PCR Primers for IL-17Gene

The primer sequence of this study is listed in table (2) (11).

Table (2): primer sequences

Name of gene	Primer	Sequences
IL-17	Forward	5-TCTGGGAGGCAAAGTGCCGC-3
	Reverse	5-GGGCAGTGTGGAGGCTCCCT-3
β- actin	Forward	5- GGGCGGCACCACCATGTACC-3
	Reverse	5- GACGATGGAGGGGCCCGACT-3

Table (3): Components of quantitative real-time PCR used in *IL-17* (Transgen / China).

Template DNA	3 µl
Reagent Master Mix	25 µl 1 rxn
PCR grade water	7.5
2xqPCR Master Mix	12.5 µl
Forward Primer (10 µM)	1 µl
Reverse Primer (10 µM)	1 µl

Real Time PCR Program

The expression levels *of i*nterleukin *17 (IL 17),* and housekeeping genes (*β-actin*) were estimated by RT-PCR To confirm the expression of target gene, quantitative real time RT-PCR SYBR Green assay was used. Primers sequences for *interleukin 17 (IL 17),* as housekeeping gene (*β-actin)* were prepared according to (11) Optimal annealing temperature of qPCR reaction was found out after several try to 64˚C with a total volume of 50 µl. The reaction components were described in table (4). According to the instructions of the manufacturer of the kit (*TransStart*® Top Green qPCR SuperMix) with Catalog Number : AQ131-01.

Table (4): Thermal profile of *IL-17* gene expression

Step	Temperature	Duration	Cycles
Enzyme activation	94ºC	30 sec	Hold
Denature	94ºC	5 sec	40
Anneal	64ºC	20 sec	
extend	72ºC	20 sec	
Dissociation	1min /95 ºC-30 sec /60ºC-30sec/95 ºC		

Real Time RT-PCR analysis of *IL17* gene expression

1. **ΔCT:** The expression ratio was calculated without a calibrator sample $2^{-\Delta Ct}$ according to the following equation:
   ΔCT (test) = CT gene of interest (target, test) – CT internal control.
   Finally, the expression ratio was calculated according to the formula
   $2^{-\Delta Ct}$ = Normalized expression ratio (12).
2. **ΔΔ CT:**
   To compare the transcript levels between different samples the $2^{-\Delta\Delta Ct}$ method was used (12).
   The CT of gene of interest was normalized to that of internal control gene. The difference in the cycle threshold (Ct) values of the B actin gene (interest gene) was calculated as the following formula.
   ΔCT (test) = CT gene of interest (target, test) – CT internal control
   ΔCT (calibrator) = CT gene of interest (target, calibrator) – CT internal control. The calibrator was chosen from the control samples.
   CT values ≥ 38 were considered unreliable and neglected.
   The ΔCT of the test samples was normalized to the ΔCT of the calibrator:
   ΔΔ CT was calculated according to the following equation:
   ΔΔ CT=ΔCT (test)- ΔCT(calibrator).
   Finally, the expression ratio was calculated according to the formula.
   $2^{-\Delta\Delta Ct}$ = Normalized expression ratio (12).

Determination of IL-17 titer using Enzyme-Linked Immunosorbent Assay kit (MyBioSource, USA)

This kit was based on sandwich enzyme-linked immune-sorbent assay technology. Anti- *IL-17* antibody were pre-coated onto 96- well plates. And the

biotin conjugated anti-*IL-17*antibody were used as detection antibodies. The standards, test samples and biotin conjugated detection antibody were added to the wells subsequently, and washed with wash buffer. HRP-Streptavidin was added and unbound conjugates were washed away with wash buffer. TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the *IL-17* amount of sample captured in plate. Read the O.D. absorbance at 450nm in a microplate reader, and then the concentration of *IL-17* can be calculated.

Statistical analysis

According to (SAS,13) Program was used to influence of various factors in gender, age, season, family history, treatment ,incidence intensity, season, sensitive for (treatment, food and dust) and onset of disease. program was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) or T-test was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

Result and Discussion

Distribution of Asthma patients and control group according to gender

Regarding to gender, the numbers of males and females in the patients group were 48 (46%) and 27 (36%) respectively as shown in table (5). A significant difference was found in between these groups regarding the gender ($p=0.0014$: $p<0.05$). This result is identical with many studies considerable gender differences in asthma and allergic diseases in childhood . It suggests that gender is an important determinant for asthma and allergies. The impact of gender varies considerably from childhood into adolescence and adulthood. In childhood, boys are consistently found to be at increased risk of asthma, which has been explained by differential growth of lung/airway size, and immunological differences (14).

Distribution of Asthma patients and control group according to Age

The age of asthma patients and control ranged from 1-10 years, the highest percentage of the asthma cases was found in the third age group included 32 patients (more than 6 year) which reached to 43% of the total patients, followed by 37 % for the second Fage group (2-6 year) and 20 % for the first age group (less than 2 year) included 15 patients. These results are in agreement with several studies, Several studies show that as many as 50–80 percent of children who have asthma develop after 4 years difficulty diagnosis at these age group (receiving such inappropriate labels as chronic bronchitis asthma, wheezy bronchitis and recurrent pneumonia) (15). And other study when measuring the distribution of asthma in different age groups the result show 56% at age group (10–12), 44% at age group (7–9), 38% at

age group 4–6 years and 66% at more than 12 years (16). Show table (5).

Distribution asthma according to family history

The distribution of patients according to family history of disease, table (5) detected that 62 (82.67%) of 75 patients have a positive family history of asthma and 13 (17.33%) of patients have no family history. A significant difference was found in between these groups regarding the family history ($p=0.0001$: $p<0.01$). These results are in agreement with several studies, that found there were positive correlation between family history and atopy (17). Noticed other that the asthma patients with family history was 56 (52.8%) of the children. about one-third of the children had at least one parent who is a known asthmatic and without family history was 50 (47.2%) of the children (18). The results of other study provide evidence that the incidence of asthma during the first years of life is strongly related to family history of asthma.

Distribution of asthma patients according to sensitive allergen

The distribution of patients according to sensitive of disease, table (5) detected that 62 (82.67%) of 75 patients have sensitive for drug ,food, dust, animal, and other environmental factor and 13 (17.33%) of patients have no sensitive . A significant difference was found in between these groups regarding to the sensitive ($p=0.0001$: $p<0.01$). This result agreement with study in northern China show that link between mold sensitization and asthma in a cohort of patients, and this link was not found in other two common allergens, house mites and weeds (19).

Distribution of Asthma patients according to Season

The highest percentage of the asthma cases was found in winter season which reached to70.67% of the total patients, followed by 14.67% for Autumn and Spring season table (5). A significant difference was found in between these groups regarding the season ($p=0.0001$: $p<0.01$). This result agreement with study that Clarify degreement the proportion of patients with symptoms is consistently greaterin winter (20). According to Asthma UK, 75 per cent of people report that cold air triggers their asthma symptoms. Some people find that just breathing in very cold air causes symptoms. When the air hits the airways it can sometimes make them go into spasm, which causes coughing, wheezing, a tight chest and breathlessness (21).

Distribution of Asthma patients according to incidence intensity

The distribution of patients according to sensitive of disease, table (5) detected that 43 (57.33 %) of 75 patients consider mild asthmatic patient and 32(42.67 %) of patients have severe asthma. These result agreement with other study (22,23).

Table (5): Distribution of asthma according to gender, age, family history, sensitive, season, incidence intensity in patients asthma

Factors		Patients No. =75 %	Control No. =25 %	P-value
Gender	Male	48 64.00%	15 60.00%	p<0.05
	Female	27 36.00%	10 40.00%	
Age	Less than 2y	15	5	__
	2 y -6 y	28	10	
	More than 6y	32	10	
Family history	Yes	62 (82.67%)	__	0.0001 **
	No	13 (17.33 %)		
Sensitive	Yes	62 (82.67%)	__	0.0001 **
	No	13 (17.33 %)		
Season	Winter	53(70.67 %)	__	0.0001 **
	Autumn	11(14.67 %)		
	Spring	11(14.67 %)		
Incidence intensity	mild	43(57.33 %)	__	0.0298*
	sever	32 (42.67%)		

**** (P<0.01) (p<0.05).**

RNA Extraction

Total RNA was successfully extracted from all samples. The concentration of total RNA ranged from 83-188ng/ µl.

cDNA reverse transcription

The cDNA reverse transcription was conducted on the second day of RNA extraction. A common primer reaction was applied since it was needed to have cDNA for both the gene in the study and housekeeping gene.

The efficiency of cDNA concentration was assessed through the efficiency of qPCR conducted later on All steps were associated with perfect yield reflecting efficient reverse transcription.

Real time PCR quantification of *IL17* Expression

The mean Ct value of *IL-17*cDNA amplification was (22.14) in the patients asthmatic group. While the mean Ct Healthy group was(22.92).The Results are shown in table (6). There was a significant difference in the mean Ct values between the different study groups, ($P \leq 0.01$).

In the present study, quantitative RT-PCR assay analyzed the mRNA expression of *IL-17* and compared its expression between asthmatic patient group and healthy

non asthmatic group .The calculation of gene expression fold change was made using relative quantification (11).

This depends on normalization of Ct values calculating the ΔCt which is the difference between the mean Ct values of replica of *IL-17* cDNA amplification of each single case and that of the *GAPDH*.

Table (6) shows the mean of ΔCt (normalization Ct values) of each study group. ΔCt means in asthmatic patient group and Healthy non asthmatic group were (-0.11) and (0.69) respectively.

A significant difference was noticed between the study groups (P≤0.01).

Results of $2^{-\Delta Ct}$ revealed significantly higher results for the asthmatic patient group when consider with healthy non asthmatic group(P≤0.01), mean of $2^{-\Delta Ct}$ for asthmatic patient group (1.08). and healthy group a mean of $2^{-\Delta Ct}$ was(0.62)

To calculate the gene expression folds in relation to the housekeeping genes the result of $2^{-\Delta Ct}$ of each group was measured in relation to that of Healthy non asthmatic group. The results are shown in table (6). The fold of gene expression in asthmatic patient group was higher than Healthy non asthmatic group in 1.74 ± 0.09 times as shown in table (6). These results indicate a significantly increase expression of *IL-17*gene in asthmatic patients.

Table (6): Fold of *IL17*expression Depending on $2^{-\Delta Ct}$ Method

Groups	Means Ct of *IL17*	Means Ct of *B-actin*	ΔCt (Means Ct of*IL17* - Means Ct of *B-actin*)	$2^{-\Delta Ct}$	experimental group/ Control group	Fold of gene expression
Asthma patient group	22.14	22.25	-0.11	1.08	1.08/0.62	1.74 ± 0.09
Healthy group	22.92	22.23	0.69	0.62	0.62/0.62	1.00 ± 0.00
T-test	--	--	--	--	--	0.362 **
P-value	--	--	--	--	--	0.0048

**** (P≤0.01).**

In calculation of the relative expression of *IL-17* gene in all study groups the $2^{-\Delta Ct}$ results was applied. A calibrator was used and it was one of the samples of the controls with high expression of *IL-17* as shown in table (6).

The mean of $2^{-\Delta\Delta Ct}$ values of asthmatic patient group was (9.253). The mean for Healthy non asthmatic group was (5.314). There was a significant difference in between these groups regarding the mean $2^{-\Delta\Delta Ct}$, (p≤0.01), as shown in table (7).

Table (7): Fold of *IL17*expression Depending on $2^{-\Delta\Delta Ct}$ Method

Groups	Means Ct of *IL17*	Means Ct of *B-actin*	ΔCt (Means Ct of *IL17* - Means Ct of *B-actin*)	Mean ΔCt Calibrator (ct*IL17*-ct *B-actin*	ΔΔCt	$2^{-\Delta\Delta Ct}$	experimental group/ Control group	Fold of gene expression
Asthma patient group	22.14	22.25	-0.11	3.1	-3.21	9.253	9.25/5.31	1.74 ± 0.09
Healthy group	22.92	22.23	0.69	3.1	-2.41	5.314	5.31/5.31	1.00 ± 0.00
T-test	--	--	--	--	--	--	--	0.362 **
P-value	--	--	--	--	--	--	--	0.0048

**** (P≤0.01).**

When calculating, the gene expression was significantly in patient asthmatic group than control 1.74 times. as shown in table (7). The above results demonstrate the significant gene expression in Healthy non asthmatic group.

The mean Ct values in healthy non asthmatic group were higher than those of asthmatic patient group .This is important in reflecting the original mRNAs present in the samples. It is evident from these results that the patients group is associated with the highest copy number of mRNAs reflecting its higher expression.

The results show convergence of Ct values between asthmatic patient group and healthy non asthmatic group it is important evidence that *IL-17*gene expression so it is possible to use *IL-17*gene as a biomarker for the early detection of asthma disease.

These results agreed with (24). Their result indicate that *IL-17* gene expression is higher in asthmatic patient than control.

These results coincide with those reported by (25). which revealed that the mRNA expression of *IL-17* in the peripheral blood is of a significant clinical value for the diagnosis of atopic asthma patient.

Also These results agreed with (11). Reveal an increase in the serum *IL-17* concentration and *IL-17* mRNA expression in children with severe asthma compared to those with mild forms of the disease and compared with healthy group.

Therefore, the evaluation of mRNA expression of *IL-17* in the peripheral blood had important clinical value for the diagnosis and the prognosis of asthmatic disease . Figures (1) and (2) show the amplification plots and dissociation curves for *IL-17*.

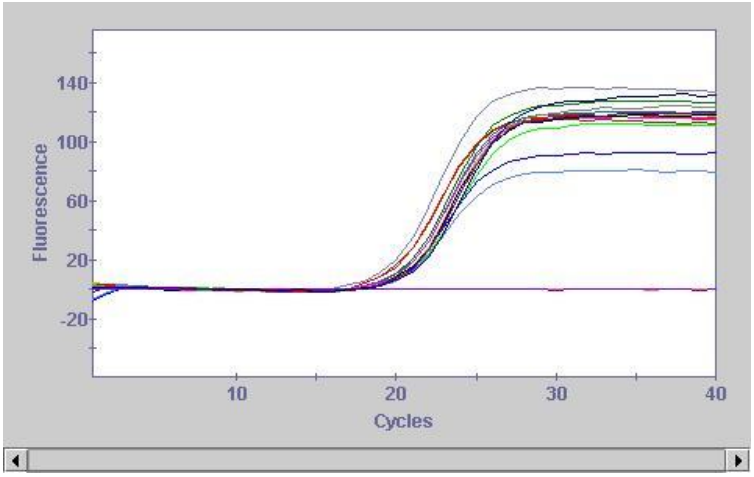

Figure (1): *IL-17* amplification plots by qPCR Samples included all study groups .Ct values ranged from 20.44 to 24.77. The photograph was taken directly from Cephoidsmartcycler qPCR machine

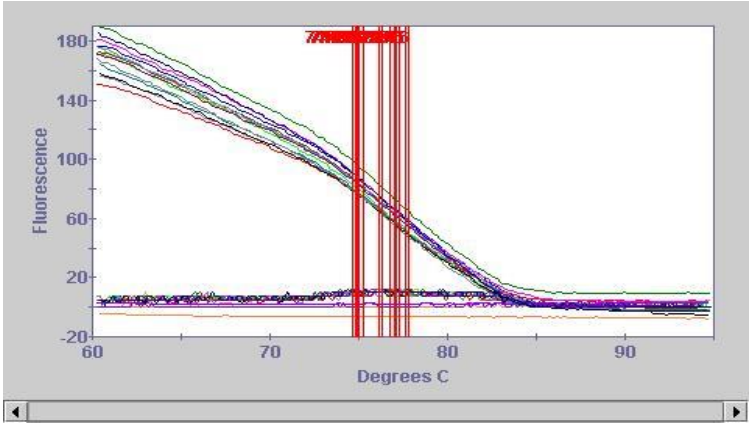

Figure (2): IL-17 dissociation curves by qPCR Samples included all study groups. Melting temperature ranged from 76.78°C to 79.96°C. The photograph was taken directly from Cephoidsmartcycler qPCR machine.

Serological Study

Evaluation the Concentrations of *IL-17* by ELISA

The results of the study evaluating the concentrations of *IL-17* in blood serum of asthmatic patient and control group. That show significant between asthmatic patients and control group (P≤0.01). Table (8). The Mean ± SE of asthma Patients(440.48 ± 59.02pg/ml) and Mean ± SE of Control group(88.03 ± 27.70pg/ml).

Table (8): Comparison between patients and control groups in concentration of *IL-17*

Group	Mean ± SE of IL-17 (pg/ml)
Patients	440.48 ± 59.02
Control	88.03 ± 27.70
P-value	0.0000001
** (P≤0.01).	

This result is identical with many studies that show Significant increase was found in the serum level of *IL-17* in asthmatic patients compared with normal control (26, 27). Were measured of serum cytokine levels in asthmatic patient and control group revealed that *IL-17* was significantly higher in the asthmatic group than the control group. And when Comparison level of cytokine (*IL-17*) between asthma patient in different forms asthma (sever and mild) and control group the result appear significantly between these groups (P≤0.01) the concentration of *IL-17* in sever asthma patient (544.97 ± 85.40), mild asthma patient (319.67 ± 76.18) and control group(88.03 ± 27.70) table (9) figure (3).Concentration of *IL-17* increase with severity form asthma more than mild form asthma and mild form asthma increase in concentration of *IL-17* more than control group and these result identical with gene expression result that previously offered. This study is identical with several studies (28,26).

Table (9): Comparison between patients (Sever, Mild) and control groups in concentration of *IL-17*.

Groups	Patients group		Control group
	Mild	Sever	
IL-17 level (pg/ml) mean ± SE	319.67 ± 76.18 [b]	544.97 ± 85.40 [a]	88.03 ± 27.70 [c]

Note : Small liter (a,b,c) indicate significant difference between groups.

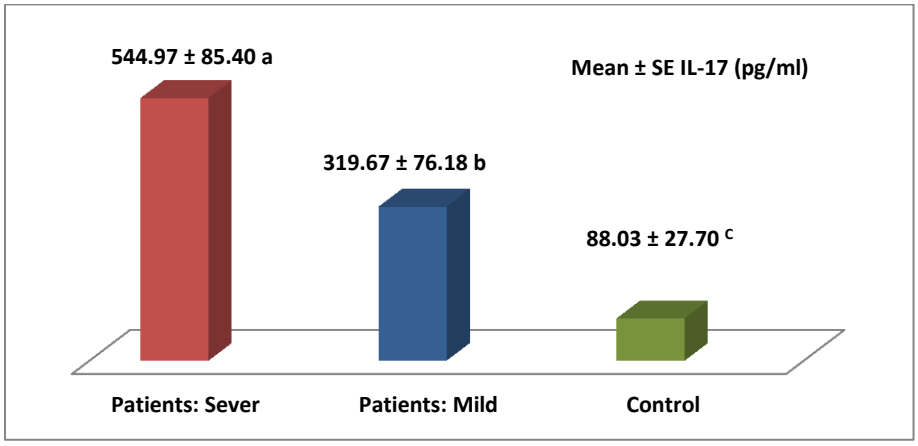

Figure (3): Comparison between patients (Sever, Mild) and control groups in concentration of *IL-17*.

Conclusion

In this study revealed that an increase *IL-17* mRNA expressions and serum *IL-17* concentrations in children with severe asthma compared to that with mild of the disease

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