Deanship of Graduate Studies
Al-Quds University

"Investigation of the Molecular Basis of Familial Mediterranean Fever-Like Disease"

Diaeddin Sami Qamhia

M.Sc. Thesis

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“Investigation of the Molecular Basis of Familial Mediterranean Fever-Like Disease”

Prepared by:

Diaeddin Sami Qamhia

B.Sc in Medical Laboratory Sciences / An-Najah National University/ Palestine

Supervisor: Dr. Fawaz Awad

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Prepared By: Diaeddin Sami Qamhia
Registration No: 21910034

Supervisor: Fawaz Awad

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The Names and Signatures of the Examining Members as Follows:

1. Head of Committee: Dr Fawaz Awad

2. External Examiner: Dr Zaidoun Salah

3. Internal Examiner: Dr Kifaya Suleiman

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Dedication

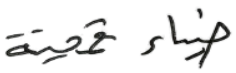
This project is wholeheartedly dedicated to my beloved parents, who have been my source of inspiration and gave me strength when I thought of giving up, who continually provide their moral, spiritual and emotional support.

To my wife, my brothers, my sister, my friends, and classmates who shared their word and advice and encouragement to finish this project.

And lastly, I dedicated this project to Almighty God, thank you for the guidance, strength, power of mind, protection and skills and for giving us a healthy life. All of these I offer to you

Declaration

I certify that this thesis submitted for the degree of master, is the result of our research, the content of the thesis is the result of work which has been carried out since the date of approval of research program. All ethics procedures and guidelines have been followed properly while preparing thesis.

Signature: 

Diaeddin Sami Qamhia

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Acknowledgment

At the end of my thesis, I would like to thank all those people who made this thesis possible and an unforgettable experience for me.

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Abbreviations

FMF: Familial Mediterranean Fever.

VUS: variants of uncertain significance.

CRP: C-reactive protein.

SAA: serum amyloid A.

IL: interleukin.

MEFV: Familial Mediterranean Fever gene.

Rho GTPase: Rho guanosine triphosphatase.

PBMCs: peripheral blood mononuclear cells.

RTq-PCR: real-time quantitative PCR.

PCR: polymerase chain reaction.

ELISA: Enzyme-linked immunosorbent assay.

MoH: Ministry of Health.

WES: Whole exome sequencing.

SPSS: Statistical Package for Social Sciences.

PYD: pyrin domain.

HIDS: Hyper Immunoglobulin A Syndrome.

TRAPS: TNF Receptor Associated Periodic Fever Syndrome.

MWS: Muckle-Wells Syndrome.

PRRs: Pattern recognition receptors.

MAS: macrophage activation syndrome.

DsDNA: double-stranded DNA.

LOF: loss-of-function.

GSDMD: Gasdermin D.

RR: relative risk.

NGS: Next generation sequencing.

NETs: neutrophil extracellular traps.

LOH: Loss of heterozygosity.

SNP: single-nucleotide polymorphism.

WES: Whole exome sequencing.

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Abstract

Background: Familial Mediterranean Fever (FMF), the most common inherited auto-inflammatory disease worldwide, is caused by gain-of-function mutations in the *MEFV* gene, which encodes the immune modulatory protein pyrin. The Familial Mediterranean Fever gene (*MEFV*) gene has been implicated as the main gene responsible for FMF. The aims of our study were to identify and characterize new genes involved in FMF and determine the prevalence of the known *MEFV* mutation associated with FMF in our patient's cohort.

Methods: clinically diagnosed FMF patients who met the Tel Hashmer's diagnostic criteria and were confirmed to be negative for the most common mutations in exon 10 of the *MEFV* gene were included in our study. DNA was extracted from 22 individuals from 7 independent families and Whole Exome Sequencing (WES) was applied to 2 patients from the same family (Father and his son).

Results: About 1422 patients (48.6% females and 51.4% males) with FMF were chosen to participate in our study, from 2018 till 2020, 763 patients were found to be negative for *MEFV* gene, and only 2 were analyzed for new genes or mutations suspecting them for FMF. Around 435 and 224 patients were found to be heterozygous and homozygous for *MEFV* gene mutation, respectively. Regarding the type of mutation, 595 patients were found to be likely pathogenic for *MEFV* gene, 222 patients were found to have variants of uncertain significance (VUS) mutation, and 605 patients were found to be pathogenic for *MEFV* gene mutation. The most common mutations found were R761H (41.09%), followed by V726A (18.82%), M694V (17.57%), M694I (5.7%), M680I (5.15%), A744S (3.51%), K695R (3.04%), P369S (2.73%), F479L (1.25%), and I692del (1.09%). Gender was not found to be significantly related to the presence of different mutations. Whole exome sequencing (WES), applied for 2 samples (a father and his son), showed 4 variant genes: *COL3A1*, *ANKRD36C*, *WNK1*, and PCDH17, that potentially may contribute to FMF-like phenotype in heterozygous variant, and MT-ND5, C1ORF109, ANKRD36B, LNP1, LRRC37A, KIR2DL4, and XKR3 in homozygous variant.

Conclusion: Several candidate genes related to the FMF-like phenotype were identified in this study, and the prevalence of known *MEFV* mutations associated with FMF was determined.

Chapter 1

Introduction and Literature Review

Familial Mediterranean fever (FMF) is the most common hereditary autoinflammatory disease [1]. It is inherited as an autosomal recessive disease, with variable presentation of recurring episodes of febrile pleuritis, peritonitis, and synovitis [2]. Although it has been classically known to affect people in the Mediterranean region like the Arabs, Armenians, Turks, Greeks, Italians, Persians, and Jews, FMF is seen worldwide due to travel and immigration that happened mainly in the twentieth century [3].

FMF is associated with a mutation in the Familial Mediterranean Fever gene (*MEFV*), which encodes for an inflammation regulatory protein, termed pyrin [4-6]. Pyrin is mainly expressed in neutrophils and monocytes [7, 8] and to a lesser extent in fibroblasts from synovium, peritoneum and skin. The expression of this protein in the aforementioned cells and tissues may explain the clinical presentation of FMF patients with serosal, synovial and cutaneous manifestations. Mutations involving the protein pyrin are suggested to be involved in a series of reactions leading to overexpression of interleukin (IL)-1β which is a potent pyrogenic cytokine and considered a hallmark of autoinflammatory disorders. However, the exact role of pyrin in the clinical manifestations of FMF as well as its implication in the activation and secretion of IL-1β through macromolecular complexes known as inflammasomes has been for many years a controversial issue [9-12]. The identification of stimuli triggering the pyrin inflammasome is a breakthrough, which may help in the discovery of a biochemical diagnostic test to distinguish between FMF disease and other SAIDs irrespective of the presence or not of biallelic mutations in the *MEFV* gene.

Most of the cases of FMF are inherited as autosomal recessive, although other phenotypes in heterozygous state have been reported in the literature [13-15]. Although sequence variants have been found throughout the gene [16] the pathogenic ones: M680I, M694V, M694I and V726A found to be clustered within the exon 10 of the gene [17].

The diagnosis of FMF has traditionally been based upon clinical criteria and confirmed by genetic testing for mutations in the *MEFV* gene. However, not all laboratories routinely screen the entire sequence of the gene for mutations, which could potentially lead to missed or incorrect diagnoses. Moreover, other genes, apart from *MEFV*, may be responsible for (FMF-like diseases), in a clinical picture resembling FMF, which includes missense mutations and small deletions [18].

Left untreated, a significant fraction of FMF patients will suffer from complications due to chronic inflammation, which include growth retardation in childhood, anemia, and splenomegaly. The major life-threatening complication of FMF is secondary amyloidosis [19] due to the deposition of Amyloid A (AA) fragments in the extracellular tissues, mainly of the kidneys, which can lead to end-stage renal failure. Colchicine treatment reduced the incidence of amyloidosis from over 60% to less than 5% even in the high incidence populations [20].

Colchicine has been reported as the first line of treatment. The anti-inflammatory action of colchicine is mainly by its action to inhibit microtubule polymerization and thereby alter the adhesion and mobility of leukocytes [21], and colchicine tend to prevents acute symptoms with most patient responding to it [22-26]. Other biological agents that target specific cytokine(s), such as interleukin (IL)-1 blocking agents (e.g., Anakinra and Canakinumab), could be considered an alternative option in a small percentage of FMF patients who do not respond to colchicine or who are intolerant to its side effects [27-29].

1.1 Familial Mediterranean fever (FMF)

1.1.1 Prevalence and Epidemiology

People living in the Mediterranean region are as higher risk of being affected by FMF, including Arabs, Turks, Sephardic, Jew sand, Armenians (Figure 1). Also, FMF theorized as the most common mono-genic auto inflammatory disorder [30]. Anywise, FMF is not limited to these ethnic groups. It could be at a lower prevalence in many other countries such as Italy, Japan, China and Greek.

People who are immigrants from the Middle East and Armenia could spread FMF to the host country. From the other side, facilitated travel between countries contributes to propagation FMF in the world [31].

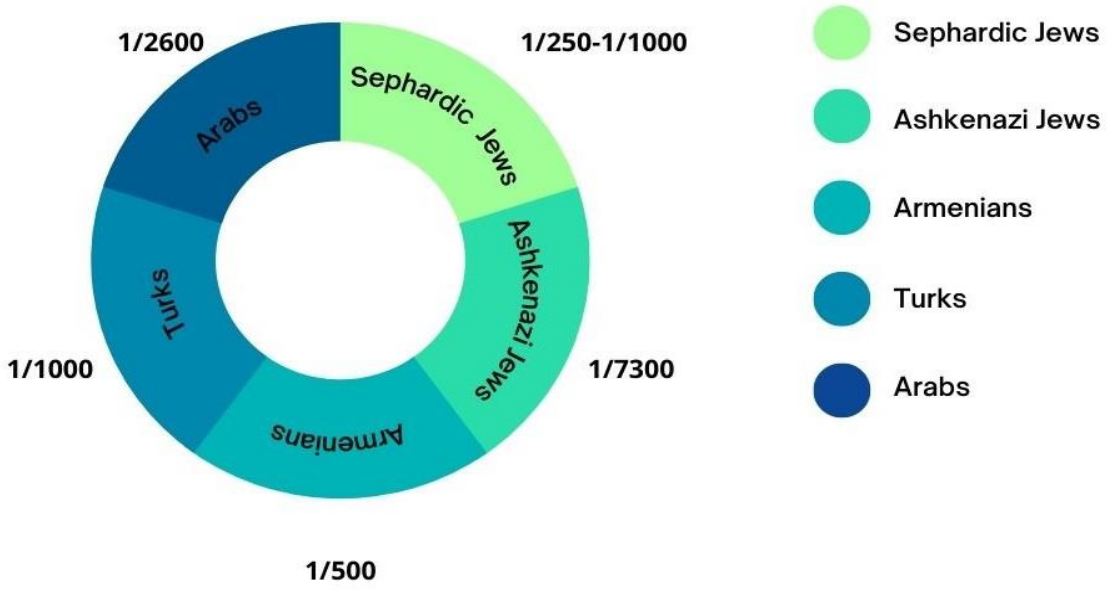

Figure1: People living in the Mediterranean region areas higher risk of being affected by FMF, including Arabs, Turks, Sephardic, Jews and, Armenians [30].

When it comes to previous Palestinian studies, Ayesh et al [32], in his study of genetic screening of familial Mediterranean fever mutation in Palestinian populations showed a 38% of homozygous genotype in their cohort.

1.1.2 Clinical Presentation

FMF is characterized by recurring febrile attacks that typically last for one to three days. These attacks may also include symptoms of peritonitis (inflammation of the lining of the abdomen), pleuritis (inflammation of the lining of the lungs), or acute synovitis (inflammation of the joints). Patients are completely healthy in between attacks, which can happen anytime from once a week to once every few months. This attack can be triggered by emotional stress, exhaustion, surgery, menstruation, strenuous exercise, and exposure to the cold [33].

The age of onset is between 2.6 and 2.7 years (1.1 and 5.3 years) [34, 35], and the time between onset and diagnosis is between 3.3 and 4.3 years [34, 36], which most likely reflects the challenges of diagnosis in very young individuals.

Abdominal attacks, arthritis, myalgia, skin manifestation (usually occurring between the knee and the ankle), vasculitis and hematuria also considered as clinical presentation of FMF. Furthermore, isolated febrile attacks can occur in young children.

1.1.3 Laboratory investigations

A leukocytosis and an increase in acute-phase reactants, such as ESR and CRP, fibrinogen, haptoglobin, C3, C4, and serum amyloid A (SAA), are signs of FMF assault. Regarding follow-up of these individuals, there is no consensus on the ideal acute-phase reactant [37]. Levels of CBC and CRP are typically checked. Comparing individuals with acute appendicitis to those who had an abdominal FMF episode, the neutrophil-to-lymphocyte ratio was considerably greater in the former group [38]. All patients should get a urinalysis every year to check for microalbuminuria [39]. The prevention of amyloidosis is reassured by a median SAA level of fewer than 10 mg/l throughout follow-up [40].

1.1.4 Etiology and Genetics of FMF

FMF is autosomal recessive, meaning that patients must inherit mutated genes from both parents that carry the mutation to have the disease. Carriers of the recessive gene are asymptomatic in most cases and associated with mutation in the *MEFV* [41].

INFEVER, an online database of autoinflammatory disorder (AID) mutations, contains to date information on about 395 *MEFV* sequence variants [42]. The majority of mutations, including the four most frequently found in FMF patients, are found in exon 10, the longest *MEFV* exon, which contains the variations M694 V, V726A, M680I, and M694I (Figure 2). Amyloidosis, colchicine resistance, and severe illness are all linked to the M694V mutation. Although homozygous E148Q mutations are infrequently discovered in patients with clinical FMF illness, the E148Q variant is prevalent in communities where the disease is uncommon [43, 44].

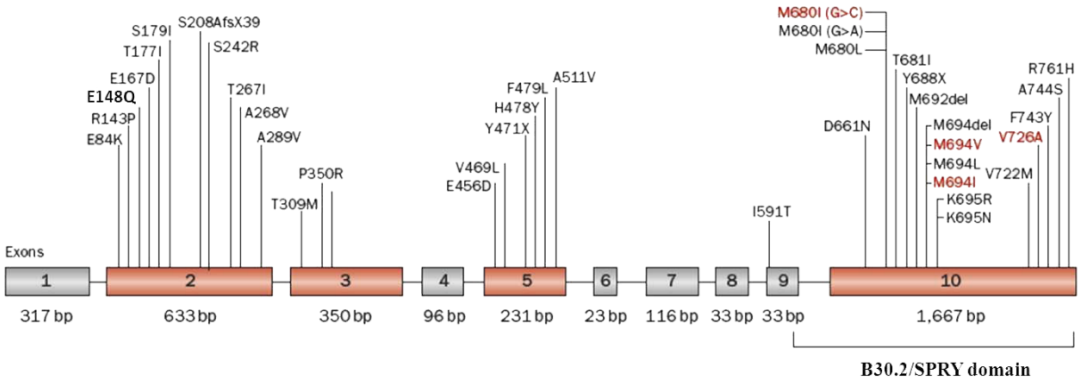

Figure 2: Schematic representation of recessively inherited FMF-associated mutations in the MEFV gene [45].

Molecular testing for common *MEFV* mutations in a large cohort of FMF-affected children revealed two mutations in 60% of patients, but no mutation was discovered in 10% of patients [46]. The majority of people who carry a single *MEFV* mutation are asymptomatic carriers of the illness; nonetheless, classical FMF can unmistakably occur in carriers with just a single *MEFV* mutation. Additionally, people who carry two mutations may not show any overt symptoms of a disease. These observations, along with the phenotypic variety of FMF disease, point to a critical involvement for extra environmental factors or phenotype modifying genes in the clinical manifestation of FMF [47-49].

According to a different study, patients with genetically heterogeneous and mutation-negative *MEFV* variants exhibit a milder severity phenotype than p.M694V homozygous patients [50]. This suggests that the disease in FMF patients without mutations may be brought on by genetic flaws either upstream or downstream of the *MEFV*-related metabolic pathway [51].

Recently, evidence-based recommendations for the genetic diagnosis of familial Mediterranean fever were released, with a focus on giving novice clinicians access to diagnostic resources for the interpretation of *MEFV* mutations [52]. The study came to the conclusion that FMF is a clinical diagnosis that genetic testing can help confirm but is not always necessary to rule out. Patients with FMF, who have two common mutant alleles, in particular the M694V, M680I, and M694I on exon 10, are thought to be at increased risk of developing a more severe form of the disease. As seen in other researches [46, 53], individuals homozygous for the M694V mutation are at risk for developing an illness with an early age start. Asymptomatic people with AA amyloidosis risk

factors should be regularly monitored in order to consider treatment, particularly if they have the M694V allele of one of the two frequently mutated *MEFV* alleles. When the E148Q variant is the only *MEFV* mutation, it does not support the diagnosis of FMF because it is frequent, has unknown pathogenic importance, and is not pathogenic [52].

The typical clinical phenotype of FMF and more severe disease are associated with these variants [54]. Most of the time, benign and likely benign variants are found on exon 2 and do not usually cause the typical FMF phenotype. Due to their unknown clinical association, up to two thirds of registered variants across the entire gene are either not classified or classified as variants of uncertain significance (VUS) [55].

In countries with a high prevalence of FMF and consanguineous marriage, people from successive generations may be mistaken for dominant inheritance, which is actually pseudo-dominant transmission. While this is the case, several *MEFV* variants have also been identified with true dominant inheritance [51, 56]. Asymptomatic carriers of the disease are those without symptoms who have one detectable pathogenic *MEFV* gene variant, and phenotype 3 refers to asymptomatic carriers who have two pathogenic variants [57].

1.1.5 Pathophysiology of FMF

The *MEFV* gene, primarily expressed in innate immune cells, codes for the 781-amino-acid protein pyrin. Pyrin is part of the inflammasome complex, an intracellular organelle required for the production of IL-1β. IL-1β maturation is controlled by pyrin through interactions with pro-caspase-1 and other inflammasome elements, such as NACHT, LRR, and PYD domain protein 3 (NLRP3). Roles for pyrin have been proposed are both pro- and anti-inflammatory. According to experimental research, pyrin is crucial in the control of both inflammation and apoptosis, and mutant pyrin results in FMF inflammation that is full-blown and marked by increased IL-1 secretion [58]. Other research using animal models revealed that gain-of-function mutations in pyrin exert their effects independently of NLRP3, perhaps via a different kind of inflammasome [59].

A member of the cytosolic pattern recognition receptors (PRRs), cytoplasmic pyrin interacts with the microtubules in the cell skeleton and triggers innate immune responses quickly by detecting

endogenous danger or foreign pathogen linked molecular patterns (DAMPs and PAMPs) [60]. Pyrin, unlike other receptors, does not directly recognize DAMPs or PAMPs; instead, it detects changes in cytoplasmic homeostasis triggered by damaging stimuli, often known as "homeostasis-altering molecular events" (HAMPs) that alter RhoA GTPase inside of cells [61]. When pyrin is activated, it combines with other cellular proteins to form the "pyrin inflammasome," a macromolecular complex that triggers caspase-1, which then triggers the release of pro-inflammatory IL-1 and IL18 from their dormant progenitors and pyroptosis via the gasdermin D pathway [62, 63].

RhoA GTPase stimulates PKN1 and PKN2 serine-threonine kinases, which bind to and phosphorylate pyrin under physiological conditions. The formation of the pyrin inflammasome is prevented by the binding of phosphorylated pyrin to inhibitory 14-3-3 proteins, which keeps pyrin in an inactive state [60].

A pro-inflammatory pyrin inflammasome is formed more easily in FMF due to mutations in the *MEFV* gene that affect pyrin's ability to interact with proteins such as microtubules, PKN, and 14-3-3. A process known as pyroptosis occurs when cells die in an inflammatory manner as a result of the pyrin inflammasome, which activates caspase-1 to convert pro-IL-1 and pro-IL-18 to their mature forms, IL-1 and IL-18, respectively. The usual febrile inflammatory attacks seen in FMF are driven by excessive pyrin inflammasome activation and the subsequent inflammation [61, 64].

The IL-1 pathway is activated by IL-1, which increases its own production and causes an inflammatory burst [65]. Interferon (IFN)-, Tumor Necrosis Factor (TNF)-, IL-4, and IL10, as well as lipopolysaccharides (LPS), can all increase the expression of pyrin [61].

Although the episodic nature of FMF is not fully understood, attacks cause the formation of neutrophil extracellular traps (NETs), which are chromatin filaments "decorated" with neutrophilic proteins and captured IL-1. These NETs prevent further IL-1 production through a negative feedback mechanism, which may help to explain why FMF attacks are self-limited [66].

1.1.6 FMF Clinical Diagnosis

FMF is still a clinical diagnosis despite improved understanding of the numerous *MEFV* gene variations. The diagnosis needs to be made based on transient febrile episodes that are accompanied by inflammation of one of the serous membranes and a response to colchicine therapy [67]. There are many alternative diagnostic criteria that have been proposed, and

specificity and sensitivity vary greatly depending on the population [35]. Valid, fact-based, clinical categorization criteria were proposed in a recent study that made use of a sizable worldwide registry of autoinflammatory diseases (AIDs) (Eurofever, 1215 patients). Two hundred and ninety one of the 498 FMF patients were classified as "gold standard," meaning they had two *MEFV* mutations, at least one of which was in exon 10 [34, 37, 39]. According to a scoring system adopted based on different signs and symptoms with different scores, if a patient has at least 60 points (**Table 1**) [34], FMF is considered to be the diagnosis. Overall sensitivity and specificity were 68 and 87%, respectively, with this rating.

Table 1: The Euro fever clinical diagnostic/classification criteria (35).

Presence	Score
Duration of episodes <2 days	9
Chest pain	13
Abdominal pain	9
Eastern Mediterranean ethnicity	22
North Mediterranean ethnicity	7
Absence of aphthous stomatitis	9
Urticarial rash	15
Enlarged cervical lymph nodes	10
Duration of episodes >6 days	13
Cutoff	⩾60

Functional abdominal pain, IBS, and recurrent infections in young children are included in the differential diagnosis. Recurrent fever-causing illnesses like HIDS (Hyper Immunoglobulin A Syndrome) and TRAPS (TNF Receptor Associated Periodic Fever Syndrome) as well as periodic

fever-causing illnesses such adenopathy, pharyngitis, and aphthae (PFAPA) syndrome are far less common. Patients may have both PFAPA and FMF since they are both prevalent in Israel [68].

As FMF only manifests as arthritis [69], particularly in youth, it is possible to confuse it with viral infections, acute articular rheumatism, juvenile idiopathic arthritis, systemic lupus erythematosus, and other rheumatic diseases. As previously mentioned, it may also be confused with illnesses in this category due to the possibility of vasculitis in FMF patients with PAN or HSP as a presentation symptom. The individuals with FMF may experience abdominal episodes that are very similar to acute abdomen presentation. Consequently, it could be confused with any factor that contributes to acute abdominal pain. It should be distinguished from renal colic and acute pelvic inflammatory illness due to the posterior peritoneum's involvement. It may also be confused with other familial periodic fevers syndromes, such as TRAPS (tumor necrosis factor receptor-related periodic syndrome), HIDS (hyperimmunoglobulin D syndrome), MWS (Muckle-Wells Syndrome), FCU (familial cold urticaria), CINCAS (chronic infantile neurologic cutaneous and articular syndrome), and PFAPA (periodic fever, aphthous stomatitis, pharyngitis and adenopatia) syndrome. The illness TRAPS is autosomal dominant. It typically starts in early infancy or adolescence. Recurrent fever episodes, rash, periorbital edema, musculoskeletal pain, and abdominal pain are some of its distinguishing features. Like FMF, the length of the episode varies, but it lasts for a longer time. It differs from FMF in that it responds to steroid therapy [70].

1.1.7 Molecular diagnosis

The sole gene known to be connected to FMF is the *MEFV* gene, which is situated on chromosome 16p13. *MEFV* is 10 kb in size and produces a 3.7 kb transcript that is assumed to be expressed in mature neutrophils as well as fibroblasts from the epidermis, peritoneum, and synovium. This suggests that *MEFV* acts as an inflammatory regulator at the level of cytoskeletal organization [71, 72]. Atopic illnesses, such as asthma and allergic rhinitis, may be less common among people with FMF than in the general population, according to a number of studies. This finding is intriguing because it is theoretically consistent with such a mechanism [73].

We try not to focus much on genotype/phenotype correlations with our patients since we believe that clinical severity and colchicine response are not solely determined by the specific *MEFV* mutation. Only minor genotype/phenotype correlations have been found [72]. The M694V mutant

homozygosity has been linked to a relative resistance to colchicine, [74] and we have occasionally seen this as well. Similar to this, it is claimed that some mutations are more likely to cause amyloidosis than others. On the other hand, there is ongoing discussion regarding whether the E148Q mutation in exon 2 should be regarded as a genuine mutation or a benign polymorphism because it typically results in a mild or occasionally even silent phenotype [75]. To induce a specific phenotype, it is likely that additional environmental factors or modifying genes work in concert with changes in *MEFV* [72]. One such component has been suggested to be isoforms of the amyloid SAA genes [76]. Pyrin is thought to have a natural role that involves suppressing certain aspect of the immune response in order to manage inflammation. A full-scale inflammatory response of the serosal membranes, which is essentially an FMF attack, would allegedly occur from a lack of normal pyrin protein (caused by disease-causing mutations in *MEFV*); this is why the condition is classified as autoinflammatory as opposed to autoimmune [77].

Depending on the clinical condition and the patient's ethnicity, diagnostic molecular testing uses a variety of testing methods. There have been discovered more than 50 disease-causing mutations in *MEFV* so far, the bulk of which are missense mutations [78]. Eighty five percent of individuals with classic FMF are believed to have one of the five most common mutations, which are p.M680I, p.M694V, p.M694I, p.V726A (all in exon 10), and p.E148Q [78, 79]. Only minor insertions or deletions as well as point mutations in the gene's coding regions can currently be found using sequencing techniques. Since just one disease-causing mutation has been found in an individual, it is feasible that another disease-causing mutation exists in a noncoding intronic region or a gene regulatory region that affects messenger RNA splicing or expression [80].Additionally, research suggests that single and multiexon *MEFV* copy number changes (i.e., significant deletions or duplications) do not contribute to the pathogenesis of FMF; as a result, multiplex ligation-dependent probe amplification and other gene rearrangement techniques are not typically advised for FMF testing [78, 80].

As it is plainly more specific than the other laboratory analytes like erythrocyte-sedimentation rate and C-reactive protein, molecular genetic diagnostic testing is performed to provide a confirmation of the FMF diagnosis [81].

However, in people with high-risk ethnic backgrounds and exhibiting classical symptoms, it is not unusual to find only a single-mutant allele based on our experience and in the medical literature. As a result, it is our finding that a sizeable portion of patients have a clinical diagnosis that cannot

be fully explained in terms of molecular genetics [80]. In these circumstances, we typically accept the DNA findings as supporting FMF and assume that the second mutation is not discovered because of its rarity or because it is located outside of the investigated gene areas. This is similar in many ways to circumstances that can occur during targeted and whole-gene analyses of CFTR mutations in patients who exhibit cystic fibrosis-like symptoms [82].

A 4–6-month trial of colchicine medication to see if there is a reduction in attack frequency and intensity is another effective diagnostic strategy in the case that two alleles are not found by either targeted mutation analysis or whole gene sequencing in a person with a strong clinical suspicion for FMF. Due to the fact that FMF and gout are the only two disorders that are responsive to colchicine, a positive response effectively establishes the diagnosis regardless of DNA results. Instead of investing in costly DNA testing, which usually proves inconclusive, we frequently prefer to use the colchicine trial because it is secure and reasonably priced.

1.1.8 Complications of FMF

Complications can occur if FMF is not treated. Inflammation can lead to amyloidosis:

AA amyloidosis: AA is a serum amyloid with a normal sequence (no mutations). A protein that is mostly produced in the liver as an acute-phase reactant in response to certain cytokines, Amyloidosis is a rare condition that only some people with high blood amyloid A levels experience.

Systemic AA amyloidosis is a chronic consequence of a number of chronic inflammatory diseases, such as rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, cancer, and diseases that increase the risk of recurrent infections. Because of the use of increasingly potent anti-inflammatory and immunosuppressive medications, the prevalence of AA in rheumatoid arthritis and other chronic arthritides has decreased protein lytic particles of the acute-phase reactant serum amyloid A (SAA) that are deposited outside of cells as amyloid fibrils cause organ damage [83].

1.2 Pyrin Protein

Pyrin, ‘marenostrin’ or TRIM20, which exist in several isoforms both in cytoplasm and nucleus. It is unclear how exactly pyrin works in the nucleus. A coiled-coil domain, two B-box zing finger domains, an N-terminal PYD, and a C-terminal B30.2 domain are all features of the protein Pyrin, which is expressed by the *MEFV* gene [84]. Pyrin recognizes cellular proteins that have been post translationally modified by bacterial toxins during infection.

The innate sensing of Clostridium difficile toxin B, Clostridium botulinum toxin C3, Vibrio parahaemolyticus effector protein VopS, and Histophilus somni IbpA are among the physiological roles of pyrin [85]. These bacterial toxins affect the switch-I region of RhoA to render it inactive, and they also target the Rho subfamily of proteins involved in the reorganization of the actin cytoskeleton. Pyrin activation and inflammasome assembly are the effects of this alteration.

Pyrin is regulated by the actin-depolymerizing cofactor Wdr1 in mice, where it is also engaged in IL-18-driven autoinflammation [86]. So, pathogenic cytoskeletal signaling may control pyrin's ability to activate inflammasomes.

The ability of the presented inflammasome to cause caspase-1 activation, which encourages the maturation of the proinflammatory cytokines IL-1 and IL-18 and the triggering of inflammatory cell death, is what unites them all despite differences in their structural makeup and pattern recognition (pyro ptosis). Because it involves cell swelling and lysis, pyro ptosis differs from apoptosis morphologically. Gasdermin D (GSDMD) is broken down by caspase-1, and this results in the N-terminal pore-forming domain being moved to the cell membrane and the release of pro-inflammatory cytokines during pyro ptosis [87, 88]. Apoptosis can also be brought on by the direct binding of LPS to caspases 4 and 5 in human cells and 11 in mouse cells, which causes the oligomerization of the caspase and the cleavage of GSDMD.As a result, pyro ptosis is crucial in boosting the immune system's defenses against an infection [89].

Early structural investigations revealed a nuclear function for pyrin because it has a bZIP transcription factor domain (266–280) and two overlapping nuclear localization signals [90]. Numerous hypotheses were further reinforced by a study that showed that pyrin's N-terminal segment interacts with the p65 subunit of NF-B and that a mutant protein lacking a domain encoded by exon 2 did in fact translocate to the nucleus [91, 92]. Later research looking into the location and function of pyrin, however, discovered that the N-terminal half of pyrin colocalizes with both

microtubules and the actin cytoskeleton and that full-length pyrin is primarily found in the cytosol [93]. The localization of pyrin was also demonstrated to depend on the kind of cell that was expressing it, and more study is required to determine the potential cell-type specific roles of pyrin [94].

The four functional domains of Pyrin are PYD, bBOX, CC, and B30.2/SPRY. The -helical, coiled-coil domain (420-440) and the B-box (370-412) may be involved in the oligomerization of pyrin [10]. The proline serine threonine phosphatase-interacting protein (PSTPIP1/CD2BP1), a protein crucial for the cytoskeleton's structure, was also demonstrated to interact with these two domains [95]. Pyogenic arthritis, pyoderma gangrenosum, and acne (PAPA) are a dominantly inherited autoinflammatory condition that is brought on by missense mutations in PSTPIP1/CD2BP1 [96] (Figure 3).

Since the majority of the FMF-associated mutations cluster in the pyrin C-terminal B30.2 domain and because this domain is crucial for the molecular processes causing FMF, it is important to note the significance of this region. According to in vitro overexpression studies, Caspase-1 and the pyrin B30.2 domain directly interact, according to in vitro overexpression studies. Controversial findings were found in research examining the impact of FMF-associated mutations on B30.2's affinity for binding caspase-1 [97, 98].

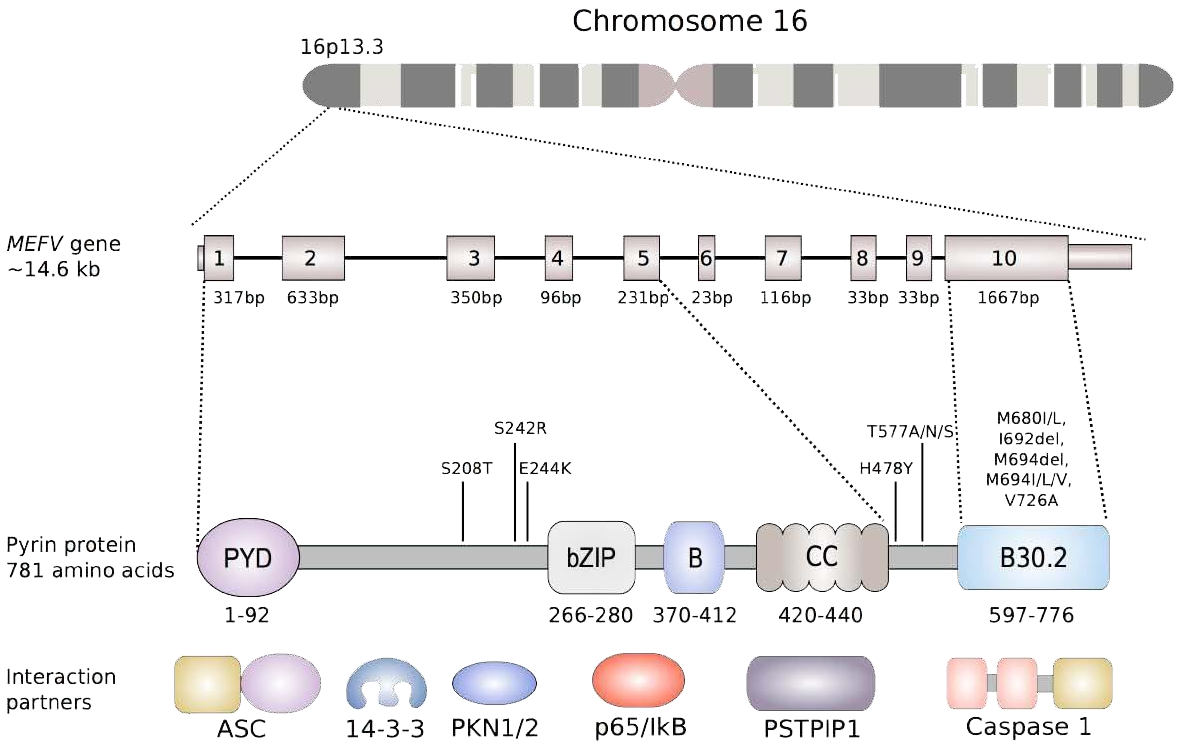

Figure 3: Schematic representation of the *MEFV* gene and the encoded pyrin protein [96].

Exon 10 of the MEFV gene, which encodes the B30.2 domain, is the site of the most frequent FMF-related mutations (B30.2). When compared to mutations in exons 2, 3, and 5, which more frequently exhibit an autosomal-dominant pattern of inheritance, mutations in the B30.2 domain are more likely to be passed down in an autosomal-recessive manner.

1.3 FMF-like diseases:

1.3.1 Definition

Mutations in a single gene (*MEFV*) are thought to be the cause of FMF. Recently, it has become established that heterozygous mutation carriers can also experience FMF-like disease, which is a moderate or incomplete form of the disease. A single mutation has been found in roughly 1/5 of symptomatic cases among Armenians, who have a relatively high carrier rate for *MEFV* mutations. Due to the varied penetrance and phenotypic diversity of FMF-like disease, one cannot rule out the impact of other modifier genes and/or environmental variables [99].

Part of the findings of a study conducted in 2012 at the national center for FMF in Sheba Medical Center, Tel-Hashomer were 57 individuals with p.M694V homozygous FMF, 60 genetically diverse patients and 47 *MEFV* genetic-negative patients. Patients who met the inclusion criteria for their research were randomly and sequentially selected. In the genetically diverse control group, the distribution of *MEFV* mutations is depicted in Table 2 as being genetically heterogeneous. According to the frequency of this genotype in the studied FMF population [100], there were almost 33% of patients in this group who were homozygous for the p.M694V allele.

Table 2: Distribution of various *MEFV* genotypes in the genetically heterogenous control group.

Mutations	Rate N (%)
p.M694V homozygous	20 (33.3%)
p.M694V heterozygous	10 (16.6%)
p.M694V compound heterozygous	16 (26.6%)
p.V726A Homozygous	3 (5%)
p.V726A Heterozygous	1(1.6%)
p.V726A/p.K695R	1 (1.6%)
p.V726A/p.E148Q	3 (5%)
p.E148Q Homozygous	2 (3.3%)
p.E148Q Heterozygous	4 (6.6%)

Table 3 displays the demographic characteristics.

Table 3: Demographic parameters of *MEFV* mutation-negative FMF patients (3).

	Mutation-negative (N = 47)	Heterogeneous controls (N = 60)	P value*	p.M694V homozygous (N = 57)	P value#
Females (%)	31 (65.9%)	30 (50%)	0.117	36 (63.2%)	0.838
Age of disease onset (yrs)	19.61 ± 15.3	12.35 ± 10.36	**0.011**	5.76 ± 6.4	**0.0001**
Average diagnosis delay (yrs)	9.95 ± 11.2	6.91 ± 8	0.373	7.59 ± 9.5	0.234
Positive family history of FMF	21 (44%)	44 (73.3%)	**0.003**	45 (78.9%)	**0.0005**

*Mutation -negative Vs. genetically heterogeneous controls.

#Mutation -negative Vs.p.M694V homozygous.

Bold Values = statically significant

Patient with no mutations started their disease at a much older age than the two control groups, and their family history of FMF was significantly less common. Comparing the two patient groups, the mutation-negative individuals' disease severity was significantly less severe compared to the other group, as measured by the increased severity score (Table 4) [50].

Table 4. Severity of FMF in *MEFV* mutation -negative patients (3).

Severity score	Mutation-negative (N = 47)	Heterogeneous controls (N = 60)	P value*	M694V homozygous (N = 57)	P value#
Mild	27 (57.4%)	23 (38.3%)		1 (1.8%)	
Moderate	9 (19%)	6 (10%)	**0.0049**	5 (8.8%)	**0.0001**
Severe	11 (23.4%)	31 (51.7%)		51(89.4%)	

*Mutation -negative Vs. genetically heterogeneous controls. #Mutation -negative Vs.p.M694V homozygous. Bold Values = statically significant.

1.3.2 FMF with heterozygous mutation in *MEFV*

The possibility of an explanation for a specific FMF phenotype in simple heterozygous patients does not rule out the association of additional mutations or polymorphisms in relevant genes that work in concert to affect the course of FMF-like disease, which runs parallel to the mounting evidence linking a single *MEFV* mutate allele with only mild FMF symptoms [99]. Despite the complexity of the ongoing linkage analysis made more difficult by the presence of two consanguineous marriages and family members with atypical or uncertain phenotypes, the coexistence of two inheritance patterns in the same family raises the possibility of a heterogeneous genetic basis for FMF in carriers of a single mutation.

From literature one of the articles found one candidate loss of heterozygosity (LOH) region in sporadic cases and the absence of a common *MEFV* haplotype encourage further investigation of genetic variations in the genes of the inflammatory pathway acting in conjunction with *MEFV* and modifying the severity of the kaleidoscopic clinical phenotype in simple carriers [99].

The hypothesis that FMF might develop in heterozygotes was recently sparked by the discovery of such Mediterranean individuals who presented with typical FMF signs, typically responded well to colchicine, and carried a single heterozygous *MEFV* mutation. Since no cellular or chemical mechanisms were offered to explain it, there was no direct evidence to back up this theory [101].

After a thorough investigation and the exclusion of the presence of a second mutant gene, the causation of heterozygosity was proposed. In fact, a number of organizations examined the full code [4, 102-106] by genomic *MEFV* sequence [78]. Transcript size anomalies were ruled out by *MEFV* RNA analysis in patients, and allele silencing was ruled out by SNP analysis following cDNA sequencing [78, 107].

Additionally, no copy number variation was found using the multiplex ligation probe amplification method (MLPA) [80, 108]. Most genetically unexplained patients from at-risk groups have FMF manifestations that are unrelated to *MEFV*, according to a population genetics-based study using Hardy-Weinberg equilibrium to evaluate fitness [104]. In affected siblings with a single *MEFV* mutated allele, the presence of several haplotypes for the second allele consistently disproved the existence of an additional, unexplained mutation [78, 109]. Last but not least, genotype research in a small number of families with an autosomal dominant disease mode of inheritance supported the hypothesis that heterozygosity may be the cause of the illness [105, 108, 109]. A significant issue for genetic counselling, nevertheless, has not yet been resolved: due to the high frequency of *MEFV* mutations in at-risk groups, some of these patients exhibiting symptoms suggestive of FMF may also be heterozygotes by coincidence. When there is no other explanation, it may be easy to assume heterozygosity is causal; however, doing so may be dangerous because it could result in incorrect diagnosis.

1.3.3 *CARD15* mutation in FMF

Other studies observe a similar frequency of *CARD15* mutations in FMF and in ethnically matched control subjects, but a higher frequency of erythematous attacks that resemble erysipelas and scrotal attacks, as well as a tendency toward partial colchicine resistance in FMF patients who contain *CARD15* mutations. While *CARD15* mutation does not appear to be a risk factor for FMF, it does appear to have the potential to alter the disease's phenotype [48].

The comparable prevalence of *MEFV* genotypes in FMF patients with and without *CARD15* mutations further supports the conclusion that CARD15 does not function as a susceptibility gene in FMF. Even though FMF is regarded as an autosomal-recessive disorder, bearers with a single *MEFV* mutated allele exhibit clinical symptoms [108]. The interaction of one proinflammatory gene with another in these patients may be the cause of the disease.

In these cases, the disease's onset may be explained by how one proinflammatory gene interacts with another. As with another inflammatory gene, *TNFRSF1A*, which was recently described by Marek-Yagel and colleagues, in which their findings demonstrated that *CARD15* does not enrich for patients who contain just one mutated allele (Table 5) [110].

Table 5: Demographic and Genetic Characteristics of FMF Patients with and Without *CARD15* Mutations.

Symptoms and Signs	Patients Without CARD Mutation (M−, *n* = 93)	Patients with CARD Mutation (M+, *n* = 10)
Age of onset, mean ±αSD (yr)	4.12 ± 3.75	3.20 ± 2.73
Male (%)	44 (47.3)[b]	7 (70)
Homozygous or compound heterozygous[c]	64 (69)	9 (90)
M694V/M694V[c]	32 (34.4)	5 (50)
Heterozygous[c]	25 (26.9)	1 (10)
FMF in family	69 (74.2)	6 (60)
Origin		
NA	50 (53.8)	5 (50)
Iraqi	11 (11.8)	3 (30)
Arab/Druze	6 (6.5)	0

NA, North African Jewish origin.

[a]In all features studied the P value was statistically insignificant.

[b]The numbers in parentheses indicate percentages.

[c] Relates to *MEFV* genotyping.

As demonstrated, FMF is the only condition where the CARD15 mutation has an effect on the disease phenotype [48]. However, the existence of CARD15 mutations in FMF patients seems to be linked to a specific phenotype and a trend toward a more severe disease. CARD15 mutations are not linked to an increased susceptibility to develop FMF [48].

1.3.4 Other possible hypotheses of the causes of FMF-like disease

Another possible explanation of the cause of the FMF-like disease is that this condition is linked to the unidentified upstream or downstream genetic flaws in a part of the same metabolic pathway as pyrin. By choosing this option, patients with genetically negative FMF are anticipated to exhibit phenotypic characteristics that are quite similar to those of patients with genetically positive FMF. Another hypothesis is that while first appearing to fit the clinical criteria for FMF, this subset of FMF is actually made up of various autoinflammatory illnesses, whose phenotypes, when carefully and extensively defined, are likely to vary from those of *MEFV*-mutated FMF.

Objectives of the Thesis

The objectives of our study were the following:

1. **To determine the prevalence of the known *MEFV* mutations in a cohort of patients clinically diagnosed with FMF.**

   This was accomplished by analyzing the genetic data for approximately 1400 patients clinically diagnosed with FMF according to their *MEFV* genotype (negative, heterozygous, and homozygous).

2. **To identify and characterize new mutations/ genes involved in FMF.**

   The search for new mutations/ genes involved in FMF was accomplished by performing exome analysis for two patients with a very well-characterized FMF phenotype but without known biallelic mutation in the *MEFV* gene.

Chapter 2

Materials and methods

2.1 Participants

Written informed consent was obtained from all the individuals participating in the study according to the local protocols and the principles of the declaration of Helsinki. The study has been approved by the ethics committee of Al-Quds University.

Patients were mainly recruited from the pediatrics clinic of Dr. Abu Alsaoud at Al-Maqassed Charitable Hospital in Jerusalem. Adult patients were recruited from the rheumatology clinic of Ramallah Governmental Hospital. We included in this study patients from all ages clinically diagnosed with FMF, who meet the established set of Tel Hashomer's diagnostic criteria (Table 6) [111] and confirmed to be negative or heterozygous for the most common mutations in exon 10 of the *MEFV* gene during routine molecular diagnosis.

Table 6: Tel HaShomer Criteria for the Diagnosis of Familial Mediterranean Fever*

Major criteria:	**Minor criteria:**
1.Recurrent febrile episodes with serositis	1.Recurrent febrile episodes
2.Amyloidosis of AA type without predisposing disease	2.Erysipelas-like erythema
3.Favorable response to colchicine treatment	3.Familial Mediterranean fever in a first-degree relative

* Diagnosis requires ≥ 1 major criterion or ≥ 2 minor criteria.

2.2 Data Collection

This study involved clinical data collection related to FMF through direct interview as well as providing blood sample for DNA extraction. Data collection involved demographic information, history of symptoms, investigations, and other medical conditions. Thanks to the tight collaboration with the Palestinian Ministry of Health (MoH) molecular genetics lab (Ramallah), we had access to *MEFV* genotyping data from 2018-2020 for approximately 1400 patients clinically diagnosed with FMF.

2.3 DNA extraction

DNA extraction was performed using the salting out method [112]. Firstly, 5 mL blood sample in EDTA tube was received and labelled with the test code, and then poured into 50 mL falcon tube. The rest of the tube is filled with RBC's lysis' buffer, mixed by inversion and then left on ice for at least 1 hour; meanwhile, the centrifuge is pre-cooled at 4C. The sample is then centrifuged at 2000 rpm for 10 minutes, the supernatant was discarded carefully and the pellet was washed with RBC's lysis buffer. Fifteen ml of RBC's lysis' buffer was added and centrifugation was done again at the same speed and time (2000 rpm for 10 minutes). Supernatant was carefully discarded and the pellet was washed again in RBC's lysis buffer. After that, 3 mL DNA lysis' buffer, 5mg/mL proteinase K and 20% SDS were added. The mixture was then incubated in the shaker at 37C for 48 hours with gentle shaking. After 48 hours the tube was removed from the shaker and 1 ml of 6M NaCl was added and vortexed at high speed until the formation of white foam. The mixture was then centrifuged at 3000 rpm for 20 minutes at 25C. The supernatant was transferred without the salt-protein deposit into another labelled tube. The supernatant containing tube was then centrifuged at 3000 rpm for 20 minutes at 25C, and the resulting supernatant was then transferred into a 15mL Falcon tube. Cold 100% EtOH was added 2:1 into the tube, and the tube was inverted a couple of times until a string white mass appears (DNA). The mass was then hooked into a clean Pasteur pipette and washed with 70% EtOH and left for 5 minutes to dry.

Finally, the DNA was transferred into a new labelled screw-cap Eppendorf tube containing 200-300 microliters of 0.02% sodium azide. The mixture was left at RT for 24 hours, or on a 37C hot-plate for 1 hour. DNA was them measured by nanodrop, and stored in the assigned sample box in -20C freezer.

2.4 Whole exome sequencing (WES)

The coding sequences and flanking intronic regions (WES) of 2 individuals presenting with FMF-like symptoms were analyzed at LC sciences (Houston, TX, USA) with an average sequencing coverage of 50x. The WES data were analyzed using an in-house bioinformatics pipeline at the Istishari Arab Hospital (IAH). In brief, after sequencing, data were uploaded onto the IAH server and reads were aligned to the reference human genome (hg19). The final list of variants was annotated by Annovar. Variants with low coverage, synonymous, predicted benign (SIFT, PolyPhen-2, REVEL) were filtered out. All genetic inheritance patterns were evaluated.

Chapter 3

Results

3.1 Genotypic and demographic characteristics of the FMF patients in our cohort

Using the *MEFV* genotyping data of the MoH molecular genetics lab, we first classified the clinically diagnosed FMF patients of our Palestinian cohort according to their *MEFV* genotype (negative, heterozygous, and homozygous / compound heterozygous). Overall, we have found that 435 of 1422 patients (30.6%) are carriers of a heterozygous mutation in the *MEFV* gene, 224 of 1422 patients (15.8%) are carriers of a homozygous / compound heterozygous mutation in the *MEFV* gene and 763 of 1422 patients (53.7%) are negative (no FMF-causing mutation was identified) (**Figure 4**).

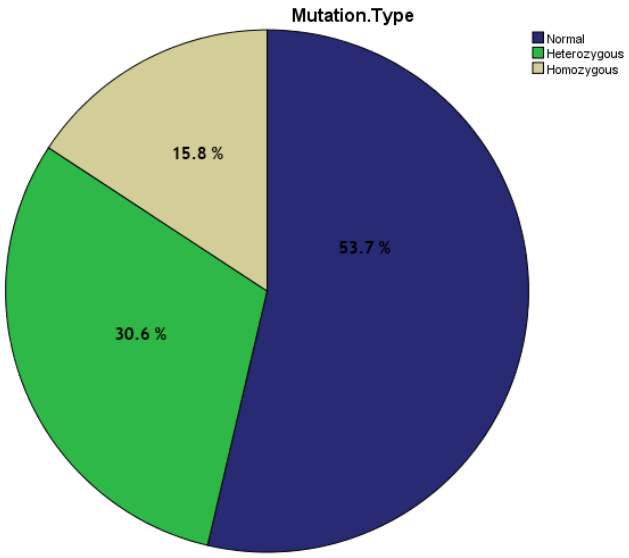

Figure 4: Distribution of the FMF patients in our cohort according to their *MEFV* genotype.

On the other hand, when considering the gender distribution within our cohort, we found no significant difference between males and females. Out of 1422 patients, 689 (48.6%) were female, while 730 (51.4%) were male (**Figure 5**).

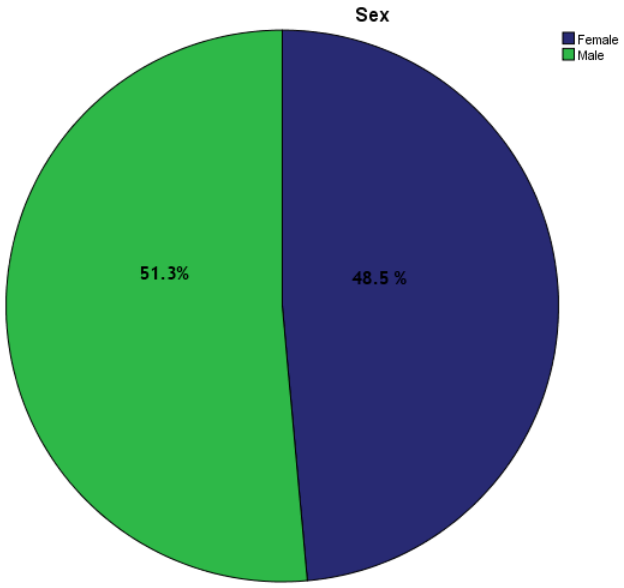

Figure 5: Gender distribution within our FMF cohort.

When looking at the allele frequency of the tested FMF-causing mutations in our Palestinian cohort, we found that the R761H mutation is the most prevalent occurring in 41.09% of cases followed by V726A, M694V, M694I, and M680I, with respective allele frequencies of 18.82%, 17.57%, 5.70%, and 5.15%. Collectively, these five mutations constitute about 88% of all identified mutations. The remaining 12% of tested mutations consist of rare variants, including A744S, K695R, P369S, F479L, and I692del (**Table 7**).

Table 7: Allele frequency of the tested FMF- causing mutations in our Palestinian cohort.

Variant Classification	MEFV Variation	Number of Patients	Percentage (%)
Likely pathogenic	R761H	526	41.09
Pathogenic	V726A	241	18.82
Pathogenic	M694V	225	17.57
Pathogenic	M694I	73	5.70
Likely pathogenic	M680I	66	5.15
VUS	A744S	45	3.51
Likely pathogenic	K695R	39	3.04
VUS	P369S	35	2.73
Likely pathogenic	F479L	16	1.25
Likely pathogenic	I692del	14	1.09
	Total	1280	100.0

When considering the *MEFV* gene variants classified according to their pathogenicity (Pathogenic, Likely pathogenic, and VUS), we found that the most prevalent category is "Pathogenic" with a frequency of 42.1% of the tested variants followed by "Likely pathogenic" with 51.6%, and the category "VUS" with 6.2% (**Figure 6**).

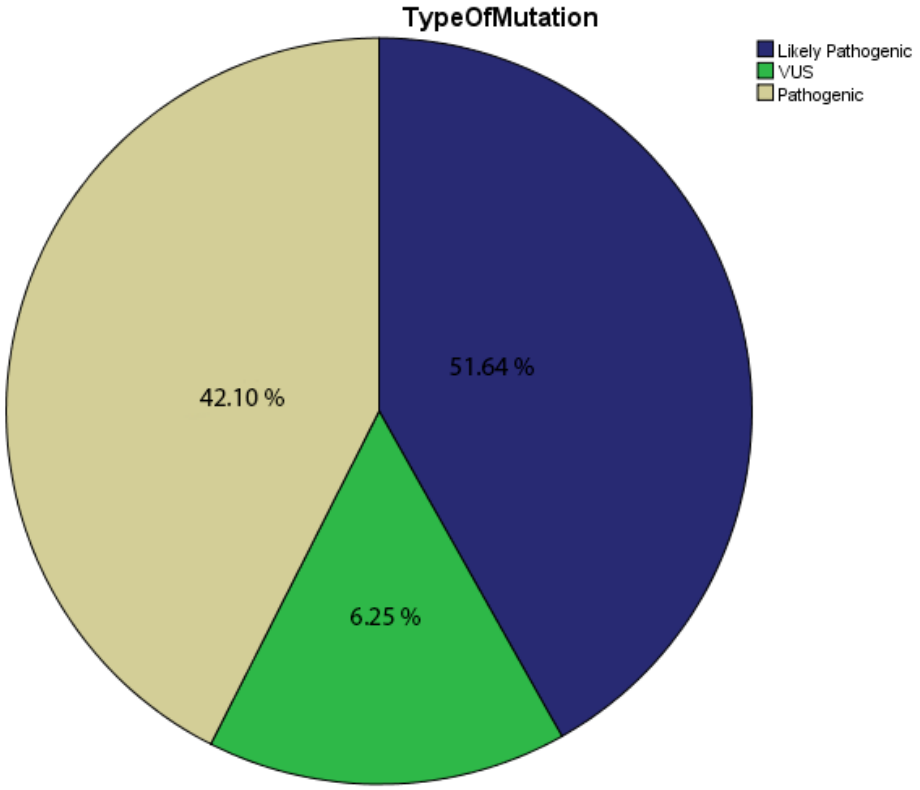

Figure 6: Distribution of the *MEFV* mutations according to their pathogenicity (pathogenic, likely pathogenic and VUS).

3.2 Selected patients phenotypic features

From 1280 patient we could reach to seven families, from them, two patients with known negative or unaffected *MEFV* mutations were chosen from a pool of seven families, and after taking into account the patients' phenotypic characteristics (see table 8), we sequenced the whole exome to determine whether they had a novel gene mutation.

Table 8: Phenotypic features of the patients.

Clinical Feature	N Total = 7	Percentage
Requiring bed rest	6/7	85.71 %
Continuation of activity	1/7	14.28 %
Symptom free intervals	7/7	100 %
Spontaneous remission	6/7	85.71 %
Temperature changes	1/7	14.28 %
Psychological Stress	1/7	14.28 %
Fever	6/7	85.71 %
Sometimes Isolated	4/7	57.14 %
Generalized Abdominal Pain	5/7	71.42 %
Localized Abdominal Pain	2/7	28.57 %
Diarrhea	4/7	57.14 %
Constipation	2/7	28.57 %
Vomiting	5/7	71.42 %
Joint Pain	6/7	85.71 %
Arthritis Monoarticular	1/7	14.28 %
Lower Limb Pain	3/7	42.85 %
Myalgia	3/7	42.85 %
Thoracic Pain	1/7	14.28 %
Conjunctivitis	1/7	14.28 %
Headache	5/7	71.42 %
Adenopathy	1/7	14.28 %
Splenomegaly	1/7	14.28 %

When we looked at their pedigree, we saw that both the father and his son were impacted, as shown in **Figure 7**, thus we chose these patients since we noticed that they had several affected individuals.

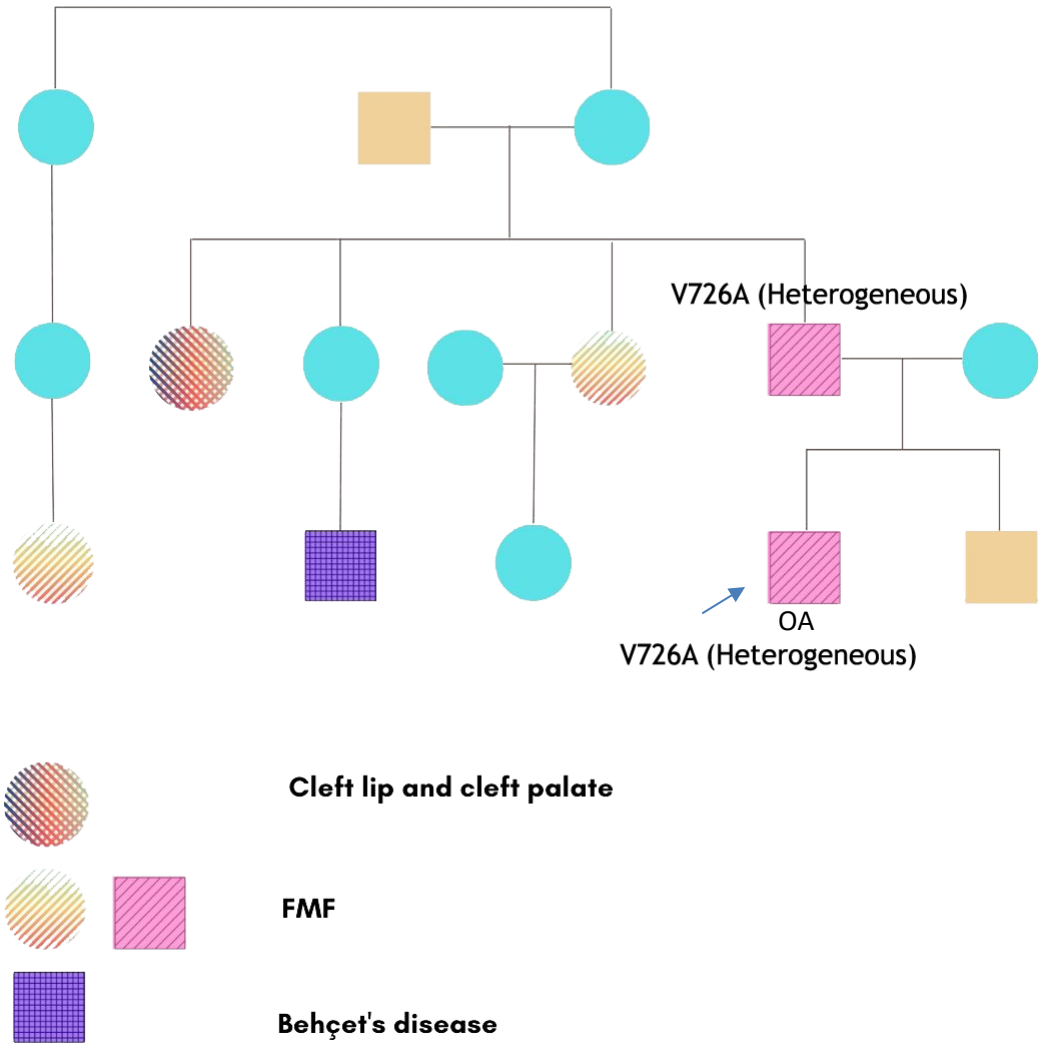

Figure 7: Pedigree for the selected family (father and his sun effected). The proband patient (OA) is indicated an arrow.

From the other side, the family had a history of Behcet disease and hereditary recurrent fever. Furthermore, we took the clinical feature for the son at the age of onset of his crisis of 1 year, age at diagnosis 4.5 years, frequency of crisis before treatment 1 per month or more, patient already had 3 crises, duration of crises 1 week also patient requiring bed rest, symptom free intervals too and he had spontaneous remission.

Patient has a fever, widespread abdominal discomfort, diarrhea, vomiting, arthropathy, monoarticular arthritis, pain in the lower limbs, myalgia, and headache. In the lab test, the ESR was 46 millimeters during the first hour of the crisis and 22 millimeters between crises. The CRP was zero between crises and 195 mg/L during the crisis see **table 9**.

Table 9: Phenotypic features of the selected patient.

Clinical Feature	Yes/No
Requiring bed rest	Yes
Continuation of activity	No
Symptom free intervals	Yes
Spontaneous remission	Yes
Temperature changes	No
Psychological Stress	No
Fever	Yes
Sometimes Isolated	Yes
Generalized Abdominal Pain	Yes
Localized Abdominal Pain	No
Diarrhea	Yes
Constipation	No
Vomiting	Yes
Joint Pain	Yes
Arthritis Monoarticular	Yes
Lower Limb Pain	Yes
Myalgia	Yes
Thoracic Pain	No
Conjunctivitis	No
Headache	Yes
Adenopathy	No
Splenomegaly	No

3.3 Whole Exome Sequencing (WES) Analysis Based on an Autosomal Dominant Mode of Inheritance

Given that the presence of a heterozygous *MEFV* mutation (p.V726A) alone does not fully explain the FMF phenotype within this family in addition to the lack of phenotype-genotype family segregation for this mutation has prompted us to further investigate the molecular basis of the FMF-like disease in this family. To this end, we have performed WES for both the proband patient (OA) and his affected father.

Our primary objective was to identify potential genes that may contribute to the FMF-like phenotype within this family. Thus, we initiated our investigation by applying a filtering strategy, grounded in the autosomal dominant mode of inheritance, which is the most possible pattern based on the family's pedigree analysis. This comprehensive filtering approach comprised the following steps:

1. **Initial Filtering Steps:** We began by excluding variants with a read depth below 5, along with those presenting an allele frequency (AF) exceeding 0.00001 in databases like gnomAD or our in-house Palestinian population database at the IAH. Additionally, we eliminated variants situated in non-coding regions and synonymous variants that do not affect splicing.

2. **Autosomal Dominant Criteria:** In line with the presumed autosomal dominant inheritance pattern, we retained solely heterozygous variants shared between the proband son and his affected father. Following this filtering approach, we concluded a **short list of 34 variants**, comprising 23 missense, 7 frameshift, 2 in-frame, 1 variant with unknown effects, and 1 non-sense variant (Table 10).

3. **Manual Assessment:** Subsequently, we conducted a thorough manual review of these shortlisted variants, guided by specific criteria:
    - **AF in Diverse Databases:** To enhance the accuracy of our findings, we manually checked the AF of each shortlisted variant across a wide array of public databases beyond gnomAD and the in-house Palestinian population database. These encompassed Middle Eastern populations (GME Variome), 4.7KJPN, ExAC, Turkish Variome, 1000 Genomes Project, Iranome, and Franklin Community Frequency, which was accessed through Genoox's Franklin platform (https://franklin.genoox.com). As a result, variants within the genes *ZNF862*, *FAM20C*, *HRCT1*, *KRT4*, and *TTC6* were excluded from further analysis due to their relatively high prevalence in these databases. Moreover, variants initially absent from gnomAD, such as those within *ATP6* and *LOC100129307*, were subsequently revealed to possess an AF of 30% and 0.20%, respectively, prompting their exclusion. In adherence to the rarity anticipated in autosomal dominant disorders, we retained 25 ultra-rare variants for further scrutiny (Table 10).

- **Tissue-Specific Gene Expression:** Another significant criterion involved the prioritization of genes predominantly expressed in cells or tissues associated with inflammation and innate immunity, such as the blood, bone marrow, and lymphatic system. This criterion led us to the identification of 6 ultra-rare variants within genes expressed in the blood and/or bone marrow (*COL3A1*, *ANKRD36C*, *GCLC*, *CTSD*, *WNK1*, and *PCDH17*), which warranted further consideration (Table 10).

- **Aggregated Prediction Classification for Missense Variants:** this aggregated prediction classification tool, which is available on Franklin website, relies on the ensemble methods such as SIFT, REVEL and MetaLR, to assign different weights to the different *in-silico* tools. Our aim was to rank and prioritize missense variants based on their predicted effects on protein structure and function. Consequently, among the 6 ultra-rare variants situated within genes expressed in the blood and/or bone marrow, variants within the GCLC and CTSD genes were predicted to be benign, leading to their exclusion from further assessment.

Collectively, this analysis has enabled us to refine our focus on a smaller set of just four variants within the *COL3A1*, *ANKRD36C*, *WNK1*, and *PCDH17* genes that potentially may contribute to the FMF-like phenotype within this family. Consequently, these selected variants have been considered for further comprehensive *in-silico* assessment and meticulous scrutiny.

In-Depth In-Silico Analysis and Literature Review of the Best Candidate Genes

Having identified the potential candidate genes through our meticulous filtering and manual assessment, we then performed a comprehensive *in-silico* analysis and literature review to gain a deeper understanding of their functional implications and potential contributions to the FMF-like phenotype within this family.

1. COL3A1, c.4340A>G (p.Tyr1447Cys)

The c.4340A>G (p.Tyr1447Cys) missense variant is located within the Collagen Type III Alpha 1 Chain gene (*COL3A1*), which encodes a critical component of the extracellular matrix and is

involved in the formation of collagen fibers. Collagen type III is particularly abundant in blood vessels, skin, and internal organs, contributing to their structural integrity.

The variant results in the substitution of a tyrosine (Tyr) residue with a cysteine (Cys) residue at position 1447. Tyrosine and cysteine have different chemical properties, potentially leading to changes in protein structure, stability, and interactions. The variant's aggregated prediction classification is "Uncertain." This classification implies that existing computational tools and databases do not strongly predict whether this variant is pathogenic or benign.

COL3A1 is known to be involved in collagen fibril formation, contributing to tissue strength and elasticity. Given that collagen is a crucial component of skin, blood vessels, and other tissues, a variant in COL3A1 could impact tissue integrity, repair, and inflammation. Variants in *COL3A1* are associated with Ehlers-Danlos syndrome, vascular type. This disorder affects connective tissues and can lead to vascular fragility, skin hyperextensibility, and joint hypermobility. While the phenotype is not identical to FMF, the involvement of collagen and connective tissue-related pathways suggests a potential link.

2. ANKRD36C, c.5629dup (p.Leu1877fs), c.5627_5628insA (p.Leu1877fs), andc.5623_5624del (p.His1875fs)

The *ANKRD36C* gene encodes a protein with ankyrin repeat domains that mediate protein-protein interactions involved in cellular processes. Among the variants, the frameshift mutations c.5629dup (p.Leu1877fs), c.5627_5628insA (p.Leu1877fs), and c.5623_5624del (p.His1875fs) likely result in truncated proteins with altered or disrupted function. ANKRD36C's roles in protein-protein interactions, transcriptional regulation, and cytoskeleton organization are pivotal in immune response modulation [113], making it a compelling candidate to investigate further for its potential impact on the FMF-like phenotype.

3. WNK1, c.2229dup (p.Thr744fs)

WNK1 encodes a kinase involved in ion transport and regulation of blood pressure. Variants within WNK1 might influence intracellular signaling pathways associated with immune responses and inflammation [114]. The c.2229dup (p.Thr744fs) variant results in a frameshift that could yield a

truncated or non-functional protein. WNK1's roles in immune cell function and inflammation modulation underscore its significance as a candidate gene [115].

4. PCDH17, c.2738C>G (p.Ser913Cys)

PCDH17 belongs to the cadherin superfamily, which is vital for cell adhesion and tissue development. The missense variant c.2738C>G (p.Ser913Cys) could impact protein folding and cell adhesion properties, potentially affecting immune cell interactions and signaling [116].

3.4 WES Analysis Based on an Autosomal Recessive Mode of Inheritance

Although autosomal dominant inheritance is the most likely pattern for the OA family, other inheritance patterns cannot be excluded and therefore, we also analyzed the WES for the autosomal recessive inheritance pattern. The following processes made up this thorough filtering method (**homozygous variants**):

1. Initial Filtering Steps: We began by excluding variants with a read depth below 5, along with those presenting an allele frequency (AF) exceeding 0.01 (1%) in databases like gnomAD or our in-house Palestinian population database at the IAH. Additionally, we eliminated variants situated in non-coding regions and synonymous variants that do not affect splicing.

2. **Autosomal Recessive Criteria:** In line with the presumed autosomal recessive inheritance pattern, we retained solely homozygous variants shared between the proband son and his affected father. Following this filtering approach, we concluded a short list of 12 variants, comprising 8 missense, 1 frameshift, 1 in-frame and 2 splicing variants. (Table 11).

3. Manual Assessment: Subsequently, we conducted a thorough manual review of these shortlisted variants, guided by specific criteria:

- AF in Diverse Databases: We confirmed the AF of each nominated variant across a wide range of public datasets outside of gnomAD and the internal Palestinian population database to improve the accuracy of our findings. Middle Eastern populations (GME Variome), 4.7KJPN, ExAC, Turkish Variome, 1000 Genomes Project, Iranome, and Franklin Community Frequency were included in this (which could be accessed via

Genoox's Franklin platform, available at https://franklin.genoox.com). As a result, variants within the genes MT-ND5, C1ORF109, ANKRD36B, LNP1, LRRC37A, KIR2DL4, and XKR3 genes that potentially may contribute to the FMF-like phenotype within this family (Table 11).

- Tissue-Specific Gene Expression: Another important criterion involves prioritizing genes expressed primarily in cells or tissues involved in inflammation and innate immunity, such as the blood, bone marrow, and lymphatic system. This criterion enabled us to identify one extremely rare variant in genes expressed in blood and/or bone marrow (DNAJB11), deserves closer consideration (Table 11).

In-Depth In-Silico Analysis and Literature Review of the Best Candidate Genes:

After carefully selecting the potential candidate genes and evaluating them manually, we conducted a thorough in-silico analysis and literature review to better understand their potential functional implications and contributions to the FMF-like phenotype in this family.

1. MT-ND5, c.514A>G (p. Ile172Val)

MT-ND5 Benign in ClinVar, Mode of inheritance in the family is not compatible with mitochondrial disease, function of the gene MT-ND5 product is a subunit of the respiratory chain Complex I that is supposed to belong to the minimal assembly of core proteins required to catalyze NADH dehydrogenation and electron transfer to ubiquinone (coenzyme Q10). Site of gene expression Cardiomyocytes - Muscle contraction (mainly). Non-specific - Cellular respiration (mainly).

2. C1ORF109, c.-43C>T

C1ORF109 Promotes the release of the shuttling protein RSL24D1/RLP24 from the pre-ribosomal particles, which is a step in the cytoplasmic maturation of pre-60S ribosomal particles. It works with SPATA5, SPATA5L1, and CINP 2. Site of gene expression Squamous epithelial cells - Keratinization (mainly).

3. ANKRD36B, c.3178A>G (p. Lys1060Glu), c.3149G>T (p. Arg1050Leu),

c.3127C>G (p. Arg1043Gly), c.3125A>G (p. Tyr1042Cys), c.3065G>A (p. Arg1022His)

The ANKRD36B gene works On Cell differentiation, Gene regulation expression, Protein- protein interaction from the other hand the site of gene expression Neuronal – Transcription.

Non-specific - Receptor signaling (mainly).

4.LNP1,exon3:c.194_195insTCCTAGAAGGCATTCTCATGAGGACCAGGAATTCCGATGCCGATCGTCTGACCGTCT (p.S80_H81insSDRLPRRHSHEDQEFRCRS)

The LNP1 is involved in the mTOR cell signaling pathway and contributes to the creation and maintenance of the tubular endoplasmic reticulum network, which is primarily where genes are expressed in neurons, the retina, the testis, spermatocytes, and spermatids.

5.LRRC37A,c.4133T>C(p.Leu1378Pro)

ADP-ribosyl transferase, the catalytic subunit of the cholera toxin, is activated allosterically by LRRC37A. oligodendrocytes and the testis are the primary sites of gene expression.

6. KIR2DL4, c.810dup(p.Met271AsnfsTer108)

The gene's function affects both the amount of membrane expression and the activation of cytotoxic activity. It has been observed in homozygous state in the other two persons in the IAH exome database: Turkish Vairone (53.7212%) and Mexican DB (16.2839%). NK-cells and T-cells are enriched at the spleen's location of gene expression.

7.XKR3,c.1324C>A(p.His442Asn)

Observed in homozygous state in other 5 individuals in the IAH exome database , which is a putative membrane transporter. location of gene expression testis and cell type enriched (Early spermatids).

Excluded homozygous variants:

1. **DNAJB11**, **c.1012+13_1012+14insTTGTGTGTGTGTGT.**

Splicing Variation Type, Likely Benign in ClinVar Common variant in 4.7KJPN (59.72%), The DNAJB11 gene encodes a soluble glycoprotein of the endoplasmic reticulum (ER) that acts as a cofactor of GRP78 (HSPA5; 138120), a heat shock protein chaperone required for the proper folding, assembly, trafficking, and degradation of proteins, disorder related to DNAJB11

Polycystic kidney disease 6 with or without polycystic liver disease (AD). Most importantly, the AF is very high in 4.7KJPN and therefore, based on our criteria (AF in diverse databases), this variant was excluded. In conclusion, this variant should not be listed as a potential candidate.

GENOMIC COORDINATE	GENE	VARIANT COORDINATES	AMINO ACID CHANGE	VARIATION TYPE	AGGREGATED PREDICTION CLASSIFICATION	AGGREGATED PREDICTION*	GNOMAD ALLELE FREQUENCY	RELATED DISORDER (OMIM) AND MODE OF INHERITANCE	FUNCTION OF THE GENE	SITE OF GENE EXPRESSION	REMARKS
chrM:8701 A>G	ATP6	ENST00000361899: c.175A>G	p.Ala59Thr	MS	Uncertain	0.36	30.32%	N/A	Provides information for making a protein that is essential for normal mitochondrial function.	Localized to the Nucleoli In addition localized to the Nucleoplasm,Cytoplasmic expression in all tissues.	Detected in blood by mass spectrometry
chr1-179078403 TG>T	ABL2	NM_007314.4: c.1998del	p.Lys667fs	FS	N/A	N/A	0	N/A	Regulates an HSF1-dependent transcriptional program required for lung adenocarcinoma brain metastasis.	Localized to the Nucleoplasm In addition localized to the Vesicles,	N/A in blood
chr2-189876439 A>G	COL3A1	NM_000090.4: c.4340A>G	p.Tyr1447Cys	MS	Uncertain	0.55	0	Ehlers-Danlos syndrome, vascular type (AD).(MIM: 130050). Polymicrogyria with or without vascular-type EDS (AR) .(MIM: 618343).	Provides instructions for making type III collagen.	Tissue enhanced (cervix, gallbladder, placenta, smooth muscle),Connective tissue - ECM organization	Detected in blood by mass spectrometry
chr2-214181998 G>A	SPAG16	NM_024532.5: c.454G>A	p.Asp152Asn	MS	Uncertain	0.44	0	N/A	Influences MMP-3 regulation and protects against joint destruction in autoantibody-positive rheumatoid arthritis.	Expressed in the cytoplasm in most cell types,Localized to the Plasma membrane, Cytosol	N/A in blood

chr2-96521477 A>AG	ANKRD 36C	NM_001310154.3: c.5629dup	p.Leu1877fs	FS	N/A	N/A	0	N/A	are modular protein domains that mediate protein-protein interactions and are involved in a wide range of biological processes, including transcriptional regulation, signal transduction, and cytoskeleton organization	Tissue enhanced (bone marrow)	Exome Sequencing Identifies Abnormalities in Glycosylation and ANKRD36C in Patients with Immune-Mediated Thrombotic Thrombocytopenic Purpura (PMID: 33184803)
chr2-96521479 A>AT	ANKRD 36C	NM_001310154.3: c.5627_5628insA	p.Leu1877fs	FS	N/A	N/A	0	N/A	Modular protein domains that mediate protein-protein interactions and are involved in a wide range of biological processes, including transcriptional regulation, signal transduction, and cytoskeleton organization	Tissue enhanced (bone marrow)	
chr2-96521482 ATG>A	ANKRD 36C	NM_001310154.3: c.5623_5624del	p.His1875fs	FS	N/A	N/A	0	N/A	Modular protein domains that mediate protein-protein interactions and are involved in a wide range of biological processes, including transcriptional regulation, signal transduction, and	Tissue enhanced (bone marrow)	

									cytoskeleton organization		
chr2-98128069 ATG>A	ANKRD 36B	NM_025190.4: c.3250_3251del	p.His1084fs	FS	N/A	N/A	0	N/A	Modular protein domains that mediate protein-protein interactions and are involved in a wide range of biological processes, including transcriptional regulation, signal transduction, and cytoskeleton organization	Neuronal - Transcription (mainly)	N/A in blood
chr3-141289870 G>A	RASA2	NM_006506.5: c.980G>A	p.Gly327Asp	MS	Benign	0.1	0	N/A	Target that boosts T cell proliferation and in vitro cancer cell-killing capacity when it is knocked out	Neurons - Mixed function (mainly)	N/A in blood
chr3-47163935 C>A	SETD2	NM_014159.7: c.2191G>T	p.Asp731Tyr	MS	Uncertain	0.4	0	Intellectual developmental disorder, autosomal dominant 70 (AD). (MIM:620157). Luscan-Lumish syndrome (AD) . (MIM:616831). Rabin-Pappas syndrome (AD) . (MIM:620155).	Initiation, progression, prognosis and treatment of solid tumors.	General nuclear expression.	N/A in blood
chr3-49336052 G>T	USP4	NM_003363.4: c.1529C>A	p.Pro510Gln	MS	Uncertain	0.62	0	N/A	To remove monoubiquitination and polyubiquitinati	Cytoplasmic expression in most tissues,Bone	N/A in blood

									on, including K48 and K63 conjugated ubiquitin chains, from target proteins	marrow - Cell proliferation (mainly),Localized to the Plasma membrane, Cytosol	
chr3-9911865 G>A	CIDEC	NM_001321142.2: c.349C>T	p.Gln117*	NS	N/A	N/A	0	? Lipodystrophy, familial partial, type 5(AR).(MIM:615238).	Unilocular lipid droplet formation and optimal energy storage in human	Cytoplasmic expression in most tissues with extracellular positivity, including adipocytes.	N/A in blood MutationTaster: Deleterious (1), SpliceAI Benign (0), fitCons Benign (0.49), DANN Deleterious (1), BayesDel Deleterious (Strong) (0.6)
chr6-106536283 C>G	PRDM1	NM_001198.4: c.250C>G	p.Pro84Ala	MS	Uncertain	0.67	0	N/A	Directly regulates both HGAL and LMO2--overexpression of PRDM1 down-regulates HGAL and LMO2; PRDM1 directly binds to promoters of both HGAL and LMO2 and represses genetic transcription. PRDM1 is implicated in the etiology of therapy-induced second malignancies after Hodgkin's lymphoma.	General nuclear and cytoplasmic expression.	N/A in blood
chr6-41617471 A>G	MDFI	NM_005586.4: c.374A>G	p.Lys125Arg	MS	Benign	0.06	0.0032%	N/A	Negatively regulating their transcriptional activities.	Cytoplasmic expression in several tissues,Epithelium - Extracellular	N/A in blood

										exosomes (mainly)	
chr6-53363720 T>C	GCLC	NM_001498.4: c.1748A>G	p.Asn583Ser	MS	Benign	0.07	0	Hemolytic anemia due to gamma-glutamylcysteine synthetase deficiency (AR) . (MIM: 230450). {Myocardial infarction, susceptibility to. (MIM: 608446).	Encodes the catalytic subunit of the enzyme glutamate cysteine ligase	Cytoplasmic expression at variable levels,Liver - Metabolism (mainly)	Detected in blood by mass spectrometry
chr7-149558860 A>G	ZNF862	NM_001099220.3: c.2611A>G	p.Thr871Ala	MS	Benign	0.03	0	N/A	Involved in transcriptional regulation.	Cytoplasmic and membranous expression in several different tissue types,Localized to the Mitochondria	Middle Eastern populations (GME Variome): 3 Alleles of 1,983 N/A in blood
chr7-286468 G>GGACAGGT GAGCCCTTCCT TCCTCCCTCCA TCCGC	FAM20C	NM_020223.4: c.986_956+31insAC AGGTGAGCCCTT CCTTCCTCCCTC CATCCG	Unknown	EU	N/A	N/A	0	Raine syndrome (AR) . (MIM: 259775).	Regulates Bone Resorption and Breast Cancer Bone Metastasis through Osteopontin and BMP4.	Localized to the Golgi apparatus In addition localized to the Nucleoplasm,Serous glandular cells - Salivary secretion (mainly)	4.7KJPN: 34.52% 3,294 Alleles of 3,294 ExAC: 24.4306% 2,832 Alleles of 11,592 294 homozygote. Detected in blood by mass spectrometry Locally secreted
chr7-57188262 T>C	ZNF479	NM_001370129.1: c.860A>G	p.His287Arg	MS	Uncertain	0.35	0	N/A	May be involved in transcriptional regulation.	Early spermatids - Spermatogenesis (mainly)	N/A in blood
chr8-10470428 C>A	RP1L1	NM_178857.6: c.1180G>T	p.Val394Phe	MS	Benign	0.07	0	Occult macular dystrophy (AD) .(MIM: 613587)	The major cause of autosomal dominant occult macular	Photoreceptor cells - Visual perception (mainly)	N/A in blood

								Retinitis pigmentosa 88 (AR) . (MIM: 618826)	dystrophy (OCMD)		
chr9-35906598 ACCC>A	HRCT1	NM_001039792.2: c.317_319del	p.Pro106del	IF deletion	N/A	N/A	0	N/A	Promotes tumor metastasis and the growth of gastric cancer by activating the ERBB2-MAPK pathway.	Enterocytes - Absorption (mainly)	In-frame deletions in a repetitive region with no known function. ExAC: 1.6419%, 331 Alleles of 20,160, 3 homozygote. Turkish Variome: 1.295%, 18 Alleles of 1,390, 0 homozygote. 4.7KJPN: 0.17% N/A in blood
chr10-25314304 A>G	THNSL1	NM_024838.5: c.2152A>G	p.Met718Val	MS	Benign	0.05	0	N/A	Functional associations with biological entities	Early spermatids - Spermatogenesis (mainly)	N/A in blood
chr10-61029768 C>T	FAM13C	NM_198215.4: c.694G>A	p.Gly232Ser	MS	Uncertain	0.33	0	N/A	is an independent prognostic marker in prostate cancer.	Oligodendrocytes - Unknown function (mainly)	N/A in blood
chr11-1785062 C>A	CTSD	NM_001909.5: c.28G>T	p.Ala10Ser	MS	Benign	0.06	0	Ceroid lipofuscinosis, neuronal, 10 (MIM: 610127) (AR)	Provides instructions for making an enzyme called cathepsin D.	Macrophages - Innate immune response (mainly)	Submitted in ClinVar by Invitae: This variant is not present in population databases (gnomAD no frequency). This variant has not been reported in the literature in individuals affected with CTSD-related conditions. ClinVar contains an entry for this variant (Variation

											ID: 957664). In summary, it has been classified as a Variant of Uncertain Significance. Detected in blood by mass spectrometry and proximity extension assay Upregulated in disease :Acute myeloid leukemia, Lung cancer
chr11-60264949 GC>G	MS4A12	NM_017716.3: c.159del	p.I54Sfs*17	FS	N/A	N/A	0	N/A	Promoting malignant cell processes.	Enterocytes - Absorption (mainly)	N/A in blood
chr12-53207583 C>CCACCAAA GCCACCAGTG CCGAAACCAG CTCCGAAGCC GCCGG	KRT4	NM_002272.4: 259_260insCCGGC GGCTTCGGAGCT GGTTTCGGCACT GGTGGCTTTGGT G	p.Gly86_Gly 87insAlaGly GlyPheGlyAl aGlyPheGlyT hrGlyGlyPhe Gly	IF insertion	N/A	N/A	0	White sponge nevus 1 (AD) (MIM: 193900).	Provides instructions for making a protein called keratin 4.	Suprabasal keratinocytes - Cornification (mainly)	Benign & likely benign in ClinVar; the variant was found in 5 of our samples uploaded on Franklin; common in 1000 genome & 4.7KJPN Detected in blood by mass spectrometry
chr12-974361 A>AC	WNK1	NM_213655.5: c.2229dup	p.Thr744fs	FS	N/A	N/A	Failed?? (0)	Pseudohypoaldo steronism, type IIC (MIM: 614492) (AD); Neuropathy, hereditary sensory and autonomic, type II (MIM: 201300) (AR)	Provides instructions for making multiple versions (isoforms) of the WNK1 protein.	Adipocytes & Endothelial cells - Mixed function (mainly)	Submitted in ClinVar by Invitae: This variant occurs in an exon with poor sequence conservation and a high frequency of truncating variants in the general population. The effect of this variant on the WNK1 protein is uncertain. This variant is not present in population databases (ExAC

											no frequency). This variant has not been reported in the literature in individuals with WNK1-related conditions. It has been classified as a Variant of Uncertain Significance. Detected in blood by mass spectrometry
chr13-58240908 C>G	PCDH17	NM_001040429.3: c.2738C>G	p.Ser913Cys	MS	Uncertain	0.39	0	N/A	Tumor suppressor inhibiting Wnt/beta-catenin signaling and metastasis in breast cancer	Adipocytes & Endothelial cells - Mixed function (mainly)	Detected in blood by mass spectrometry andproximity extension assay Upregulated in disease:Acute myeloid leukemia, Myeloma, Chronic lymphocytic leukemia
chr13-78236514 C>T	LOC100 129307	NM_001310140.1: c.248G>A	p.Arg83Gln	MS	N/A	N/A	0.20%	N/A	Encodes a potent cyclin-dependent kinase inhibitor.	N/A	N/A in blood
chr14-38222349 G>A	TTC6	NM_001310135.3: c.3223G>A	p.Gly1075Arg	MS	Deleterious	0.71	0.00%	N/A	No function available	Prostatic glandular cells - Transcription (mainly)	Iranome: 0.1877% (3 Alleles of 1,598); Franklin Community Frequency: 12 cases with different associated phenotypes N/A in blood
chr14-50154884 G>A	POLE2	NM_002692.4: c.38C>T	p.Ala13Val	MS	Benign	0.1	0	N/A	facilitates the malignant phenotypes of glioblastoma through promoting	Nuclear expression in several tissues, including lymphoid tissues.	N/A in blood

									AURKA-mediated stabilization of FOXM1.		
chr15-48441425 C>G	MYEF2	NM_016132.5: c.1522G>C	p.Gly508Arg	MS	Uncertain	0.34	0	N/A	Inhibits the transcription of the myelin basic protein gene (MBP) by binding to the proximal MB1 element 5′-TTGTCC-3′ of the MBP promoter	Ciliated cells - Cilium assembly (mainly)	N/A in blood
chr15-90127621 G>T	TICRR	NM_152259.4: c.1039G>T	p.Ala347Ser	MS	Benign	0.02	0	N/A	Normal DNA replication and S/M checkpoint function.	Tissue enhanced (bone marrow, esophagus, lymphoid tissue)	N/A in blood
chr20-58348405 C>G	PHACTR 3	NM_080672.5: c.823C>G	p.Pro275Ala	MS	Benign	0.14	0	N/A	Functions as a regulatory subunit of protein phosphatase-1.	Tissue enriched (brain)	N/A in blood

Table 10. Lists the candidate genes (Heterozygous variants) with its chromosomal location and specific location of the variant detected in our study in addition to expression data of this gene. Aggregated Prediction was taken from Franklin website which relies on the ensemble methods such as REVEL and MetaLR which assigns different weights to the different in-silico tools. This might cause scenarios where the aggregated prediction can be 'benign' while several tools would give the variants 'deleterious' score, or vice-versa. Aggregated prediction score ranges: Benign supporting 0-0.15, Pathogenic Supporting 0.7-0.8, Pathogenic Moderate 0.8-0.9, Pathogenic Strong 0.9-1.0. Abbreviations: MS: Missense, FS: Frameshift, EU: Unknown effect, NS: Nonsense, IF: In-frame, AR: Autosomal recessive, AD: Autosomal dominant.

Genomic Position	Gene	VARIANT COORDINATES	AMINO ACID CHANGE	Variation Type	Aggregated Prediction Classification	Aggregated Prediction*	gnomAD Allele frequency	Remarks	RELATED DISORDER (OMIM) AND MODE OF INHERITANCE	Function of the gene	Site of gene expression
chrM-12850 A>G	MT-ND5	ENST00000361567.2: c.514A>G	p.Ile172Val	MS	Uncertain	0.39	0.02%	Benign in ClinVar, Mode of inhertince in the family is not compatible with mitochondrial disease	Leber optic atrophy (Mi). (MIM: 535000). Leigh syndrome due to mitochondrial complex I deficiency MELAS syndrome (Mi). MIM:(540000). MERRF syndrome (Mi). MIM: 545000. Parkinson disease 6, modifier of.	The MT-ND5 product is a subunit of the respiratory chain Complex I that is supposed to belong to the minimal assembly of core proteins required to catalyze NADH dehydrogenation and electron transfer to ubiquinone (coenzyme Q10).	Cardiomyocytes - Muscle contraction (mainly). Non-specific - Cellular respiration (mainly). N/A IN BLOOD
chr1-38157704 G>A	C1ORF109	NM_001350757.2: c.-43C>T		Splicing	N/A	N/A	0.82%	1.423 % Turkish Variome	N/A		Squamous epithelial cells - Keratinization (mainly). N/A IN BLOOD
chr2-98128143 T>C	ANKRD36B	NM_025190.4: c.3178A>G	p.Lys1060Glu	MS	N/A	N/A	0		N/A	Cell differentiation,Gene regulation expreesion,Protein protein interaction	Neuronal – Transcription. Non-specific - Receptor signaling (mainly). N/A IN BLOOD
chr2-98128172 C>A	ANKRD36B	NM_025190.4:c.3149G>T	p.Arg1050Leu	MS	N/A	N/A	0.00%	Observed homozygous in 1 individuals gnomAD homozygous count : 1	N/A	Cell differentiation,Gene regulation expreesion,Protein protein interaction	Neuronal – Transcription. Non-specific - Receptor signaling (mainly). N/A IN BLOOD

chr2-98128194 G>C	ANKRD36B	NM_025190.4: c.3127C>G	p.Arg1043Gly	MS	N/A	N/A	0		N/A	Cell differentiation,Gene regulation expreesion,Protein protein interaction	Neuronal – Transcription. Non-specific - Receptor signaling (mainly). N/A IN BLOOD
chr2-98128196 T>C	ANKRD36B	NM_025190.4: c.3125A>G	p.Tyr1042Cys	MS	N/A	N/A	0		N/A	Cell differentiation,Gene regulation expreesion,Protein protein interaction	Neuronal – Transcription. Non-specific - Receptor signaling (mainly). N/A IN BLOOD
chr2-98128256 C>T	ANKRD36B	NM_025190.4: c.3065G>A	p.Arg1022His	MS	N/A	N/A	N/A (Failed)		N/A	Cell differentiation,Gene regulation expreesion,Protein protein interaction	Neuronal – Transcription. Non-specific - Receptor signaling (mainly). N/A IN BLOOD
chr3:100,170,601-100,170,602	LNP1	NM_001085451:exon3:c.194_195insTCCTAGAAGGCATTCTCATGAGGACCAGGAATTCCGATGCCGATCGTCTGACCGTCT	p.S80_H81insSDRLPRRHSHEDQEFRCRS	IF Insertion	N/A	N/A	Common Variant (Occurance= 7599)	Benign in ClinVar	Neurodevelopmental disorder with epilepsy and hypoplasia of the corpus callosum. (AR).MIM(618090).	Involved in mTOR cell signaling pathway.Plays a role in tubular endoplasmic reticulum network formation and maintenance	Neurons - Mixed function (mainly). Retina & Testis - Cilium (mainly). Spermatocytes & Spermatids - Spermatid development (mainly). N/A IN BLOOD
chr3-186302391 A>ATTGTGTGTGTGTGT	DNAJB11	NM_016306.6: c.1012+13_1012+14insTTGTGTGTGTGTGT		Splicing	N/A	N/A	0	Likely Benign in ClinVar; Common variant in 4.7KJPN (59.72 %)	Polycystic kidney disease 6 with or without polycystic liver disease (AD). MIM:(618061)	The DNAJB11 gene encodes a soluble glycoprotein of the endoplasmic reticulum (ER) that acts as a cofactor of GRP78 (HSPA5; 138120), a heat shock protein chaperone required for the proper folding, assembly, trafficking, and degradation of proteins.	Non-specific - Protein processing (mainly). Non-specific - Metabolism (mainly). Plasma cells - Protein processing (mainly). Detected in blood by mass spectrometry.
chr17-44408776 T>C	LRRC37A	NM_014834.4:c.4133T>C	p.Leu1378Pro	MS	Benign	0.04	0.13%	gnomAD homozygous count : 43	N/A	as an allosteric activator of the cholera toxin catalytic subunit, an ADP-ribosyltransferase.	Testis - Transcription (mainly). Non-specific - Mixed function (mainly). Oligodendrocytes - Unknown function (mainly). N/A IN BLOOD.

chr19-55324674 C>CA	KIR2DL4	NM_001080772.2: c.810dup	p.Met271AsnfsTer108	FS	N/A	N/A	0	Observed in homzygous state in other 2 individuals in the IAH exome database; Common variant in Turkish Variome (53.7212%); Common variant in Mexican DB (16.2839%)	N/A	influences both levels of membrane expression and activation of cytotoxic function.	Spleen - Immune response (mainly). Group enriched (NK-cells, T-cells). N/A IN BLOOD.
chr22-17264565 G>T	XKR3	NM_001386955.1: c.1324C>A	p.His442Asn	MS	Benign	0.08	0	Observed in homzygous state in other 5 individuals in the IAH exome databa	N/A	which is a putative membrane transporter.	Testis - Spermatogenesis (mainly). Cell type enriched (Early spermatids). N/A IN BLOOD.

								se; Reported as Benign By UniProt VAR_053743; Common variant in 1000 Genomes (64.8163%)			

Table 11. Lists the candidate genes (Homozygous variants) with its chromosomal location and specific location of the variant detected in our study in addition to expression data of this gene. Aggregated Prediction was taken from Franklin website which relies on the ensemble methods such as REVEL and MetaLR which assigns different weights to the different in-silico tools. This might cause scenarios where the aggregated prediction can be 'benign' while several tools would give the variants 'deleterious' score, or vice-versa. Aggregated prediction score ranges: Benign supporting 0-0.15, Pathogenic Supporting 0.7-0.8, Pathogenic Moderate 0.8-0.9, Pathogenic Strong 0.9-1.0. Abbreviations: MS: Missense, FS: Frameshift, IF: In-frame, AR: Autosomal recessive, AD: Autosomal dominant.

Chapter 4

Discussion

FMF is a common hereditary disease classically known to affect people in the Mediterranean region [1]. It is inherited in an autosomal recessive fashion, and it usually is associated with a mutation in the *MEFV* gene, localized to the short arm of chromosome 16 [2, 117, 118].

Armenians, Sephardic Jews, Arabs, and Turks are particularly susceptible to FMF. However, it is not just limited to these ethnic groups. About 100,000–150,000 patients are thought to be affected globally[119]. The prevalence of FMF varies among different populations, in which it occurs in 1/250-1/1000 among Sphardic Jews, 1/73000 among Askhenazi Jews, 1/500 among Americans, 1/1000 among Turkish, and 1/2600 among Arab population [120].

In our study, 48.6% of our cohort were female, while 51.4% were male with no significant difference. In previous studies, *MEFV* mutations were distributed similarly in males and females, and the proportion of individuals without a detectable *MEFV* mutation was higher in female patients, but this finding was unrelated to any of the clinical observations [121].

Most of the cases of FMF are inherited as an autosomal recessive, although other phenotypes in heterozygous state have been reported in the literature [13-15]. Although sequence variants have been found throughout the gene [16], the undefined pathogenic ones: M680I, M694V, M694I and V726A found to be clustered within the exon 10 of the gene [17]. Moreover, one mutated *MEFV* allele could be only identified in about 30% of the patients with a clinical diagnosis of FMF. In our study,30.6% were carriers of a heterozygous mutation in the *MEFV* gene, 15.7% were carriers of a homozygous / compound heterozygous mutation in the *MEFV* gene and 53.7% were negative (no FMF-causing mutation was identified). This in contrast to other studies were about 10-20% of FMF patient's lack *MEFV* mutation [101, 122], and this variability in the mutation negative percentage could be attributed to different prevalence of FMF among different studies, and prevalence of FMF greatly varies among different populations. This also might be related to misdiagnosis and the number of tested mutations.

As FMF causes, the majority of studies to yet have exclusively looked at *MEFV* gene mutations. Almost all of the sequence variants in the *MEFV* gene are single-nucleotide changes, although the four most prevalent and clearly harmful ones—M680I, M694V, M694I, and V726A—are grouped inside exon 10 [17]. The sequence variant E148Q in exon 2's detrimental nature is debatable. An Iranian study of *MEFV* gene variant alleles showed that the most frequent variants were E148Q (18.3%), followed by P369S (3.1%), V726A (2.2%), A744S (1.3%), and F479L, M694V, and R761H (0.8%), and eventually K695R (0.4%), respectively[123]. On the other hand, a Turkish study showed that the most frequent mutation was R202Q, followed by M694V, E148Q, M680I, R761H, V726A and R354W [124]. In addition, a study in Azerbaijan showed that the most frequency *MEFV* gene variant allele was M694V followed by R761H, and V726A[125]. In our research, R761H mutation was found to be the most prevalent occurring in 41.09% of cases followed by V726A, M694V, M694I, and M680I, with respective allele frequencies of 18.82%, 17.57%, 5.70%, and 5.15%. This can be explained by different genomic imprints of different populations studied, and thus the difference in prevalence rates of the variable mutations. It was originally thought that this sequence variation was a disease-causing mutation with low penetrance and mild symptoms, but the absence of segregation of E148Q with the illness phenotype and its similar frequency in patients and controls revealed that it is actually a benign polymorphism [17], and thus it was excluded from our study.

Two interesting patients with multiple affected individuals were selected from seven family after screening 1280 patient upon selective criteria and pedigrees analysis in addition to having a fever, the patient also suffers from myalgia, headaches, diarrhea, vomiting, arthropathy, monoarticular arthritis, pain in the lower limbs, and a fever. In the laboratory experiment, the ESR ranged from 22 millimeters between crises to 46 millimeters during the first hour of the crisis. During the crisis, the CRP increased from 0 between crises to 195 mg/L.

As part of Whole Exome Sequencing (WES), heterozygous analysis has enabled us to refine our focus on a smaller set of just four variants within the COL3A1, ANKRD36C, WNK1, and PCDH17 genes that potentially may contribute to the FMF-like phenotype within a specific family. On the other hand, homozygous variant analysis showed that the genes MT-ND5, C1ORF109, ANKRD36B, LNP1, LRRC37A, KIR2DL4, and XKR3 potentially may contribute to the FMF-like phenotype within a specific family. Other studies showed that compound heterozygous variant have been confirmed, with a different variant of *MEFV* gene [126].

The aforementioned variants COL3A1, ANKRD36C, WNK1, and PCDH17, which represent heterozygous variant genes, have also been linked to other important structures or functions in the body. For instance, COL3A1 plays an important role in the formation of Collagen type III. On the other hand, ANKRD36C encodes ankyrin protein that is important for cellular processing, and pivotal in immune response modulation, making it an important candidate to investigate further for its potential impact on the FMF-like phenotype given it immunological response. Moreover, WNK1 is involved in ion transport and regulation of blood pressure as it encodes protein kinase. In addition, PCDH17 regular cell adhesion and tissue development function.

Meanwhile, homozygous variants, MT-ND5, C1ORF109, ANKRD36B, LNP1, LRRC37A, KIR2DL4, and XKR3, have also being linked to other functions. For instance, MT-ND5 plays a role in muscle contraction and cellular respiration. While C1ORF109 is involved in squamous keratinization. These findings suggest that different genes play a role in FMF-like disease, as well as playing a pivotal role in an important cellular function.

Several autoimmune and inflammatory conditions have been linked to FMF and MEFV gene mutations, such as IgA vasculitis, polyarteritis nodosa, and multiple sclerosis. Homozygosity for the M694V MEFV mutation can be an aggravating factor in the phenotypic picture of multiple sclerosis [2, 117, 118]. This could suggest an association between FMF contributing genes and other diseases.

In conclusion, our study determined the prevalence of the known *MEFV* mutations associated with FMF in our patients' cohort. This was accomplished by analyzing the genetic data of our clinically diagnosed FMF patients' cohort according to their *MEFV* genotype (negative, heterozygous, and homozygous). Moreover, we identified and characterized new genes involved in FMF. In addition, multiple variant genes have been linked to FMF disease, while playing a role in important cellular structure and function, suggestive their relation to other disease process which needs to be investigated more.

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العنوان: دراسة الأساس الجزيئي للمرض الشبيه بحمى البحر الأبيض المتوسط العائلية.

اعداد: ضياء الدين سامي محمد نور قمحية

اشراف: د. فواز عواد

ملخص

يعتبر مرض حمى البحر الابيض المتوسط احدى الامراض الوراثيه التي تسببها طفره جينيه متنحيه نادره ، يعاني الاشخاص المصابون بالمرض من التهاب المفاصل ، التهاب في الصدر نتيجه لوجود التهاب في تجويف الرئه ، يصيب المرض بشكل رئيسي الأشخاص المنتمين الى بيئه البحر الابيض المتوسط والشرق الاوسط.

تعتمد المضاعفات الصحيه للمرض على العلامات المصاحبه لها ، في بعض الحالات يمكن ان يتطور الداء النشواني نتيجه للمرض ، حيث يمكن ان تتراكم البروتينات الغير قابله للذوبان تدريجيا وتسبب تلف الاعضاء الرئيسيه نتيجه لهجمات الحمى المتكرره ،مثلا ذا كنت مصابا بالداء النشواني قد تفشل كليتك فجاه بسبب تلف الكبيبات ، والذي يمكن ان يؤدي الى الفشل الكلوي ،كما يمكن ان يكون سبب العقم عند النساء هي العدوى التي تحدث مع المرض حيث يمكن ان تدمر الاعضاء التناسليه وهذا يزيد من احتماليه العقم وصعوبة الحمل والتهاب المفاصل ،حيث ينتج الم المفاصل والكاحلين والوركين عن التهاب ناتج عن دوار البحر.

سبب حمى البحر الابيض المتوسط العائليه هي طفره جينيه تنتقل من الاباء الى الابناء ،حيث تسبب عدم قدرته الجسم على تنظيم الالتهاب ولهذا السبب غالبا ما يعاني الأفراد المصابون بهذه الحالة من نوبات من الحمى والألم. التهاب الجلد والأعضاء الداخلية والمفاصل. طفح جلدي أحمر ملتهب على الجسم ، وخاصة تحت الركبتين. الصداع والحمى. التهاب الرئة هو حالة شائعة يمكن أن تحدث نتيجة مجموعة متنوعة من الأسباب. الشائع حدوث ألم شديد في الصدر بسبب التهاب التامور. التهاب السحايا عدوى خطيرة للغاية تصيب السحايا ، وهي الأغطية الواقية للدماغ والنخاع الشوكي. يمكن أن تكون قاتلة إذا لم يتم علاجها بسرعة. التهاب المفاصل هو مرض مناعي ذاتي شائع يمكن أن يسبب الألم والالتهابات في المفاصل. يمكن أن تكون خطيرة للغاية ويمكن أن تتطلب جراحة أو دواء لمعالجتها.

كما يعتبرمرض حمى البحر الابيض المتوسط اضطراب وراثي التهابي ينتشر في المناطق المحيطة بحوض البحر الأبيض المتوسط ومناطق الشرق الأوسط ، بما في ذلك الأتراك والعرب والأرمن،حيث ان 90٪ من المصابين بحمى البحر الأبيض المتوسط تظهر عليهم الأعراض الأولى للاضطراب قبل بلوغهم سن العشرين. عادة ما يتم تشخيص المرض في مراحل الطفولة المبكرة،مع الاخذ بعين الاعتبار ان المرض يصيب الرجال والنساء على حد سواء ،وتعتبر مرضا وراثيا متنحيا. هذا يعني أن إصابة شخص ما به تتطلب منه أن يرث نسختين من الجين المسؤول عن العدوى، انتقال الجينات بين الوالدين مهم في تطور الأمراض الوراثية. إذا كان أحد الوالدين مصابا بالمرض ، فيجب أن يكون الآخر مصابا به أيضا أو يحمل جينات المرض.

التحليل الكامل للاكسوم الذي تم خلال هذا البحث اظهر وجود 4 متغيرات جينية (*COL3A1, ANKRD36C, WNK1,* and PCDH17) والتي قد من المحتمل ان تكون مسؤولة عن النمط الظاهري الذي يشبه حمى البحر الابيض المتوسط في المتغيرات متباينة الازدواج و متغيرات جينية اخرى (MT-ND5, C1ORF109, ANKRD36B, LNP1, LRRC37A, KIR2DL4, and XKR) في المتغيرات متماثلة الزايجوت.