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MONITORING THE ACTIVATION OF IMMUNE RESPONSE IN FAMILIAL MEDITERRANEANFEVER AMONG EGYPTIAN PATIENTS

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ABSTRACT

Background: Familial Mediterranean fever (FMF) is a systemic autoinflammatory disorder characterized by seemingly unprovoked recurrent episodes of fever and serosal, synovial, or cutaneous inflammation **(Djouher Ait-Idir et al., 2017).** It is mainly diagnosed clinically and the most widely accepted criteria for diagnosis of typical cases is the Tel Hashomer criteria **(Ryan et al., 2010). Methods:** Tests used to diagnose 40 patients with FMF disease clinically include physical exam, review of your family medical history; serological tests such as total Leukocytes Count, fibrinogen, erythrocyte sedimentation rate serum Amyloid A protein, C-reactive protein and Cytokines (IL-10, 1L-1B), and molecular analysis of mutations with strip test technique. **Results:** This study revealed that the most common mutations were M694I (26.4%), E148Q (20.8%), V726A (17.0%), and M680I (G/A) (17.0%). The CRP levels (mg/dl), Fibrinogen (mg/L), Amyloid A protein (mg/L) as well as ESR (mm/hrs.) increased comparing to the baseline level by the values 633.64%, 303.82%, 1258.78%, and 500.2% (p<0.001) postoperatively. Evaluation of IL-1b represented by 100.59% (p< 0.0001) was recorded, while IL-10 not changed compared with control group. **Conclusion:** There was a positive correlation between IL-10 and IL-1b in M694I mutation and positive correlation between CRP and Fibrinogen in E184Q mutation.

KEYWORDS: Familial Mediterranean fever (FMF), CRP, Fibrinogen.

INTRODUCTION

Familial Mediterranean fever (FMF) is a systemic autoinflammatory disorder and its prevalent among multiple populations from the eastern Mediterranean basin, particularly Jews, Armenians, Turks, and Arabs. FMF caused by recessively inherited mutations in MEFV, which encodes pyrin, and most of the mutations are present in the C-terminal end of the protein encoding B30.2 domain **(Belkhir et al, 2007).** Although FMF mainly affects people living in the Mediterranean. The highest prevalence was in Turks, the prevalence is 1 in 400–1 in 1000. Israel ranks second in terms of prevalence, with more than one per 1000 non-Ashkenazi (Sephardic) Jews; Ashkenazi Jews have a lower prevalence. Armenia comes in second with 1 per 500 people. Other Middle Eastern countries, such as Jordan, Lebanon, and Syria, have many cases of FMF, but the exact prevalence is unknown **(Ben-Chetrit and Touitou, 2009).**

FMF is characterized by short recurrent bouts of fever and localized inflammation usually involving the peritoneum, pleura, joints, or skin. FMF inflammation is mediated by a massive influx of polymorphonuclear leucocytes into the affected tissues, neutrophilia, and a rapid acute-phase response. In some patients, progressive systemic AA amyloidosis can lead to kidney failure and death. FMF is caused by recessively inherited mutations in MEFV, which encodes pyrin, and most of the mutations are present in the C-terminal end of the protein encoding B30.2 domain. Pyrin is expressed in granulocytes, monocytes, dendritic cells, and synovial fibroblasts. Pyrin regulates caspase-1 activation and consequently interleukin-1β production through the interactions of its N-terminal PYRIN domain and C-terminal B30.2 domain with an adaptorprotein, apoptosis-associated speck like protein with a caspase-recruitment 2 domain (ASC) and caspase-1 respectively.

Whether the normal function of pyrin is to inhibit or activate IL-1β secretion, the inflammatory symptoms of FMF are thought to be triggered by IL-1β which may be abnormally induced by mutations in the C-terminal B30.2 domain of pyrin. Thus, the blockade of IL-1β may prove to be a fascinating adjunctive therapy for FMF. Indeed, in several case reports, colchicine resistant FMF patients have shown immediate and sustained resolution of symptoms when treated with the IL-1 receptor

antagonist, anakinra. Whether the normal function of pyrin is to inhibit or activate IL-1β secretion, the inflammatory symptoms of FMF are thought to be triggered by IL-1β which may be abnormally induced by mutations in the C-terminal B30.2 domain of pyrin. Thus, the blockade of IL-1β may prove to be a fascinating adjunctive therapy for FMF. Indeed, in several case reports, colchicine resistant FMF patients have shown immediate and sustained resolution of symptoms when treated with theIL-1 receptor antagonist, anakinra. Thus, IL-1 Trap may also be expected to ameliorate the symptoms in FMF. IFN-α may be another adjunctive therapy forFMF since early administration of IFN-α injections at the onset of attack has shown reduction of attack length and/or severity in some cases of FMF. The aim of this research is to find a correlation between mutational type and serological markers.

SUBJECTS AND METHODS

Clinical data and diagnosis of familial Mediterranean patients

This study was carried out on patients admitted to the Ain shamsUniversity, Faculty of Medicine, Genetics Unit from the period of Dec. 2016 to 2018. Forty patients of hospitalized patients aging from 3 to 25 years, and the gender (21 males and 19 females) were suspected as Familial Mediterranean Fever (FMF). In addition to 32 normal subjects were collected from persons never had FMF before (14 males and 18 females) matched age with patients from 5 to 32 years. The study complies with the research ethics committee in the pediatric hospital, Faculty of Medicine, Ain Shams University.

A) Patients

They were subjected to clinical examination such as chest, cardiac and abdominal examination, musculoskeletal signs suggestive of disease activity, skin rash. As well as clinical history such as age of onset, duration of disease and frequency of attacks, history of being bed ridden and/or school absence, assessment the clinical pattern of FMF disease, detailed medication history of colchicine including start date, dose, perceived side effects and response to treatment and also, family history of similar conditions. Patients were initial diagnosed as FMF according to the classification criteria and divided into three groups. Sure FMF; certain clinical diagnosis in the presence of two major criteria or one major and two minor criteria; probable FMF; clinical diagnosis considered probable in the presence of one major and one minor criterion or two minor criteria; and non-FMF; clinical diagnosis considered unlikely in the presence of only one minor and no major criteria. Putting in our consideration that, response to colchicine was defined as complete, incomplete, or absent. Severity of the disease was determined according to the score: 3-5 Mild; 6-8 moderate; >9 severe disease.

B) Specimen collection

Six ml of blood samples were collected from familial Mediterranean patients and normal subjects visiting Medical and clinical Genetics Unit, Ain Shams University Hospital located at Cairo, Egypt. Four ml were collected on EDTA as anticoagulated factor, mixed well and left for three minutes at room temperature then divided into two parts. First part used to measure ESR and total leukocytes count, second part were centrifuged for 5 minutes at 3000Xg at room temperature to collect a plasma for fibrinogen measurements. Other two ml were collected without anticoagulant and centrifuged for 5 minutes at 5000Xg to collect serum for remaining serological tests as Amyloid A protein,C-reactive protein using spectrophotometric technique, IL-10, IL1b using ELISA technique as well as genotyping tests using the FMF Strip Assay. Serum and plasma samples were barcoded and labelled with the patient information and stored at -20 until the date of testing. Leucocyte counting and ESR on whole blood samples were ran on the same day.

C) Serological diagnosis

Blood samples were collected from patients and controls to determine levels of IL-10 and IL-1B with ELISA technique, Hematological assessment for total leucocytes and ESR. Fibrinogen and Amyloid A protein levels were determined by spectrophotometer. Finally, mutational assay was done by Test strip technique through PCR amplification and reverses hybridization. Double marker requisition forms were filled with the patient information, which include all of the following clinical information: date of birth; diagnosis; specimen type and molecular study test.

D) Molecular analysis

Buffy coat samples were prepared and brought to room temperature, and then the sample was mixed well carefully by inverting. DNA extracted by GENxTRACT resin (Vienna, Austria). Genomic DNAs were quantified by using UV-Vis spectrophotometer (Quawell Q5000, Quawell Technology, UnitedStates) and all genomic DNA samples were measured at 260/280nm to determine the purity and concentrations of purified DNA. PCR was carried out in 20μl scale according to the manufacture products (Vienna Lab Diagnostics GmbH Gaudenzdorfer Guertel 43-45. A-1120 Vienna, Austria) with thermal cycle (Berkin Elmar 3800 USA). Initial denaturation at 94°C for 2 minutes, followed by 35 cycles (denaturation at 94°C for 15 sec, annealing at 58°C for 30 sec, extension at 72°C for 30 sec.), and final extension at 72°C for 3 minutes. The samples were hold at 4°C. Amplification products were stored on ice or at2-8°C for further use. Hybridization reaction was done according to the manufacture products that was carried out by FMF StripAssay® method Vienna Lab Diagnostics GmbH Gaudenzdorfer Guertel 43-45. A-1120 Vienna, Austria.

Statistical analysis

Data were analyzed using SPSS software (SPSS Inc., Chicago, IL, USA). Results were expressed as the mean ± standard deviation (SD) for continuous variables. For quantitative data, the Mann–Whitney U rank-sum test

compared two independent groups. Comparisons for categorical variables were evaluated using the chi-square test.

RESULTS

1- Demographic results

The present prospective study was conducted on 40 patients suffering from familial Mediterranean fever (21 males and 19 females) and the median age of the studied patients at the time of analysis was 13 years ranging from 3-25 years (<6 years 13 patients, 6-12 years 17 patients and > 12 years 10 patients). They were admitted to the Genetic Research Unit of Ain Shams university Hospitals (GRU-ASUH). The main reasons for exclusion were the absence of periodic fever syndrome (drug fever,

infections, and neoplastic diseases). In addition to 32 healthy subjects as a control group (20 males and 12 females) with median age of 12 years ranging from 5-32 years (<6 years 8, 6-12 years 18 and > 12 years 6 subjects) as represented in Table (1). The majority of cases were between 6-12 years (42.5%), and (56.3) for FMF patients and control respectively. Moreover, among 32 control group 18 subjects are rural (56.25%) and 14 are Urban (43.75%), while FMF patients represented as 31 rural (77.5%) and 9 patients are Urban (22.5%). Moreover, no significant difference was detected for gender, age and residency distribution between FMF patients and control groups. This data reflects homogeneity between both groups.

Table 1: Demographic distribution of studied patients.

Parameters		Control group (N=32)	FMF patients (N=40)	χ^2 P value	Significance
Gender N, (%)	Male	20 (62.5%)	21 (52.5%)	χ^2 0.725 P=0.394	NS
	Female	12 (37.5%)	19 (47.5%)		
Age N, (%)	<6 years	8 (25.0%)	13 (32.5%)	χ^2 1.347 P=0.510	NS
	6-12 years	18 (56.3%)	17 (42.5%)		
	>12 years	6 (18.8%)	10 (25.0%)		
Residency	Rural	18 (56.25%)	31 (77.5%)	χ^2 3.693 P=0.063	NS
	urban	14 (43.75%)	9 (22.5%)		

2- Clinical characterization of FMF patients

Among 40 selected FMF patients clino-pathological characterization represented that the mean duration of the disease is 9.45 ± 3.76 years and the mean age of the onset of symptoms was of the disease was 6.15 ± 2.62 years (30 patient <12 years and 10 patients > 12 years). The frequency of febrile attack /month among FMF patients was >4 times/month (23 patients; 57.5%), 4-10 times /month (13; patients; 32.5%) and >10 times/month (4 patients; 10.0%). Duration of each fever attack/hour ranging from <12 to >24 hours through which 8 patients (20%), 9 (22.5%), 20 patients (50%) suffering from

attack for <12hr, 12-24hr and >12 hr respectively and three patients (7.5%) can't able to determine duration of attack. Family history among FMF patients represented that those 17 patients without family history of the disease among parents or grandparents (42.5%), while 23 patients with confirmed that one at least of nearest relatives have FMF history (57.5%). Moreover, treatment with a Colchicine drug has positive response to 10 patients (25%) while represented negative response to one patient (2.5%) and 29 (72.5%) patients not treated with such drug as represented in Table 2.

Table 2: Clinical characterization of FMF patients.

Parameters	Classes	FMF patients
Age at onset (years), N, (%)	<12	30 (75.0%)
	≥12	10 (25.0%)
Frequencies of febrile attack (per month) N, (%)	<4	23 (57.5%)
	4-10	13 (32.5%)
	>10	4 (10.0%)
Duration of fever attack (hours), N, (%)	<12	8 (20%)
	12-24	9 (22.5%)
	>24	20 (50%)
	Missed	3 (7.5%)
Family history of periodic fever N, (%)	Yes	23 (57.5%)
	No	17 (42.5%)
Response to colchicine treatment N, (%)	+Ve	10 (25.0%)
	-Ve	1 (2.5%)
	ND	29 (72.5%)

3- FMF phenotype Description

The main clinical characteristics of the 40 FMF patients concerning major mono symptom were as follows: the most common symptom was fever observed in 38 (95.0%). The other frequent symptoms were abdominal pain (33, 82.5%), arthritis/arthralgia (30, 75%) and Weakness / Fatigue (16, 40%). Moreover, skin eruption was seen in 8 patients (20.0%), chest pain in 2 patients (5.0%). Other minor symptoms as vomiting, diarrhea and erythema were identified in number of patients representing 5 (12.5%), 3 (7.5%) and 2 (5.0%) respectively as in Table (3).

Table 3: Mono and combined phenotyping among FMF study population.

Phenotypes/Symptoms	N	%
Fever	38	95.0
Abdominal pain	33	82.5
arthritis/Arthralgia	30	75.0
Weakness / Fatigue	16	40.0%
Skin eruption	8	20.0%
Chest pain	2	5.0%
Vomiting	5	12.5%
Diarrhea	3	7.5%
Erythema	2	5.0%
Combined	40	100.0

4- Classification of FMF patients depending on clinical data

There are at least three types of criteria for clinical diagnosis of FMF. In this thesis, we are concern about symptoms of patients based on clinical investigations of Tel-Hashomer criteria. The FMF group (n=40) were sub classified into 33 had typical FMF (82.5%) and 5 had incomplete FMF (12.5%). The remaining 2 patients (5.0%) were classified as non-FMF. Our results showed also that according to Tel-Hashomer criteria FMF patients were represented in sure (definitive) FMF group for 38 patients (95.0%), followed probable FMF patients 2 (5.0%). Moreover, according to Eurofever/PRINTO clinical + genetic criteria all FMF patients were diagnosed as a confirmed disease Table (4).

Table 4: Distribution of FMF phenotype among FMF group.

FMF group	Tel-Hashomer criteria			Eurofever/PRINTO clinical + genetic criteria
	typical FMF33 (82.5 %)	incomplete FMF5 (12.5 %)	non-FMF2 (5.0 %)	40 (100%)
N, (%)	Sure FMF 38 (95.0 %)	Probable FMF2 (5.0 %)		

5- Hematological and laboratory characterization of FMF group

Clinical and laboratory features categorize into subgroups as to evaluatethe distribution of patients among the FMF criteria as shown in Table 5. ESR: The majority of FMF patients (29), represented within the range 6-20mm/hr. (72.5%), While in control groups all subjects were less than 6mm/hr. Majority of patients (35 patients; 87.5%) with total leucocyte count TLC 11≤50 in contrast to control group where (93.75%), subjects were normal count. C- reactive protein in FMF patients were +ve (>6 up to 96 mg/l) in 31 patients (77.5%) On the other hand, only one was positive among control group (3.1%). Fibrinogen level (mg/dl) with FMF were high level (100≤400) in the majority of patients (31, 77.5%) while most of control subjects were located in normal range. Serum amyloid A (sAA) protein was a normal level in all control subjects that showed a significant different compared to. 26 FMF patients (65.0%) with high level (30≤200 mg/L) and by 14 patients (35.0%) with protein level more than 200mg/L. Among 40 FMF patients there are 28 patients with only single mutation (70.0%), followed by 11 patients with double mutation (27.5%) and 1patient with triple mutation (2.5%). Mutations were classified into two groups; heterozygous which is the majority (29 patients; 72.5%) and homozygous mutation (11 patients 27.5%).

Table 5: Clinical characteristics of the studied subjects.

Variable(N, %)	FMF group N=40	Control group N=32	χ^2	P value	Significance
ESR (mm/hr)					
≤6	3 (7.5%)	32 (100%)	60.98	0.0001	
6-20	29 (72.5%)				HS
≥20	8 (20.0%)				
TLC (x10^9 cells/L)					

≤ 11	0 (0.0%)	30 (93.75%)	64.34	0.0001	
11-≤50	35 (87.5%)	2 (6.25%)			HS
>50	5 (12.5%)	0 (0.0%)			
CRP (mg/L)					
Positive	31 (77.5%)	1 (3.1%)	39.	0.0001	HS
Negative	9 (22.5%)	31 (96.9%)	83		
Fibrinogen (mg/dl)					
<100	1 (2.5%)	28 (87.5%)	53.74	0.0001	
100≤400	31 (77.5%)	4 (12.5%)			HS
>400	8 (20.0%)	0 (0.0%)			
Amyloid A (mg/l)					
<30	0 (0.0%)	32 (100%)	72.2	0.0001	
30≤200	26 (65.0%)	0 (0.0%)			HS
>200	14 (35.0%)	0 (0.0%)			
Mutations type n= (%)					
Simple	28 (70.0%)				
Doubled	11 (27.5%)				
tripled	1 (2.5%)				
Mutation homogeneity					
(%)	11 (27.5%)				
Homozygous	29 (72.5%)				
Heterozygous					
Mutation complexity					
Compound heterozygous	12 (30.0%)				
Compound homozygous	0 (0.0%)				

6- Distribution of genotyping in FMF group

In the present study, 40 FMF patients compressed 53 mutations. There were 28 (52.8%) as a single homo- or heterozygous mutations was recorded that distributed mainly in exon 10 (41; 77.4%) followed by exon 2 (11; 20.7%) and less distributed in exon 3 (1.9%). However, no mutations recorded in exon 5. Mutation M694I was the most frequent mutation, which recorded 14 times out of 53 (27.5%), followed by E148Q that was moderate frequent in 11 mutations (20.8%), and both mutations M680I (G/A) and V726A were detected in 9 times for each (17.0%). Other mutations [M680I (G/C), M694V and A744S] were less frequent distribution which repeated 3 times each (5.7%) and finally P369S found only once (1.9%). Among 11 double mutations recorded in 40 FMF patients (27.5%), the most frequent double mutation recorded was [M680I (G/A) & M694I] that recorded in three patients (7.5%), followed by [M680I (G/A) & E148Q] and [M680I (G/A) & V726A] mutations, which located in 2 patients each (5.0%). All other remaining double and triple mutations were recorded only once (2.5%). Distribution of mutations represented in Tables 6.

Table 6: Different types of mono mutation recorded by strip technique.

Lane	Exon	Type	Mutation No. (%)
1	2	E148Q	11 (20.8%)
2	3	P369S	1 (1.9%)
3	5	F479L	0 (0.0%)
4	10	M680I (G/C)	3 (5.7%)
5	10	M680I (G/A)	9 (17.0%)
6	10	I692del	0 (0.0%)
7	10	M694V	3 (5.7%)
8	10	M694I	14 (26.4%)
9	10	K695R	0 (0.0%)
10	10	V726A	9 (17.0%)
11	10	A744S	3 (5.7%)
12	10	R761H	0 (0.9%)
---	---	Total	53 (100.0%)

7- Phenotypes and mutations

Table 7 and Figure 12 showed that the combined and arthralgia phenotyping were significantly high (82% and 55% respectively) in E148Q mutation in comparison to the other mutations. Meanwhile, P369S mutation characterized mainly with abdominal pain, which is

specific to this type of mutation together with other symptoms as arthralgia and combined.

Table 7: Clinical phenotypes among the common FMF mutations.

Phenotypes(N=43)	M694I(N=14)	E148Q (N=11)	V762A (N=9)	M680I (G/A) (N=9)	X^2 P value	Sig.
Fever	7 (50%)	5 (45%)	3 (33%)	6 (67%)	8.22 0.77	NS
Abdominal pain	3 (21%)	4 (9%)	8 (89%)	5 (56%)		
Chest pain	1 (7%)	0 (0%)	0 (0%)	1 (11%)		
Arthralgia	9 (64%)	6 (55%)	7 (78%)	5 (56%)		
Combined*	14 (100%)	9 (82%)	7 (78%)	8 (89%)		

8- Serological parameters
A highly significant differences were noted between the FMF patients and non-FMF groups regarding the CRP levels (mg/dl), Fibrinogen (mg/L), Amyloid A protein (mg/L) as well as ESR (mm/hrs.) measurements at baseline (p<0.001*) by the values 633.64%, 303.82%, 1258.78%, and 500.2% postoperatively. While no significant difference was noted between the two groups regarding the total leukocytes count (cells/L) measurements (p=0.093) by 7.9% (Table 8).

Table 8: Serological factors of control and FMF cases.

Groups	CRP (mg/L)	Fibrinogen (mg/L)	Amyloid A(mg/dL)	TLC cells/ml	ESR mm/hrs.
Control group (n=32) Min. – Max. Mean ± SD. Median	2.0– 7.0 4.4 ± 1.32 4.5	49.2 – 142.0 94.2± 24.43 94.0	10.0 – 48.0 24.5 ± 10.19 24.0	3.0 – 14.0 8.14 ± 2.5 8.16	2.0 – 8.0 4.0 ± 1.49 4.0
FMF group (n=40) Min. – Max. Mean ± SD. Median	12.0 – 96.0 33.6 ± 24.63 24.0	420.0 – 595.0 380.4 ± 42.37 486.5	126.0 – 562.0 332.9 ± 107.25 326.5	4.0 – 18.3 7.5 ± 2.84 6.9	15.0 –30.0 24.1 ± 3.8 24.0
T	9.089*	12.192*	23.932*	1.719	20.117*
p-value	<0.001*(HS)	<0.001*(HS)	<0.001*(HS)	0.093 (NS)	<0.001*(HS)
% change	633.64	303.82	1258.78	7.9	500.2

9- Cytokine's characterization of FMF group
Analyze and evaluate the levels of IL-10 and IL-1b for FMF patients in relation to type of mutation status in patients and for healthy controls as well as their implication as diagnostic tools in the disease. The results revealed that serum (IL-10) levels for FMF group showed no significant change compared with control group (17.21%), while serum (IL-1b) levels for FMF group showed highly significant increase (p<0.001) compared with control group (100.6%) in Table 9.

Table 9: Control and FMF cytokines level.

Group	IL-10 (pg/L)	IL-1b (pg/L)
Control group (n=32) Min. – Max. Mean ± SD. Median	37.8– 462.24 169.6 ± 122.18 130.60	429.0 – 7654.0 1345.6± 134.75 984.0
FMF group (n=40) Min. – Max. Mean ± SD. Median	104.7– 187.3 144.7 ± 24.06 141.91	1442.5 – 3873.5 2699.1± 584.44 2837.1
T test	1.089	7.192*
p-value	0.694 (NS)	<0.001*(HS)
% Change	17.21	100.59

t: Student t-test HS: high significant at p <0.001 *: Statistically significant at p ≤ 0.05

10- Level of serological biomarkers according to the mutational type
Study the change in serological markers between two most repeated mutations [M694I] and [E148Q] compared to control group. It is clear that TLC, CRP and Fibrinogen were not significantly changed between two groups of mutations (P>0.05). On the other hand, Amyloid A protein was moderate significantly increase (P<0.01) in E184Q mutation compared with M694I mutation with 27.7% while a high significant increase in

ESR in M694Imutation (P<0.001) compared with E184Q mutation with 172.8% (Table 10).

Table 10: Serological parameters in most common single mutation.

	CRP (mg/L)	Fibrinogen (mg/L)	Amyloid A (mg/dL)	TLC cells/ml	ESR mm/hrs.
M694I (n=14)					
Min. – Max.	12.0 – 96.0	420.0 – 595.0	126.0 – 540.0	5.3 – 18.3	19.0 – 30.0
Mean ± SD.	36.0 ± 28.63	482.4 ±49.58	317.1 ± 123.47	8.0 ± 3.46	25.4 ± 3.52
Median	24.0	475.0	323.0	6.98	25.5
E148Q (n=11)					
Min. – Max.	12.0 – 96.0	425.00 – 595.0	223.0 – 562.0	4.71 – 12.56	3.5 – 15.0
Mean ± SD.	30.0 ± 29.39	500.75 ± 45.27	405.0 ± 131.76	7.0 ± 2.53	9.31 ± 465
Median	18.0	505.5	407.5	6.0	10.0
t	1.089	7.192	10.932^{*}	16.719	20.117^{*}
p-value	**0.42 (NS)**	**0.25 (NS)**	**<0.01*(S)**	**0.25 (NS)**	**<0.001*(HS)**
% Change	**20**	**3.8**	**27.7**	**12.5**	**172.8**

The results revealed that serum (IL-10) and (IL-1b) levels for most frequent mutations among FMF group showed no significant change of any single mutations or compound mutation by one way ANOVA (Kruskal-Wallistest), which represented by 4.823 and 0.631 and p-values were 0.085 and 0.753 respectively as shown in Table 11.

Table 11: Comparison between cytokines level in common mutations.

Group	IL-10 (pg/L)	IL-1b (pg/L)
M694I (n=14)		
Min. – Max.	105.8– 181.6	1442.5 – 3102.9
Mean ± SD.	145.1 ± 25.38	2451.8± 580.95
Median	142.60	2813.9
E148Q (n=11)		
Min. – Max.	125.1– 140.27	2904.8 – 2017.1
Mean ± SD.	134.3 ± 8.07	2367.5.1± 472.44
Median	137.53	2180.6
M680I(G/A) & M694I (n=3)		
Min. – Max.	104.7– 187.3	1442.5 – 3873.5
Mean ± SD.	144.7 ± 24.06	2699.1± 584.44
Median	141.91	2837.1
Kruskal-Wallis test	4.823	0.631
p-value (Sig.)	**0.085 (NS)**	**0.753 (NS)**

12- Specificity and sensitivity of cytokines in M964I mutation

Sensitivity and specificity were determined of all studied parameters in M964I mutation as shown in Figure 1 and Table 12. It is clear that IL-1b is more sensitive for this kind of mutation, which represented AUC by 0.948.
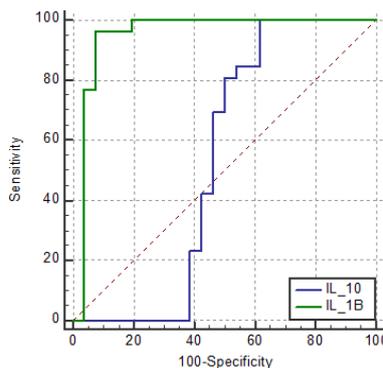

Figure 1: The Receiver Operating Characteristic (ROC) curve for studied parameters in M964I.

Table 12: Cytokines specificity and sensitivity in M964I mutations.

Baseline	AUC	95% CI	p value	sig.	Cut-off	Sensitivity	Specificity	PPV	NPV
IL-10 pg/dl	0.533	0.389 to 0.672	0.0002	HS	>1.8 *	100.00	87.50	45.5	78.9
IL-1b pg/dl	0.948	0.849 to 0.990	0.0032	HS	>1.95 *	100.00	38.46	60.5	85,8

AUC: Area Under a Curve
p value: Probability value
CI: Confidence Intervals
NPV: Negative predictive value
PPV: Positive predictive value
*: Statistically significant at p <0.05
#cut off choose according to Youden index

On the other side, M964I mutation showed that CRP, Fibrinogen and Amyloid A protein are more sensitive for this kind of mutations by measured with AUC 1.000 that represented in Table 13 and Figure 2.
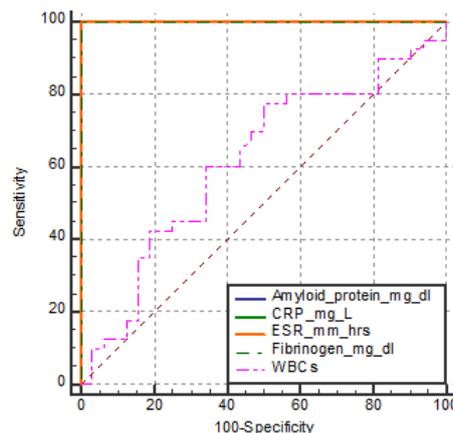
Figure 2: ROC curve for studied biomarkers to diagnose patients (n = 14) for M964I mutation.

Table 13: Sensitivity, specificity to diagnose patients (n = 14) for M964I mutation.

Baseline	AUC	95% CI	p value	sig.	Cut-off	Sensitivity	Specificity	PPV	NPV
ESR mm/hr	1.000	0.950 to 1.000	P < 0001	HS	>1.95 *	66.67	80.95	60	85
CRP mg/L	1.000	0.950 to 1.000	P < 0001	HS	>212 *	98.2	91.12	65.2	47.8
Fibrinogen mg/dl	1.000	0.950 to 1.000	P < 0001	HS	>310 *	88.89	97.14	47.1	92.3
sAA mg/dl	1.000	0.950 to 1.000	P < 0001	HS	>310 *	96.67	80.95	60	85
TLC cellex109?L	0.616	0.494 to 0.728	1.0000	NS	>1.8	55.56	71.43	45.5	78.9

AUC: Area Under a Curvep
value: Probability value
CI: Confidence Intervals
NPV: Negative predictive value
PPV: Positive predictive value
*Statistically significant atp < 0.05
#Cut off was choosed according to Youden index

DISCUSSION

Familial Mediterranean Fever (FMF) is one of the most common autoinflammatory diseases(AIDs) in the world , which is an autosomal recessive disorder characterized by recurrent acute attacks of fever accompanied by abdominal pain, arthritis, and pleurisy (**Djouher Ait-Idir et al., 2017).** Usually, FMF is restricted to Turks, Armenians, Arabs, and non- Ashkenazi Jews. In the rest of the world, it is a very rare disease (**Bar-Eli et al., 1981; Isabelle et al., 2001**). FMF is linked to a wide range of mutations in theMEFV gene (**Ben-Chetrit and Touitou 2009**). The MEFV gene's cytogeneticlocation is 16p13.3 (Genetic Home, Reference, National Institute of Health, National Library of Medicine of USA, 2019). The MEFV gene encodes a protein called pyrine, with a weight of 95 kDa. The pyrine protein is essentially responsible for the regulation of apoptosis, inflammation, and cytokines, and is mainly expressed in neutrophils, eosinophils, dendritic cells, and fibroblasts (**Shinar et al., 2012**). Pyrin is an important effector in the innate immune system and a component of the inflammasome, which leads to an overactive inflammatory response via uncontrolled interleukin-1 production (**Mansour Alghamdi., 2017**). MEFV gene mutations result in a malformed pyrin protein that cannot function properly.

As a result, pyrin is unable to play its presumed role in inflammation control, resulting in an inappropriate inflammatory response (Genetic Home Reference, National Library of Medicine, National Institute of Health of USA, 2019). MEFV gene is made up of 10 exons. Most of the mutations identified occur in exon 10. By this means, the twelve common mutations: E148Q in exon 2, P369S in exon 3, F479L in exon 5 and M680I (G/C), M680I (G/A), I692del, M694V, M694I, K695R, V726A, A744S, and R761H in exon 10 were determined. Our present study is a clinical and biochemical Cross-sectional study that included forty patients who admitted to the Genetic Research Unit of Ain Shams university Hospitals (GRU-ASUH), in the period from December 2016 to 2018. They were randomly recruited from those who were diagnosed primarily on clinical basis to have FMF then referred to be genetically tested for the most common 12 mutations in the MEFV gene.

Our study population showed that there is no sex difference among our patients; 52.5% males and 47.5% females with a male to female ratio of 1:1.1. This finding coincides with the results of **Salah et al., (2016)** who reported a male to female ratio about 1:1.03. Meanwhile, **El Haggar et al., (2014)** studied population in Delta region in Egypt and showed male to female predominance of 2:1. While **Kilic et al., (2015)** studied population in Turkey showed slight female predominance of 0.96:1. Regarding the age distribution among FMF group, the mean age of population was 18.53 ± 5.7 years through which majority of patients located between 6 and 12 years (17; 42.5%) with mean age 8.2 ± 3.9 years, followed by less than 6 years (13; 32.5%) with mean age 4.3± 1.9 years. In the meantime, the mean age of onset was 6.15 ± 2.62 years (range from 1 year to 12 years of age). Our current study showed that there was a positive family History in (23; 57.5%) in the entire study group and ranging from 11% to 20% among different mutations but without significant difference. Like the Jewish, Armenian, and Turkish populations, we found a single predominant mutation in Egyptian patients with FMF. Single mutation was found in 28 patients (70%), double mutation in 11 patients (27.5%) and triple mutation in 1 patient (2.5%). The diversity of mutations among Arabs was reported before by **Brik et al., (1999)** and could be related to the heterogeneous origin of the Egyptian population and the effect of different civilization marks such as Romans, Byzantines, and Ottomans beside the original inhabitants. The Arabs left on this country since ancient times because of its unique location at the crossroads between Africa, Europe, and Asia.

The main clinical characteristics of the 40 FMF patients concerning major mono symptom were Fever, abdominal pain, arthritis and other symptoms but the most common reported symptoms were Fever (95.0%), abdominal pain (82.5%) and Arthralgia (75.0%). Then come in succession; weakness & fatigue (40.0%). Other recorded symptomatologies were skin eruption (20.0%), chest pain (5.0%), Diarrhoea (7.5%), Vomiting (12.5%), and Erysipelas like erythema (5.0%). These results agree with **Mneimneh et al., (2016)** who reported almost similar percentages to our data; Abdominal pain (84.7%), Fever (78.2%), Arthralgia (43%), Chest pain (30.5%), Vomiting (15.3%), Diarrhea (6.2%) and Erysipelas like rash (3.3%). In addition, **Kilic et al., (2015)** demonstrated mimic percentages of symptoms similar to our findings like Fever (97.3%), abdominal pain (96.6%), Arthralgia (63.7%), Arthritis (43.2%) and Chest pain (40.7%). On the contrary, Ozturk et al., (2009) reported a different percentage of symptoms than ours; abdominal pain (90.3%), Fever (75%), Joint pains (23.6%), Diarrhea (16.6%), Vomiting (16.4%), and Chest pain (15.3%). Again, this discrepancy could be justified by the different frequencies of gene mutations among ethnic groups enrolled from the different studies. This difference necessitates a larger scale study of Egyptian FMF patients to be representative of the large Egyptian population. Regarding the clinical phenotyping of our study population, patient may present as monosymtomatic phenotype in a minority of cases; fever 4%, abdominal pain 3%, and joint pain 1%. On the other hand, combined symptomatologies were recorded to be 100.0% which, means that FMF present here with two or more symptoms at the time of presentation.

On clinical investigations depending on Tel-Hashomer criteria. The majority of FMF group (33; 82.5%) were classified as typical FMF (82.5 %) and only 5 patients (12.5%) were incomplete FMF. Meanwhile, according to these parameters (38; 95.0%) were sure FMF patients and only 2 patients (5.0%) were probable FMF patients that need another investigation to confirm from the diagnosis in this small scale.

It is demonstrated in our study that there are 28 patients with only single mutation (70%), followed by 11 patients with double mutation (27.5) and 1 patient with triple mutation (2.5%). Mutations were classified into two groups heterozygous which is the majority (29 patients; 72.5%) followed by homozygous mutation (11 patients; 27.5%). In addition, compound heterozygous mutations were recorded in 12 patients (30.0%). These findings agreed with the Egyptian study of **El Haggar et al., (2014)** and the Lebanese study of **Mneimneh et al., (2016)** but on the contrary; the Turkish study of **Kilic et al., (2015)** showed that homozygous genotype (40.5%) is two and half times more common than compound heterozygous (14.6%). This could be explained by the possibility of the higher rates of consanguinity among the Turkish study group over the Egyptian and Lebanese study groups.

Regarding the percentage frequency of different mutations, the study revealed that the most common mutations were M694I (26.4%), E148Q (20.8%), V726A (17.0%), M680I(G/A) (17.0%), M680I(G/C)(5.7%), A744S(5.7%) and M694V (5.7%) in our study

population. Similarly, a study by **El Gezery et al., (2010)** revealed that the most common alleles were M694I (34.0%) followed by E148Q (22.7%), V726A (15.6%), M680I (G/A) (12.1%) and M694V (7.8%). We can assume that the high frequency rate of E148Q mutation in our study is related to what reported by **El Haggar et al., (2014)** who assumed that E148Q mutation has a high carrier rate (>10%) and does not cause an FMF phenotype, even in homozygous cases. Some researchers have claimed that this should not be considered a mutation, but rather a polymorphism. On the contrary, results from **Salah et al., (2016)** revealed that V726A gene mutation was the most frequent mutation (19.7%); followed by M680I(G/A) mutation (11.5%), and M694V mutation was reported in one patient (1.6%). Another study by **Kilic et al., (2015)** who found that mutation E148Q was (20.2%) and M680I(G/A) was (11.3%), which is relatively goes with our study but M694V percentage frequency was (51.6%), which was significantly higher in relation to our study and others.

The symptomatology of the different mutations, the M694I (n=14) mutations, which is the most frequent one in our study population showed that abdominal pain (100.0%) and combined symptomatology (100.0%) are the most common in heterozygous mutations then comes in succession fever (60.0%) and arthralgia (70.0%), the least common was the chest pain (10.0%). Similar results were obtained in the compound mutations (n=4) in which one of them at least was M694I mutation. These findings coincide with the study done by **Mneimneh et al., (2016).**

Looking into the symptomatology of the different mutations, the E148Q (n=11) mutations, which is the second most frequent mutation in our study population showed that abdominal pain (100.0%) and combined symptomatology (100.0%) are the most common in heterozygous mutations then comes in succession fever (71.0%) and arthralgia (57.0%), the least common was the chest pain (14.0%). Similar results were obtained in the compound mutations (n=4) in which one of them at least was E184Q mutation. These findings coincide with the study done by **Mneimneh et al., (2016).**

In regard to of mutations V726A, and M680I(G/A) they present almost similarly by fever and abdominal pain in more than 90% of the study population. These findings were also supported by **Ozturk et al., (2011) & Mneimneh et al., (2016).**

By using the Multiple Logistic Regression analysis for the commonest FMF gene mutations (dependent variable) Vs symptomatology (independent variables), we identified that the most sensitive (statistically significant) independent variables (symptomatology) that predict the dependent variable (mutations) are Vomiting for V726A; Weakness, Fatigue & Myalgia for M680I; Arthralgia & Vomiting for E148Q and Vomiting for M694I.

Also, By using the same test for the different Zygosity (dependent variable) Vs symptomatology (independent variables), it revealed that the most sensitive (statistically significant) independent variables (symptomatology) that predict the dependent variable (zygosity) are; family history & arthralgia for Compound heterozygous; family history & Vomiting for Heterozygous and Arthralgia & Abdominal Pain for Homozygous.

These statistical findings are hardly explained or implemented on clinical basis because of the non-specificity of the independent variables (symptomatology) in relation to dependent variables whether mutations or zygosity.

A highly significant differences were noted between the FMF patients and non-FMF groups regarding the CRP levels (mg/dl), Fibrinogen (mg/L), Amyloid A protein (mg/L) as well as ESR (mm/hrs.) measurements at baseline (p<0.001*) by the values 633.64%, 303.82%, 1258.78%, and 500.2% postoperatively. While no significant difference was noted between the two groups regarding the total leukocytes count (cells/L) measurements (p=0.093) by 7.9%.

Analysis and evaluation of two immunologic markers which are interleukin-10 (IL-10), and interleukin-1b (IL-1b) for FMF patients in relation to type of mutation. The results revealed that serum (IL-10) and (IL-1b) levels for most frequent mutations among FMF group showed no significant change of any single mutations or compound mutation by one way ANOVA (Kruskal- Wallis test), which represented by 4.823 and 0.631 and p-values were 0.085 and 0.753 respectively.

It was shown that there is no obvious correlation as a whole was detected but through studying with particular mutation, the correlation was recorded in both major mutations while there was a positive correlation between IL-10 and IL-1b in M694I and positive correlation between CRP and Fibrinogen in E184Q.

In conclusion, the mutation spectrum in Egyptian patients with FMF is heterogeneous and necessitates a larger scale population screening and sequencing of the whole MEFV gene searching for other disease-causing mutation.

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