



Clinical Implications of S100A12 and Resolvin D1 Serum Levels, and Related Genes in Children with Familial Mediterranean Fever

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Abstract

The aim of this article was to study the role of S100A12 and resolvin D1-related genes and serum levels in the diagnosis and detection of subclinical inflammation in children with familial Mediterranean fever (FMF) during the quiescent stage of the disease. Seventy-eight children with FMF during the silent state and 60 healthy control were studied. Serum S100A12 and resolvin D1 were quantitatively measured using enzyme-linked immunosorbent assay. In addition, the levels of C-reactive protein, erythrocyte sedimentation rate, and hemoglobin were determined. The clinical severity was evaluated. The link between the Mediterranean fever (*MEFV*) gene and the genes related to the two studied biomarkers was also assessed. Correlation between S100A12 and resolvin D1 and the clinical severity was assessed. The mean serum levels of S100A12 and resolvin D1 were 847.4 and 793.3, respectively, which were highly significantly increased ($p=0.001$) compared with the controls (324.3 and 235.1, respectively). The receiver operating characteristic curve test showed that S100A12 had a sensitivity of 97.4% and specificity of 80% with cutoff value of 529.5, while resolvin D1 showed a sensitivity of 100% and specificity of 50% with cutoff value of 231.2. A correlation was detected between the clinical severity and S100A12 and resolvin D1. This study delineated that S100A12 and resolvin D1 are sensitive biomarkers to detect the degree of inflammation in children with FMF during the silent period. Consequently, we recommend adjusting the colchicine dose to ameliorate the disease's symptoms and to improve the quality of life in these patients.

Keywords

- ▶ familial Mediterranean fever
- ▶ inflammation
- ▶ *MEFV* gene
- ▶ S100A12 protein
- ▶ resolvin D1

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Introduction

Familial Mediterranean fever (FMF) is the most common monogenic autoinflammatory disease in the world; its prevalence is very high among people from the eastern Mediterranean such as Jews, Turks, Armenians, and Arabs.¹ Although it is known to be inherited autosomal recessively, a substantial number of heterozygotes are presently expressing the phenotypic characteristics.² The disease is characterized by recurrent fever episodes, which may be accompanied by serositis, arthritis, vasculitis, dermal manifestations, and long-term complications, mainly renal.³ The episodes are self-limiting lasting 12 to 72 hours, and the interval between the episodes is extremely variable from weeks to years.⁴ The most common genetic mutations encoded from exon ten and exon 2 are responsible for more than 85% of FMF cases in the Mediterranean basin.⁵ The Mediterranean fever (*MEFV*) gene encodes for protein pyrin (marennostin), mostly in neutrophils and macrophages and has a crucial role in apoptosis and inflammatory pathways. M694V is the most common mutation in Turk, Armenian, and Jewish populations, while M694I is mostly seen in the Arabic population.⁶

There has been an increasing interest in the function of *S100A12* protein and its role as an indicator of inflammation. *S100A12* was shown to be associated with active FMF. It is involved in the pathogenesis of this disease, and its release is independently regulated from inflammasome activation.⁷ It shows an excellent relation to disease activity, and serum levels are higher in patients with unstable disease state under colchicine treatment.⁸

Bioactive derivatives of omega-3 fatty acids, mainly eicosapentaenoic acid and docosahexaenoic acid, are essential for the innate immune system and have natural anti-inflammatory, pro-resolving properties.⁹ Resolvin mainly switches inflammation to the resolution phase by inhibition of endothelial migration and infiltration of leukocytes and promotion of clearance of apoptotic polymorphonuclear leukocytes. The efficacy of endogenous *resolvin* D1 has been tested for anti-inflammatory actions in several *in vivo* experimental models.¹⁰ The serum levels of *resolvin* D1 increase in both the attack and silent periods of FMF and correlate with classical biomarkers of inflammation such as highly sensitive C-reactive protein (CRP) and serum amyloid A (SAA).¹¹

Colchicine is the mainstay of FMF treatment and should be started once a clinical diagnosis is established. Its regular use prevents attacks and suppresses chronic subclinical inflammation. Anti-interleukin-1 drugs emerged as promising treatment options in patients who fail to respond or tolerate colchicines.^{12,13} This work aimed to study the role of *S100A12* and *resolvin* D1 in the diagnosis and detection of subclinical inflammation in children with FMF during the quiescent stage of the disease.

Patients and Methods

Seventy-eight Egyptian FMF children during the quiescent disease state and 60 age and sex-matched healthy controls were enrolled in this study. The diagnosis of FMF was

established according to the Tel-Hashomer criteria.¹⁴ A quiescent disease state was considered with clearance of all the signs and symptoms of an FMF attack for at least two consequent weeks. All the patients were on colchicine treatment when the samples were drawn.

The FMF patients were recruited from the Clinical Genetics Department outpatient clinic at the Medical Center of Excellence, National Research Centre. The participants did not have other systemic diseases (diabetes mellitus, chronic renal failure, malignancy and ischemic heart disease) or performed heavy exercises. Other exclusion criteria were smoking, trauma, or administration of drugs other than colchicine. This study was approved by the Research Ethics Committee of the NRC according to the World Association Declaration of Helsinki, and written informed consent was obtained from all patients' legal guardians.

Patients were subjected to detailed medical history, including demographic data, age at the onset, consanguinity, similarly affected family members with three-generation pedigree construction, meticulous clinical evaluation, and disease severity assessment using the scoring systems.^{15,16} The erythrocyte sedimentation rate, SAA, and CRP were assessed.

The blood samples from patients and controls were immediately centrifuged within 20 minutes at 3,000 g for 10 minutes and stored at -20°C for subsequent assay. Serum *S100A12* and *resolvin* D1 concentrations were evaluated using enzyme-linked immunosorbent assay kits (Human *S100* Calcium Binding Protein A12, Human *Resolvin* D1, Bioneovan Co., Ltd; Beijing, China) according to the manufacturer's instructions.

DNA Extraction

All cases were diagnosed by molecular analysis of *MEFV* gene mutation. Genomic DNA was extracted from venous blood, according to Miller et al.¹⁷ Both *MEFV* exon 2 and 10^{18,19} were individually amplified by PCR using two pairs of primers: Exon 2: F: 5'- GCCTGAAGACTCCAGACCACCCCG-3', R: 5'- AGGCC-CTCCGAGGCCTTCTCTCTG-3' Exon10: F: 5'GAGGTGGAGTT-GGAGACAA-3',R:5'-TGACCACCCACTGGACAGAT-3'

Bidirectional direct sequencing of purified PCR products was performed using the Big Dye Terminator V1.1 Cycle Sequencing kit (ABI prism, Foster City, California, United States) and an Applied Biosystems 3500DX Genetic analyzer.

Analysis of *MEFV* gene functional association and interaction with our biomarkers and their associated genes was performed using the Gene MANIA prediction server.²⁰ The genes involved in the *resolvin* D1 biosynthesis pathway (*Alox5*) and *S100A12* protein production were queried against the *MEFV* gene. Association links screening parameters included protein and genetic interactions, pathways, coexpression, and protein domain similarity.

Variation influence on the final protein structure and stability was modeled using Site-Directed Mutate web-server.²¹ A three-dimensional structure for wild-type protein was used as a reference with pdb id: 2WL1. Mutations M694I, V726A, A744S, and M680I were covered by the reference protein structure, and hence their influence was evaluated.

Statistical Analysis

The variables were expressed as mean and standard deviation or as frequencies and percentage. Mann–Whitney U test was used for the comparison of the two groups. Spearman's correlation was used to assess the correlation between S100A12 and resolvin D and between S100A12, resolvin D, CRP, and serum amyloid A. In addition, it was used to analyze the correlation between the disease phenotypes and S100A12 and resolvin D biomarkers. The receiver operating characteristic (ROC) curve was used to evaluate the sensitivity and specificity of S100A12 and resolvin D1 to estimate the efficacy of these biomarkers in detecting the degree of inflammation. A significance level of $p < 0.05$ was used in all tests. All statistical procedures were performed using SPSS version 20 for Windows (SPSS Inc, Chicago, Illinois, United States).

Results

The patients' mean age was 5 ± 3.4 years (3–13 years); there were 32 males and 46 females with a M: F ratio of 1:1.4. Their mean disease duration was 4.56 ± 2.21 years. Consanguinity was present in 22 (28%) cases. The number of attacks ranged from 2 to 3 times/week to 3 to 4 times/month. All patients were on colchicine treatment ranging from 0.5 to 3 mg/day. Twelve patients (15.4%) met the definition of refractory FMF.

The 60 controls were of matched age 5.96 ± 2.25 years (4–14 years) and gender (21 males and 39 female) (M: F 1:1.8) ($p = 0.06$ and $p = 0.47$, respectively).

The characteristics of the patients are presented in ►Table 1. The mutational distribution is presented in ►Table 2. This is a leading study on the link between the MEFV and relevant genes to the studied biomarkers to the best of our knowledge. The gene interaction network analysis using the GeneMANIA web tool showed that the MEFV gene possesses direct and indirect co-expression link evidence with genes responsible for producing biomarker S100A12, S100A8, and S100A9 (► Fig. 1).

►Table 3 showed the mutated protein structure stability. The available protein reference structure did not cover the residue location E148Q, while the remaining four mutations were subjected to the evaluation. The analysis revealed that three out of these four mutations resulted in increased structure stability (M694I, V726A, and M680I). Only A744S mutation caused reduced stability with a predicted ΔG difference from the wild type of negative 0.14 value. However, the M694I mutation, the most common mutation observed, caused increased residue solvent accessibility compared with the wild type and caused the loss of side-chain-sidechain hydrogen bond formation at the respective residue location suggesting marked structure conformational change at the mutation location. The mutation caused a positive increase in $\Delta\Delta G$ by 0.72, leading to a more stable structure than the wild type. M680I mutation shows the slightest difference in $\Delta\Delta G$ in all studied mutations with only 0.03 $\Delta\Delta G$ change due to the variation with no marked protein structural property change.

Table 1 Characteristics of patients with FMF

Variables		FMF patients (n = 78)
Mean \pm SD (range) or n (%)		
Age (y)		5 ± 3.4 (3–13)
Gender (male/female)		32/46
Disease duration (y)		4.56 ± 2.21 (5–10)
Consanguinity		22 (28)
Laboratory	ESR (mm/1st h)	29.5 ± 24.9
	Elevated ESR	56 (71.8)
	CRP (mg/dL)	22.2 ± 17.2
	Positive CRP	50 (64.1)
	Anemia	34 (43.6)
	Serum amyloid A	70 (89.7)
	Elevated liver function tests	4(5.1)
Colchicine dose (mg/d)		(0.5–3) 1.8 ± 0.74
Echo	Patent foramen oval and MVP	4 (5.1)
	Mitral regurgitation	4 (5.1)
	Subcortical epileptogenic activity	1 (1.3)
Abd. US	Mesenteric lymphadenitis	8 (10.3)
	Epididymitis, hydrocele and orchitis	1 (1.3)
	Lymphadenopathy, splenomegaly, and hernia	1 (1.3)
	Fatty liver	1 (1.3)

Abbreviations: Abd. US, abdominal ultrasounds; CRP, C-reactive protein; Echo, echocardiogram; ESR, erythrocyte sedimentation rate; FMF, familial Mediterranean fever; MVP, major vault protein; SD, standard deviation.

Table 2 Mutational distribution among the FMF patients

Gene mutations n (%)	FMF patients (n = 78)
MEFV	78
<i>Heterozygous</i>	
M694I	60 (76.9)
M680I	8 (10.2)
A744S	2 (2.6)
E148Q	2 (2.6)
V726A	2 (2.6)
<i>Homozygous</i>	
E148Q	2 (2.6)
M694I	2 (2.6)

Abbreviations: FMF, familial Mediterranean fever; MEFV, Mediterranean fever.

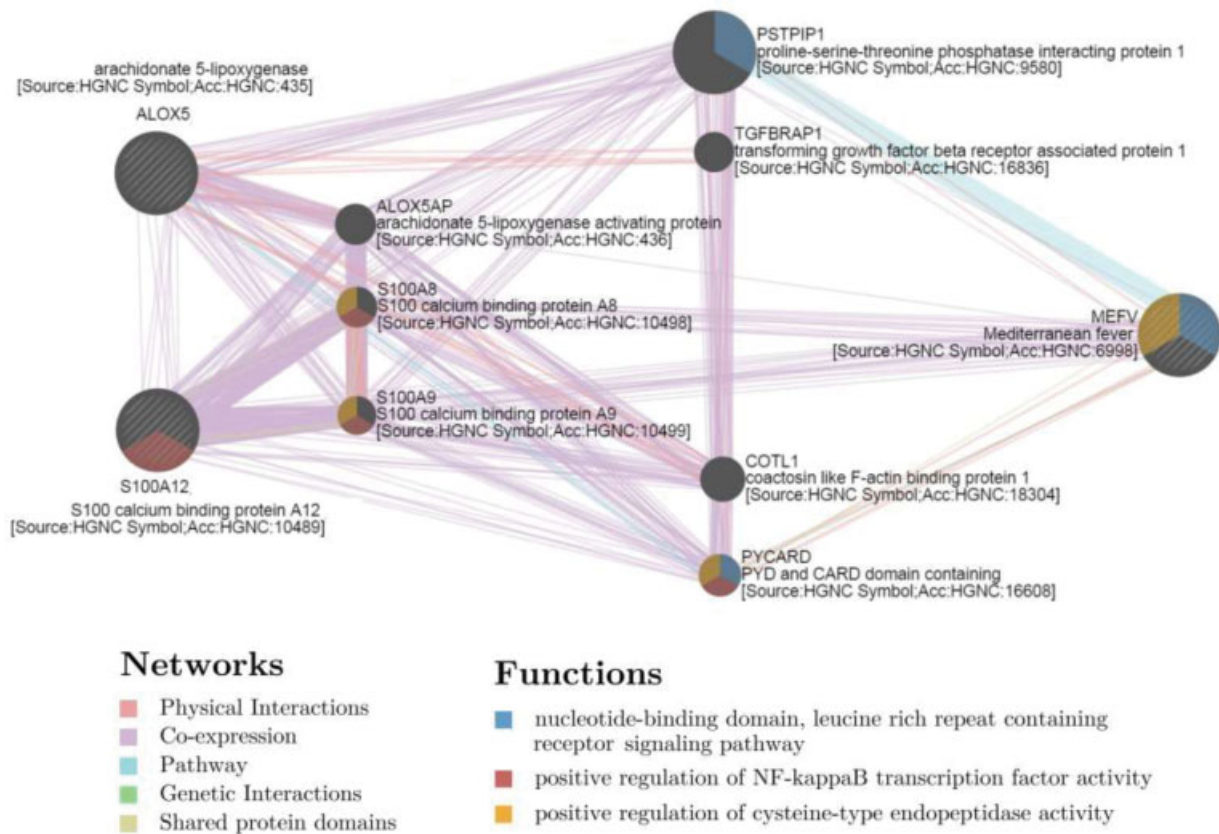


Fig. 1 Interaction network analysis among Mediterranean fever (*MEFV*) gene and genes associated with the production of S100A12 and resolvin D1. Interaction analysis showed a set of intermediate genes including (ALOX5AP, S100AB, S100A9, PYCARD, GOTL1, PSTPIP1) sharing coexpression, protein-domain similarity and pathway interaction links connecting all *MEFV*, *ALOX5*, and *S100A12* genes.

Table 3 Mutated protein structural stability analysis in FMF patients

Mutation	Mutated protein structural stability analysis in FMF patients (n = 78)								
	Residue	RSA (%)	Depth (Å)	OSP	SS	SN	SO	ΔΔG	Stability
M694I	Wild type	40.3	4	0.32	T	F	F	0.72	Increased
	Mutation	54.6	3.8	0.28	F	F	F		
V726A	Wild type	56.3	3.5	0.21	F	F	F	0.12	Increased
	Mutation	54.1	3.2	0.23	F	F	F		
A744S	Wild type	67.9	3.2	0.24	F	F	F	-0.14	Reduced
	Mutation	71.6	3.3	0.23	F	F	F		
M680I	Wild type	52.7	3.7	0.17	F	F	F	0.03	Increased
	Mutation	37.9	3.7	0.26	F	F	F		

Abbreviations: F, false; FMF, familial Mediterranean fever; OSP, occluded surface packing; RSA, relative solvent accessibility; SN, sidechain-main chain amide hydrogen bond; SO, sidechain-main chain carbonyl hydrogen bond; SS, sidechain-sidechain hydrogen bond; SSE, main chain conformational class; T, true.

The serum levels of S100A12 and resolvin D1 were significantly increased in the FMF patients during colchicine treatment compared with the control (mean: 847.4 ± 553 pg/mL, median: 726.5 and 793.3 ± 622 pg/mL, 700 vs. 324.3 ± 201.7 pg/mL, median: 272.5 and 235.1 ± 154.7 pg/mL, 261.5; $p=0.001$). Spearman's correlation test showed no significant correlation between S100A12 and resolvin D1 among cases with $r_s=0.014$ and $p=0.943$,

although there was a highly significant correlation between S100A12 and resolvin D1 among controls with $r_s=0.627$ and $p=0.003$.

On comparing the serum levels between those with heterozygous mutation of M694I gene and those with other mutations, the levels were 842.4 ± 611 pg/mL, median 723.5 and 710.7 ± 487.8 pg/mL, 572.5 vs. 864 ± 314.8 pg/mL 726.5 and 958.4 ± 841.9 pg/mL, 810; $p=0.516$ and 0.607 (► Fig. 2).

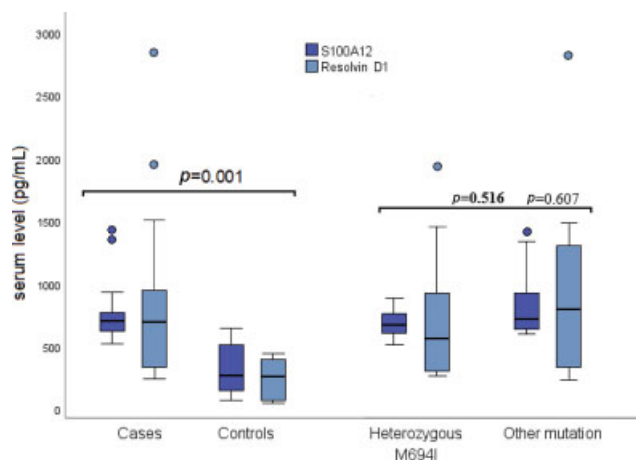


Fig. 2 Levels of S100A12 and resolvin D1 between familial Mediterranean fever patients and controls (left) and between those with M694I mutation versus other mutations (right).

Table 4 Spearman's correlation between S100A12 and resolvin D versus serum amyloid A and C-reactive protein

S100A12 (pg/mL)		Serum amyloid A	C-reactive protein
	rs	0.907 ^a	0.857 ^a
	p-Value	0.001	0.001
	Significant	HS	HS
Resolvin D (pg/mL)			
	rs	0.983 ^a	0.925 ^a
	p-Value	0.001	0.0001
	Significant	HS	HS

^aSpearman's rho.

The Spearman's correlations revealed a significant value with $p=0.001$ between the studied biomarkers and the classical inflammation markers (SAA and the C-reactive protein) (► **Table 4**) and between the disease phenotypes and both S100A12 and resolvin D1 biomarkers.

The sensitivity, specificity, and area under the curve (AUC) of S100A12 and resolvin D1 for the diagnosis of the inflammation degree in cases were 97.44%, 80%, AUC 0.96 and 100%, 50%, AUC 0.82, respectively, with $p=0.001$ (► **Fig. 3**).

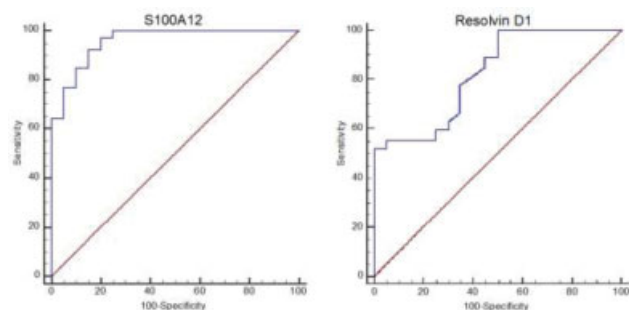


Fig. 3 The ROC curve of S100A12 and resolvin D1 was at a cutoff point of 529.5 pg/mL and 231 pg/mL respectively, their sensitivity was 97.4 and 100%, specificity 80 and 50% respectively and the areas under the curve (AUC) was 0.958 and 0.82 respectively.

Discussion

FMF is the most common autoinflammatory disease prevalent in the Middle East. There is increasing demand for new biomarkers to estimate inflammation and treatment follow-up in FMF patients. In this study, the role of S100A12 and resolvin D1 was evaluated in the quiescent period to estimate the degree of inflammation. This may assist in further studies to adjust the doses of colchicine to ameliorate disease symptoms. The present study confirmed that the level of S100A12 was significantly increased in the quiescent period of FMF patients during colchicine treatment compared with controls. This coincides with previous studies.^{10,22,23} Therefore, S100A12 can be used to detect subclinical inflammation during the attack-free period.

According to the current study, the most common mutation was heterozygous M694I (76.9%), heterozygous M680I (G/C) (10.2%), followed by E148Q, V726A, and A744S with lower frequencies. The M694V mutation could not be identified in this study in agreement with the results reported in previous work.¹⁸ Moreover, there was no significant difference in S100A12 levels between cases with heterozygous M694I mutation and those with other types of mutations. This may be due to the small number of FMF patients in the subgroups.

The current study showed S100A12 to be a reliable biomarker of inflammation during the quiescent period of FMF patients by the ROC test with high sensitivity, specificity, and highly significant p -value. Therefore, determining the level of S100A12 can help gauge the effectiveness of treatment by the detection of the degree of inflammation during the quiescent period of FMF. Resolvin regulates the pro-inflammatory state, and it actively promotes resolution via monocyte/macrophage uptake of debris and apoptotic polymorphonuclear neutrophils.^{23,24} Serhan²⁵ elucidated that resolvin was an important molecular player involved in the active termination of inflammation specialized pro-resolving mediators. When this active process fails, chronic inflammation may proceed to different disorders like rheumatoid arthritis, inflammatory bowel disease, and other autoimmune diseases. FMF is an autoinflammatory disease that primarily affects the leucocytes of the innate immune system.^{26,27} In the present study, the level of resolvin was significantly higher in the cases compared with the controls. This is in agreement with the study of Taylan et al.¹² Additionally, no significant correlation was found in relation to the types of mutations.

The ROC curve enlightened that resolvin is a good diagnostic biomarker for the degree of inflammation in the quiescent period of FMF patients, besides its role in the resolution of inflammation and may point to the presence of subclinical inflammation.

The levels of S100A12 and resolvin D correlated significantly ($p<0.001$) with the classical inflammatory biomarkers as 70 (89.7%) patients had high serum amyloid levels, which agree with Lofty et al.²⁸ and Duzova et al.²⁹ Similarly, Ben-Zvi and Livneh²⁴ affirmed that although the

patients are under treatment, both CRP and SAA increased in 30 to 90% of their patients during the episode-free period. On the other hand, Lachmann et al³⁰ and Berkun et al³¹ reported elevated SAA during the attack-free periods in only 33.3 and 25% of their FMF patients, respectively. This could be due to the existence of subclinical inflammation in FMF patients.

In addition, CRP was elevated in 50 (64.1%) cases higher than the findings of others studies.^{28,29,32} The number of patients with increased SAA was more than those with elevated CRP. This may be due to the high sensitivity of SAA in detecting the degree of inflammation. Furthermore, a correlation was detected between the clinical severity and S100A12 and resolvin D1 ($p < 0.001$). Similarly, Taylan et al¹² delineated the role of S100A12 and resolvin D1 in revealing the clinical severity and the estimation of subclinical inflammation in FMF patients.

The bioinformatics analysis for the gene interaction network revealed a coexpression link between the *MEFV* gene and the genes responsible for the production of the two biomarkers of interest. The GeneMANIA web tool suggested a hidden functional association between the *MEFV* gene and both *ALOX5* and *S100A12*. Moreover, the evaluation of those biomarkers' protein structure stability revealed that M694I, V726A, and M680I mutations resulted in an increase in their stability and helped improve the functions of the protein, contributing to the disease, while A744S mutation suppressed it.

This study's main limitation was that the control sample missed the presence of FMF patients with amyloidosis or Behcet disease.

In conclusion, S100A12 and resolvin D1 may be useful biomarkers in evaluating clinical severity and inflammation during the quiescent period of the disease in FMF patients. Further studies are needed to discover other biomarkers that might aid in diagnosing, following up, and treating FMF patients. Moreover, according to the bioinformatics analysis results, we recommend further study with a larger number of cases to determine if more genes possess coexpression link with the *MEFV* gene and the genes responsible for the production of the studied biomarkers and those affecting their structural stability.

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None.

Conflict of Interest

None declared.

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