AIM OF THE WORK

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The present study aimed at investigation of possible association between the fibulin-1 levels in asthmatic patients and its relation to asthma severity.

PATIENTS

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The study was carried out on forty five asthmatic patients and thirty age and sex-matched normal control subjects recruited from chest diseases department of the Alexandria Main University Hospital.

An informed consent was taken from all subjects prior to the onset of the study.

Inclusion criteria: asthmatic patients in different degrees of disease (mild, moderate and severe degrees).

Exclusion criteria: renal, hepatic, immunological diseases and smoking.

MATERIALS AND METHODS

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Subjects were divided into two groups:

1. Forty five asthmatic patients were classified into three groups:[191]

   Group I: Fifteen asthmatic patients (mild stage).

   Group II: Fifteen asthmatic patients (moderate stage).

   Group III: Fifteen asthmatic patients (severe stage).

2. Thirty normal healthy adults were recruited as controls with matched age and sex.

Both groups were subjected to:

1) Full history taking including :

   - Name.
   - Age.
   - Gender.
   - Relevant family history/ past history.
   - Smoking.
   - Medical history.

2) Careful general examination :

   - Head and neck examination
   - Abdominal examination
   - Extremities examination
   - Vital signs.
     - Blood pressure.
     - Pulse.
     - Temperature.

3) Local chest examination:

   - Inspection: to detect rate of respiration.
   - Palpation.
   - Percussion
   - Auscultation: to detect sound of air entry.

4) Laboratory investigations:

A- Routine laboratory investigations:

- Complete blood picture (CBC): CBCs were performed on a 4 differential automated cell counter Sysmex KX-21N.
- ALT/ AST/ urea and serum creatinine: were measured by fully automated chemistry analyzer Dimension RXL.

B- **Chest X-ray**: Standard poster-anterior (PA) chest radiographs were done.

C- **Pulmonary function test (PFT):** including forced expiratory volume in one second (FEV1%) predicted, forced expiratory volume in one second/forced vital capacity (F1V1/FVC %), peak expiratory flow rate (PEFR %) and reversibility test were done by spirometry.

D- Sampling:

Blood samples were obtained (from patients and controls) from the anticubital vein with minimal stasis and under complete aseptic technique.

Samples were centrifuged at the speed of 2000-3000 r.p.m. Serum was removed, aliquoted and stored at -20°C till it be used. Samples were centrifuged again after thawing before they were used.

Bronchoalvoelar lavage (BAL) [192] was obtained (from patients only) by means of flexible fibreoptic bronchoscopy. Specimens were collected via normal saline lavage of the segmental airways and alveolar spaces in sterile containers, centrifuged for 20 mins. at the speed of 2000-3000 r.p.m. to remove mucus and cells. Supernatant was removed, aliquot and stored at -20°C till it be used. Samples were centrifuged again after thawing before they were used.

Procedure:

Enzyme-linked immune-sorbent assay (ELISA) [193,194,195] (Figure VI).

Immunoenzyme methods having been successfully applied to the localization of intracellular antigens both at the light and electron microscope level, the same general principle was employed to detect soluble antigens and antibodies in body fluids. Immunoenzyme assays were therefore developed as alternatives to radio-immune assays.

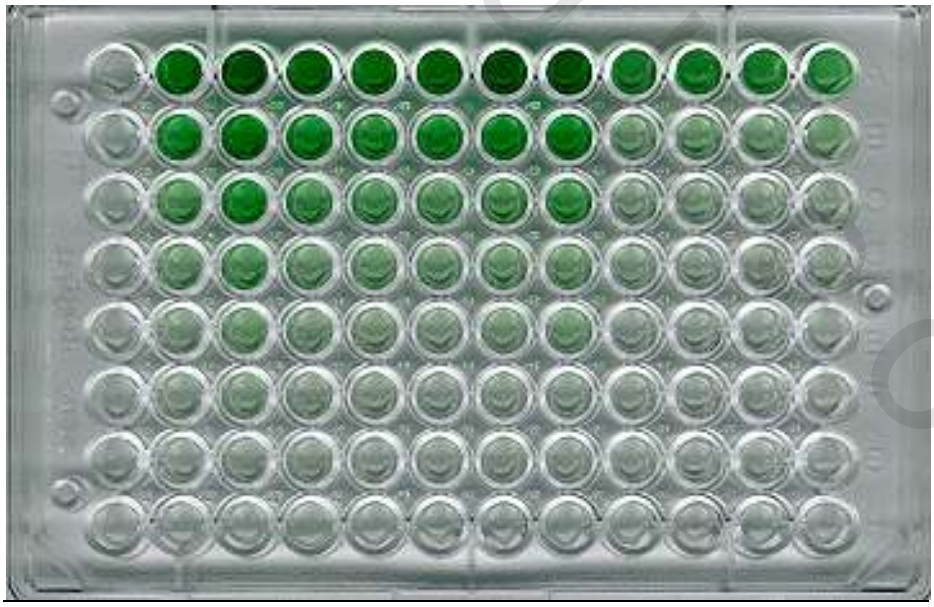


Figure (VI): Enzyme-linked immune-sorbent assay (ELISA).

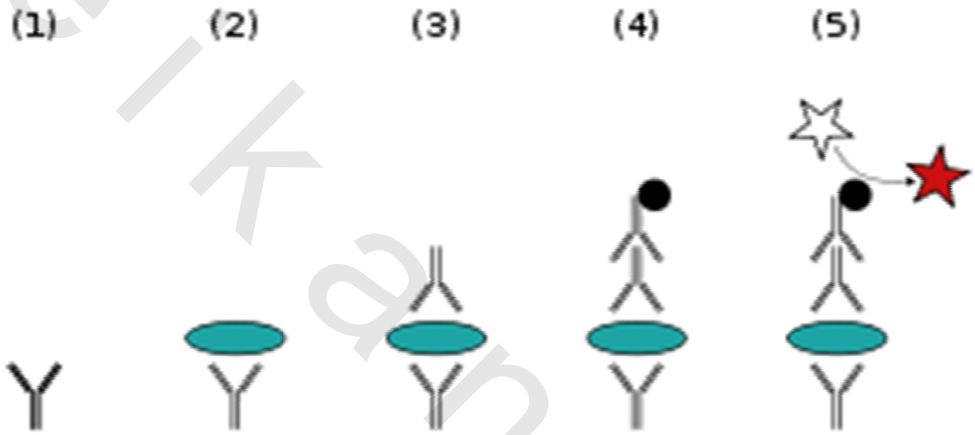
Applications:

ELISA can be performed to evaluate either:

- The presence of antibodies (Indirect ELISA).
- The presence of antigens (Direct or Sandwich ELISA).

A double-antibody ELISA (Sandwich or direct ELISA): (figure VII)

After coating the tube wall with antibody, the test serum is added followed by conjugate consisting of the initial antibody labeled with the enzyme. The reaction product is proportional to the amount of antigen in the test fluid.



A sandwich *ELISA*. (1) Plate is coated with a capture antibody; (2) sample is added, and any antigen present binds to capture antibody; (3) detecting antibody is added, and binds to antigen; (4) enzyme-linked secondary antibody is added, and binds to detecting antibody; (5) substrate is added, and is converted by enzyme to detectable form

Figure (VII): Antigen detection (direct or sandwich ELISA)

Principle of the assay:

The kit (derived from NEW YORK ,USA) assay Human FBLN1 level in the sample, use Purified Human FBLN1 antibody to coat microtiter plate wells, make solid-phase antibody, then add FBLN1 to wells, Combined FBLN1 antibody which with enzyme labeled, become antibody - antigen - enzyme-antibody complex. After washing completely, add substrate. Substrate becomes blue color at HRP enzyme-catalyzed. Reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of FBLN1

in the samples is then determined by comparing the optical density (O.D) of the samples to the standard curve.

Assay procedure:

1. Ten standard wells were set on the microtiter plate coated: 100µl Standard was added to the first and the second well, then 50µl Standard dilution was added to the first and the second well, then were mixed. 100µl from the first and the second well was taken out and was added to the third and the forth well separately, then 50µl Standard dilution was add to the third and the forth well, then were mixed. 50µl from the third and the forth well was discarded, then 50µl was taken out and was added to the fifth and the sixth well, then 50µl Standard dilution was added to the fifth and the sixth well, then was mix. 50µl from the fifth and the sixth well was taken out and was added to the seventh and the eighth well, then 50µl Standard dilution was added to the seventh and the eighth well, then was mixed. 50µl from the seventh and the eighth well was taken out and was added to the ninth and the tenth well, 50µl Standard dilution was added to the ninth and the tenth well, then were mixed. 50µl from the ninth and the tenth was discard. (density:600pg/ml, 400pg/ml , 200pg/ml, 100pg/ml, 50pg/ml).
2. Blank wells were setted separately (in blank comparison wells we didn't add sample and Enzyme Conjugate, other each step operation was same). 40µl sample dilution was added to sample well, then 10µl sample was added (sample final dilution is 5-fold), the well wall has not been touched as far as possible, and gentle mixing was done.
3. After the plate had been closed with Closure plate membrane, it was incubated for 30 mins at 37℃.
4. Wash solution was prepared: 30-fold wash solution was diluted 30-fold with distilled water and was reserved.
5. Five washes were done using the previously prepared washing solution. After final wash, the plate was inverted, and blotted dry by hitting plate onto absorbent paper or paper towels until no moisture appears.
6. Fifty µl Enzyme Conjugate reagent was added to each well, except blank well.
7. Incubate: Operation with 3.
8. Washing: Operation with 5.
9. Fifty µl Substrate A and Substrate B were added to each well, the plate was covered and was incubated for 15 mins at 37℃.
10. The reaction was stopped by adding 50µl Stop Solution to each well and mixing was done.
11. The optical density of each well was determined within 15mins by a microplate reader.

Important notes

1. Reagents hadn't been substituted from one kit lot to another. Standard, conjugate and microtiter plates are matched for optimal performance.
2. Kit reagents and materials had been allowed to reach room temperature (20-25℃) before use.
3. Kit components hadn't been used beyond their expiration date.
4. Only distilled water had been used to dilute reagent.
5. Fresh disposable pipette tips had been used for each transfer to avoid contamination.
6. Acid and sodium hypochlorite solutions hadn't been mixed.

7. Serum and BAL had been handled as potentially hazardous and capable of transmitting disease. All blood derivatives should be considered potentially infectious and good laboratory practices should be followed.
8. All samples had been disposed of in a manner that would inactivate viruses.
9. Liquid Waste: sodium hypochlorite had been added to a final concentration of 1.0%. The waste had been allowed to stand for a minimum of 30 mins to inactivate the viruses before disposal.

Calculation:

The standard curve had been drawn on graph paper with the standard density had been taken as the horizontal, the OD value for the vertical. The corresponding density according to the sample OD value had been found out by the Sample curve, multiplied by the dilution factor if any.

Assay range:

20pg/ml -800pg/ml.

Statistical analysis

Data were analyzed using SPSS software package version 18.0 (SPSS, Chicago, IL, USA). Quantitative data was expressed using Range, mean, standard deviation and median while Qualitative data was expressed in frequency and percent. Qualitative data was analyzed using Chi-square test also exact tests such Fisher exact was applied to compare the four groups. Not normally distributed quantitative data was analyzed using Mann Whitney test for comparing the four groups. Pearson coefficient was used to analyze correlation between any two variables. P value was assumed to be significant at 0.05.

RESULTS

RESULTS

Subjects submitted to this study were divided into two groups:

1. Forty five asthmatic patients were classified into three groups:[14]

   Group I: Fifteen asthmatic patients (mild stage).

   Group II: Fifteen asthmatic patients (moderate stage).

   Group III: Fifteen asthmatic patients (severe stage).

2. Thirty normal healthy adults were recruited as controls with matched age and sex.

Table (4) shows comparison between the four studied groups according to demographic data. The control group included 10 males (33.3%) and 20 females (66.7%) with a mean age of 28.30 ± 5.21years (ranging from 19.0 – 40.0 years). The asthmatic patients include 2 male (13.3%) and 13 females (86.78%) with a mean age of 46.20 ± 10.42 (ranging from 26.0 – 62.0 years) in mild stage, 1males (6.7%) and 14 females (93.3%) with a mean age of 45.53 ± 14.60 (ranging from 26.0 – 72.0 years) in moderate stage, 2 males (13.3%) and 13 females (86.7%) with a mean age of 42.33 ± 10.24 (ranging from 22.0 – 56.0 years) in severe stage.

The gender predilection shows that females are much more affected with bronchial asthma with statistical significant difference ($p<0.05$).

According to age, the results show that mild and moderate stages of asthma increased in age between 40-60 years than between 20-40 years with statistical significant difference ($p<0.05$) while severe stage of the disease increased in age between 20-40 years than between 40-60 years with statistical significant difference ($p<0.05$).

Table (4): Comparison between the four studied groups according to demographic data

	Mild (n=15)		Moderate (n=15)		Severe (n=15)		Control (n=30)		Test of sig.	p
	No.	%	No.	%	No.	%	No.	%		
Age (years)										
<20	0	0.0	0	0.0	0	00	1	3.3		
20 – 40	5	33.3	6	40.0	8	53.3	29	96.7	χ^2= 35.801*	MCp <0.001*
40 – 60	9	60.0	7	46.7	7	46.7	0	0.0		
>60	1	6.7	2	13.3	0	00	0	0.0		
Min. – Max.	26.0 – 62.0		26.0 – 72.0		22.0 – 56.0		19.0 – 40.0			
Mean ± SD.	46.20 ± 10.42		45.53 ± 14.60		42.33 ± 10.24		28.30 ± 5.21		F= 17.723*	<0.001*
Median	47.0		50.0		40.0		28.0			
p_1	<0.001*		<0.001*		<0.001*					
p_2	0.235		0.372							
p_3	0.766									
Sex										
Male	2	13.3	1	6.7	2	13.3	10	33.3	χ^2= 5.034	MCp= 0.187
Female	13	86.78	14	93.3	13	86.7	20	66.7		

P : p value for comparing between the four studied groups
p_1: p value for Post Hoc test (LSD) for comparing between control with each other group
p_2: p value for Post Hoc test (LSD) for comparing between severe with each other group
p_3: p value for Post Hoc test (LSD) for comparing between mild and moderate
F: F test (ANOVA)
χ^2: Chi square test
MC: Monte Carlo test
*: Statistically significant at $p \leq 0.05$

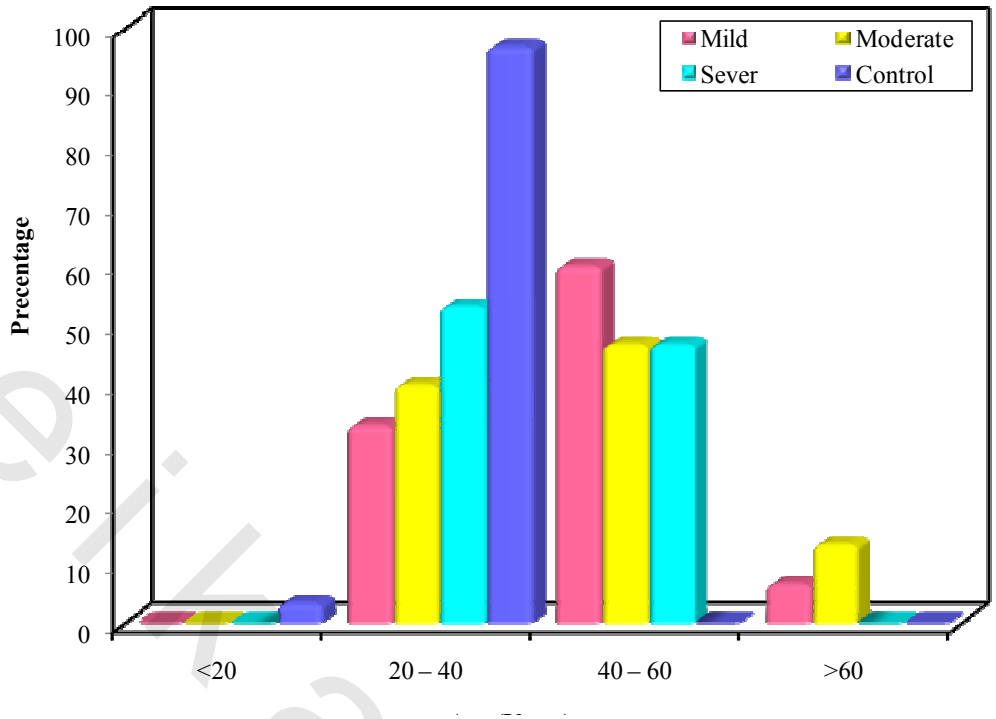


Figure (VIII): Comparison between the four studied groups according to age

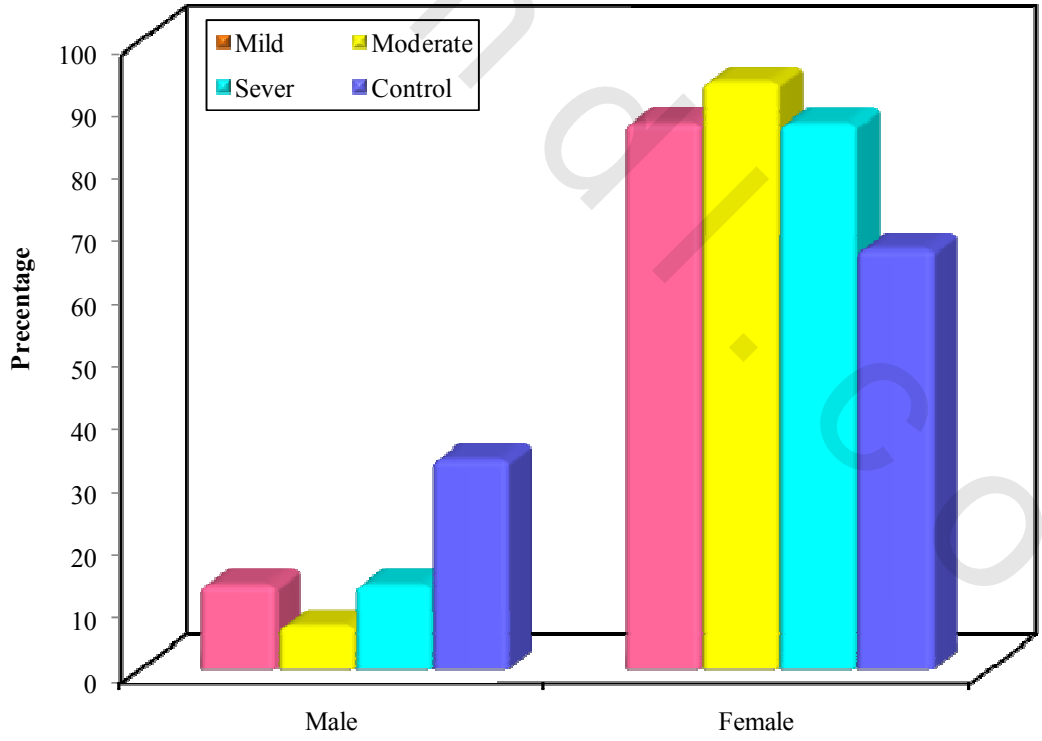


Figure (IX): Comparison between the four studied groups according to sex

Table (5) shows comparison between the three asthmatic groups according to pulmonary functions. The first group included 15 patients in mild stage with a mean FEV1 of 100.28 ± 10.04 % (ranging from83.67 – 122.0%), PEFR of 65.69 ± 11.46 L/Sec (ranging from 45.50 – 82.50 L/Sec) and F1V1/FVC of 111.83 ± 13.94% (ranging from 96.0 – 142.40%). The second group included 15 patients in moderate stage with a mean FEV1 of 73.34 ± 5.18% (ranging from 60.06 – 78.30 %), PEFR of 43.51 ± 16.92 L/Sec (ranging from 20.30 – 73.0 L/Sec) and F1V1/FVC of 94.17 ± 11.42 % (ranging from 71.80 – 122.30 %).The third group included 15 patients in severe stage with a mean FEV1 of 43.90 ± 12.73 % (ranging from 25.0 – 57.0 %), PEFR of 18.68 ± 5.86 L/Sec (ranging from 11.0 – 31.0 L/Sec) and F1V1/FVC of 56.73 ± 20.81 % (ranging from 40.0 – 107.0%).

FEV1, PEFR and F1V1/FVC were increased in mild stage than in moderate and severe stage.

Table (5): Comparison between the three asthmatic groups according to pulmonary functions

	Mild (n=15)	**Moderate (n=15)**	**Sever (n=15)**	**Test of sig.**	**p**
FEV1					
Min. – Max.	83.67 – 122.0	60.06 – 78.30	25.0 – 57.0		
Mean ± SD.	100.28 ± 10.04	73.34 ± 5.18	43.90 ± 12.73	F= 123.495*	<0.001*
Median	99.0	75.30	43.0		
$^{Sch}p_1$		<0.001*	<0.001*		
$^{Sch}p_2$		<0.001*			
PEFR					
Min. – Max.	45.50 – 82.50	20.30 – 73.0	11.0 – 31.0		
Mean ± SD.	65.69 ± 11.46	43.51 ± 16.92	18.68 ± 5.86	F= 55.066*	<0.001*
Median	69.20	44.90	19.0		
$^{Sch}p_1$		<0.001*	<0.001*		
$^{Sch}p_2$		<0.001*			
F1V1/FVC					
Min. – Max.	96.0 – 142.40	71.80 – 122.30	40.0 – 107.0		
Mean ± SD.	111.83 ± 13.94	94.17 ± 11.42	56.73 ± 20.81	$^{KW}\chi^2$= 25.744*	<0.001*
Median	110.40	97.0	48.0		
$^{MW}p_1$		0.002*	<0.001*		
$^{MW}p_2$		0.001*			

p : p value for comparing between the three studied groups
p_1: p value for comparing between mild with each other group
p_2: p value for comparing between moderate and severe
F: F test (ANOVA)
χ^2: Chi square for Kruskal Wallis test
Sch: Post Hoc Test (Scheffe)
MW: Mann Whitney test
*: Statistically significant at $p \leq 0.05$

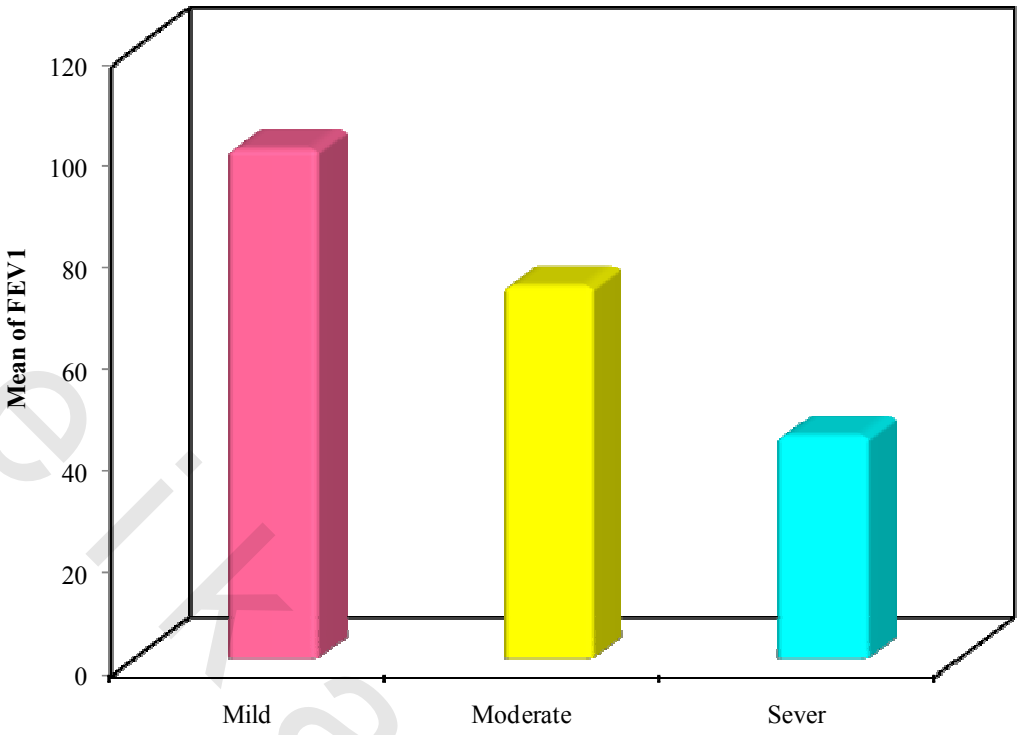


Figure (X): Comparison between the three asthmatic groups according to FEV1

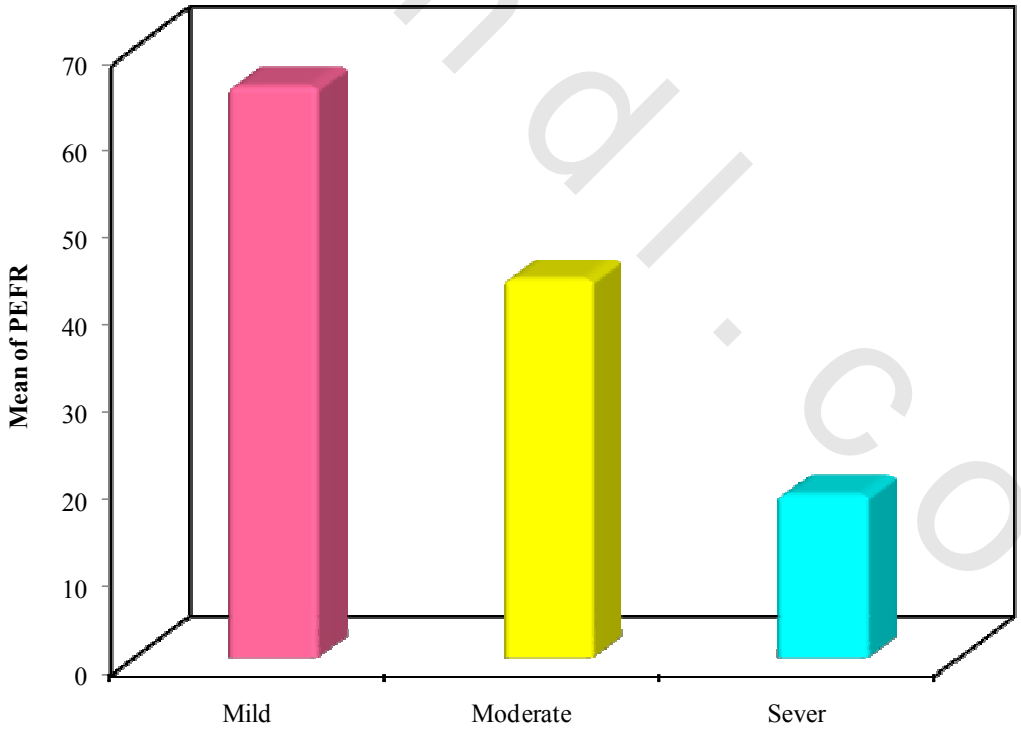


Figure (XI): Comparison between the three asthmatic groups according to PEFR

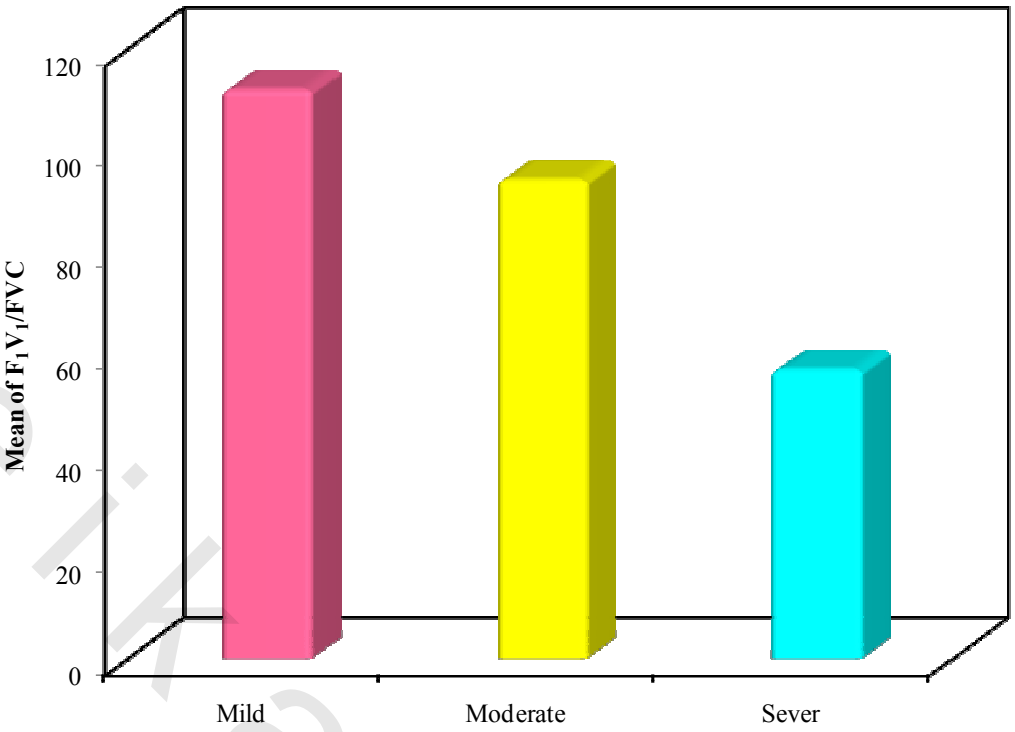


Figure (XII): Comparison between the three asthmatic groups according to F1V1/FVC

Table (6) shows estimation of serum levels fibulin-1 in both asthmatic patients and control group. The mean level of Serum fibulin-1 in asthmatic patients was 244.10 ± 98.28 pg/ml (ranging from 170.50-577.0 pg/ml) in mild group, 217.97±121.16 pg/ml (ranging from 45.50-454.50 pg/ml) in moderate group, 172.20±53.85 pg/ml (ranging from 101.50-265 pg/ml) in severe group compared to a mean level of 187.23±67.97 pg/ml (ranging from 49.50-426.50 pg/ml) in control group.

According to our results, we found that fibulin-1 increased in serum of asthmatic patients than in controls with statistical significant difference ($p<0.05$) but without relation to asthma severity. Fibulin-1 (FBLN-1), a secreted glycoprotein, presents normally in serum and assists in stabilizing the ECM, but in cases of bronchial asthma, its level increased.

Table (6): Comparison between the four studied groups according to serum fibulin-1 in pg/ml

	Mild (n=15)	Moderate (n=15)	Sever (n=15)	Control (n=30)	$^{KW}\chi^2$	p
Serum fibulin-1 in pg/ml						
Min. – Max.	170.50 – 577.0	45.50 – 454.50	101.50 – 265.0	49.50 – 426.50		
Mean ± SD.	244.10 ± 98.28	217.97±121.16	172.20 ± 53.84	187.23 ± 67.97	9.498*	0.023*
Median	237.0	202.50	151.50	178.0		
p_1	0.002*	0.492	0.433			
p_2	0.010*	0.418				
p_3	0.383					

p: p value for comparing between the four studied groups
p1 : p value for Mann Whitney test for comparing between control with each other group
p2 : p value for Mann Whitney test for comparing between severe with each other group
p3: p value for Mann Whitney test for comparing between mild and moderate
KW 2: Chi square for Kruskal Wallis test
*: Statistically significant at $p \leq 0.05$

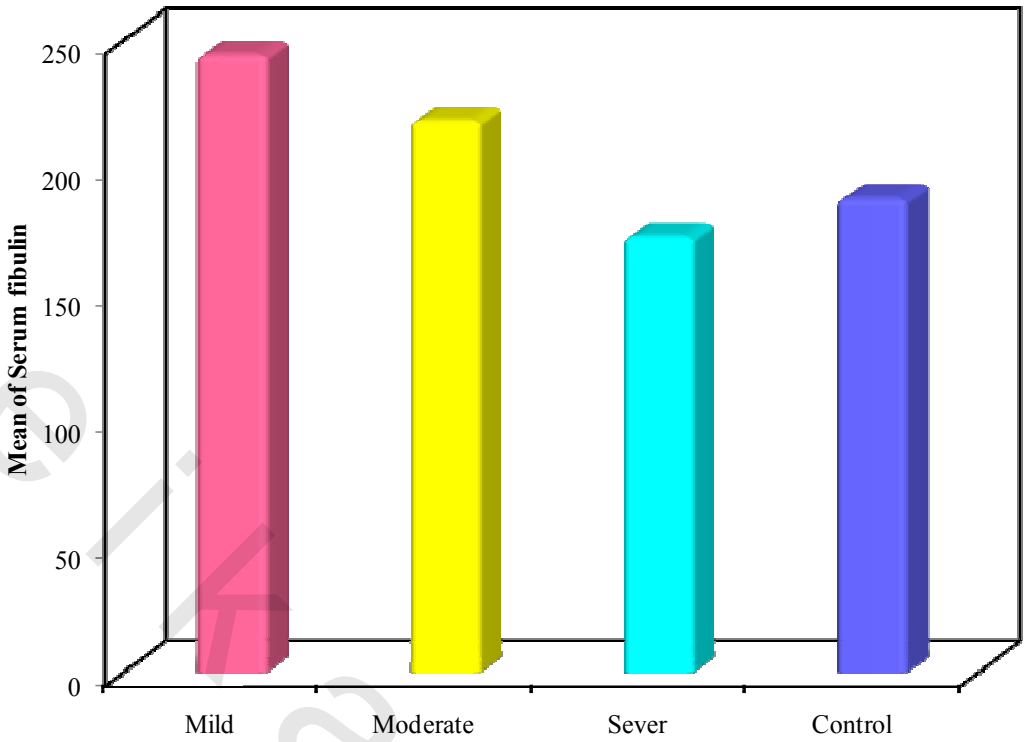


Figure (XIII): Comparison between the four studied groups according to serum fibulin-1 in pg/ml

Table (7) shows estimation of BAL fibulin-1 in the three asthmatic groups. The mean level of BAL fibulin-1 was 507.0 ± 152.27 pg/ml (ranging from 290.50 -750.0 pg/ml) in mild group, 692.81 ± 207.14 pg/ml (ranging from 260.50 – 960.0 pg/ml) in moderate group, 702.0 – 127.67 pg/ml (ranging from 550.0 -1050.0 pg/ml) in sever group.

It was found that fibulin-1 increased in BAL of severe degree bronchial asthma than in mild and moderate degrees with statistical significant difference ($p<0.05$).

Table (7): Comparison between the three asthmatic groups according to BAL fibulin-1 in pg/ml.

	Mild (n=15)	**Moderate (n=15)**	**Sever (n=15)**	**F**	**p**
BAL fibulin-1 in pg/ml.					
Min. – Max.	290.50 – 750.0	260.50 – 960.0	550.0 – 1050.0		
Mean ± SD.	507.0 ± 152.27	692.81 ± 207.14	702.0 – 127.67	6.612*	0.003*
Median	500.0	750.0	665.0		
p_1		0.004*	0.002*		
p_2		0.880			

F: F test (ANOVA)
p_1: p value for Post Hoc test (LSD) for comparing between mild with each other group
p_2: p value for Post Hoc test (LSD) for comparing between moderate and severe
*: Statistically significant at $p \leq 0.05$

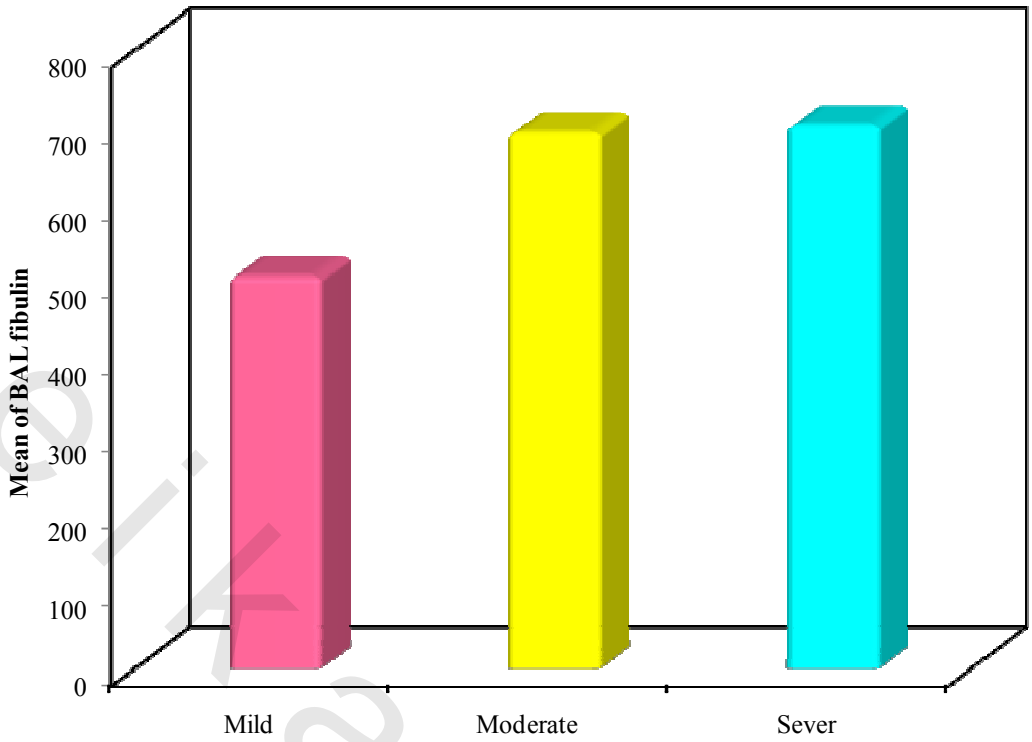


Figure (XIV): Comparison between the three asthmatic groups according to BAL fibulin-1.