

INTRODUCTION

1.1 Historical Notes

Familial Mediterranean Fever (FMF) was first reported at 1908 by description of a 16 years old Jewish girl with recurrent fever, abdominal pain and leucocytosis (*Janeway et al 1908*). FMF was described as a separate nosological entity by *Shepard Siegal in 1945*, he described a set of 10 cases under the name benign paroxysmal peritonitis. *In 1948, Reimann* reported his first three cases of recurrent peritonitis. Reimann was impressed by the periodicity of the symptoms of this and other diseases and therefore introduced the term "periodic disease". *In 1952, Mamou and Cattani* described the disease in North Africa, in Jews of Sephardic extraction. They were the first to mention the entity's familial occurrence and the lethal nephropathy that may affect the patients. *Heller and his group* established a detailed clinical description of the condition. This included the recessive inheritance of the disease, the arthritis of FMF and the nature of amyloid nephropathy. They suggested the name "Familial Mediterranean Fever" which was later accepted by the international community (*Heller et al 1961*). *Ehrenfeld and his group* published a series of 55 FMF patients, stressing the fact that many of these patients did not develop amyloidosis (*Ehrenfeld et al 1961*). *In 1972, Goldfinger* reported the suppressive effect of colchicine on the attacks in five patients. Since then, colchicine has become the undisputed treatment for this disease. *Barakat et al (1984)* referred to FMF as recurrent hereditary polyserositis. *Matzner and Brzezinski (1984)* identified a chemotactic inhibitor in synovial fluid as a possible clue to understanding the pathogenesis of FMF. *In 1997*, two consortia cloned the gene that causes FMF, 28 disease mutations were identified so far in the gene

(*International FMF consortium, French FMF consortium*). It is called *MEFV* and encodes the pyrin protein, which is mainly expressed in neutrophil.

1.2 Racial and ethnic distribution

Familial Mediterranean Fever occurs predominantly in populations inhabiting or originating from the Mediterranean basin. The vast majority of patients are Arabs, Jews, Armenians, and Turks. In these populations (*Arabs, Jews, Armenian and Turks*) the frequency of FMF disease is 1 in 200 and the carrier state may be as high as 1 in 6. *Dode and his colleagues* investigated a large series of unselected and unrelated patients of various ethnic backgrounds with clinical suspicion of FMF to determine the spectrum of mutations, 62% of Sephardic Jews, North African Arabs, Armenian and Turkish patients were homozygous or compound heterozygous for *MEFV* mutations. In Other populations surrounding the Mediterranean such as Greek, Italian, Portuguese, Kurdish and Lebanese mutations were also found (*Dode et al 2000*).

1.3 Inheritance

Surveys of large numbers of FMF patients and their families have revealed that the disease is inherited as a single-gene autosomal recessive trait. Two points support a recessive inheritance: First is in the vast majority of affected families the disease occurs in members of one generation, and second is the high prevalence of consanguinity can explain the occurrence of the disease in two or more successive generations. Molecular studies were reported by *Aksentijevich et al (1999)* explain the unusual instance of dominantly inherited FMF as a recessive inheritance of the Glu148Gln mutation which has a high frequency and reduced penetrance among Ashkenazi Jews. On the other hand, *Booth and*

his group reported a comprehensive analysis of *MEFV* families from different ethnic groups. Their findings are compatible with the existence of dominantly inherited FMF, and demonstrate that this syndrome has a heterogeneous genetic basis (*Booth et al 2000*). Although there have been occasional reports suggesting that FMF can also be inherited dominantly, these have tended to be discounted on the basis that asymptomatic FMF carriers are very common in certain population and therefore give rise to pseudo-dominant inheritance.

1.4 Sex Ratio

Kastner and his group reported 60% of cases in males, suggesting that in females, mutation penetrance may be incomplete (*Kastner et al 1998*). The risk for male patients of developing renal amyloidosis was four folds higher than that for female patients was reported by *Cazeneuve et al (2000)*.

1.5 Age at onset

The bouts customarily begin in early childhood where they begin in 50% of patients during the first decade of life and in 80% before the age 20. Once they begin, the bouts do continue throughout life (*Matzner et al 1995*). *Kastner and his group* reported that typical episodes of FMF first appear during childhood or adolescence, although the disease may be evident even in infancy (*Kastner et al 1998*). *Cazeneuve and his group* concluded that the onset of FMF in individuals with the Met694Val homozygous genotype was earlier than those with another *MEFV* genotype. The age of onset was lower in-patients with renal amyloidosis than in those without this complication (*Cazeneuve et al 2000*).

1.6 Inciting factor

Inciting factors are unclear, patients may enjoy long periods without symptoms followed by frequent attacks. In some patients, attacks may be precipitated by physical activity or emotional stress (*Kastner et al 1998*). Menstruation and diet have been reported to increase susceptibility to FMF attacks but the mechanisms by which these and others constitutional factors modulate clinical expression of the disease are not known (*Booth et al 2000*).

1.7 Clinical Manifestations

Temperature as high as 38 to 40°C is one of the most consistent and characteristic features of the disease and is present in almost all cases. Fever may occur without concomitant evidence of serositis, but this is unusual. In 20 to 30% of the cases the rise in temperature is preceded by chills. Typically, the duration of fever is brief, lasting between 12 hours and three days (*Sohar et al 1967*).

More than 95% of patients experience abdominal pain and signs of peritonitis during acute attacks (*Kastner et. al 1998*). After 6 to 20 hours the signs and symptoms subside (if patient is not operated on) and within 24 to 48 hours, the attack is usually over, and the patients return to normal state as before the attack. Examination of the exudate, which is formed in the peritoneum during the acute inflammation, reveals fluid rich neutrophils (*Sohar et. al 1967*).

Pleuritic pain occurs during acute attacks in 75% of patients. Pleural exudate, which can be aspirated, contains numerous neutrophils and resolves within 48 hours. The pleuritic

attack usually disappears within 12 to 72 hours but it may also last as long as seven days (*Sohar et al 1967*).

Arthritis is a common and important manifestation of FMF. It usually affects large joints, the knee in particular, and effusions are common (*Langevitz et al 1999*). Also as many as a third of FMF patients experience transient erysipelas-like skin lesions that appear typically on the lower leg, ankle, or dorsum of foot. These lesions subside spontaneously within 24 to 48 hours (*Langevitz et al 1999*). *Barzali and his group* studied seven patients with FMF in whom Erysipelas Like Erythema (ELE) developed. He suggests that ELE belongs to the spectrum of neutrophilic dermatosis and supports a pathogenesis that involves abnormal inhibition of inflammatory cascade in FMF (*Barzali et al 2000*).

Although FMF is not an autoimmune disease, several vasculitis, such as Polyarteritis Nodosa (PAN), Henoch Schonelin Purpura (HSP) and non-specific purpura are encountered in FMF more commonly than in the general population. Bechets disease (BD) is a form of vasculitis found in several ethnic groups also affected by FMF. *Schwartz and his colleagues* conducted a retrospective study in which FMF patients, also suffering from BD (FMF-BD) were examined. They found that the prevalence of BD was higher in FMF patients than in population known to be rich in BD ($p < 0.001$). FMF-BD cases and FMF or BD controls were comparable in most demographic, clinical and laboratory aspects. In most cases of patients with both diseases, the FMF was of moderate severity and the BD of limited extent. He suggested that BD should be included among the vasculitis complicating FMF in FMF patients with a genetic background of BD (*Schwartz et al 2000*). *Tekin and his group* reported clinical and laboratory findings and mutation results in 23 children with FMF-associated vasculitis. HSP, PAN and protracted febrile attacks were found in 11, 2 and 10 FMF patients respectively. *MEFV* mutations were also studied and they found that

most children with FMF-associated vasculitis had identifiable mutations in *MEFV* (*Tekin et al (2000)*). *Oguzkurt and his group* described a rare case of PAN involving the hepatobiliary system in a child with FMF. They suggest that hypersensitivity, genetic or autoimmune mechanism is the cause of occurrence of those two systemic diseases together (*Oguzkurt et al 2000*).

Majeed and his colleagues explored the incidence of acute scrotum swelling in a large group of Arab children with FMF. They found that among 175 boys with FMF, 9% developed episodes of scrotal swelling. He suggested that inflammation of tunica vaginalis resulting in scrotal swelling is another feature of serositis seen in FMF (*Majeed et al 2000*).

Cattan and his group studied the incidence of inflammatory bowel disease (IBD) in non-Ashkenazi Jewish patients with FMF and they showed that inflammatory bowel disease was particularly frequent and severe in non-Ashkenazi Jewish patients with FMF. He postulated that the inflammatory processes of FMF and IBD are additive, resulting in increased severity of the FMF disease in the new patients (*Cattan et al 2000*).

Self limited pericarditis with pericardial effusions, conjunctivitis, aseptic meningitis and other forms of serositis have been reported as manifestations of this disease but are unusual. However the most serious complication of FMF is systemic amyloidosis of the AA (amyloid A) type, which was first described by *Mamou and Cattan* in 1952 (Fig1. 7). Development of amyloidosis is not directly related to the severity or frequency of inflammatory attacks.

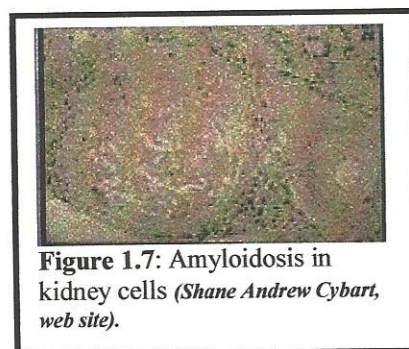


Figure 1.7: Amyloidosis in kidney cells (*Shane Andrew Cybart, web site*).

Amyloidosis presents clinically as a persistent heavy proteinuria, usually in childhood, and leads to end-stage renal disease. While a substantial proportion Turkish and Israeli patients develop amyloidosis, this complication has been rare among patients in the United States, Armenian and Arab children. The genetic or environmental factors that explain these differences in the incidence of amyloidosis remain unclear. In Israel, 90% of patients who develop amyloidosis do so after experiencing typical attacks of FMF (phenotype I); However, amyloidosis may occur in asymptomatic siblings of FMF patients, or it may precede the onset of typical FMF attacks (phenotype II) (*Langevitz et al 1999*).

The onset of amyloidosis is independent of the patient's age and duration of his/her disease. Amyloidosis, when present, develops before the age of 40 years in the vast majority of cases and only 12% of the affected patients will live over the age of 40. The probability of developing amyloidosis after this age is extremely rare. Patients who have reached 40 years can safely be assured that this serious complication is not likely to occur. The disease becomes milder, or even disappears, as the patients grow older.

Cazeneuve and his group suggested a role of other genetic and/or environmental factors for developing renal amyloidosis, because of the variable risk for patients carrying the same *MEFV* mutation to develop renal amyloidosis. They studied SAA1, SAA2 and apoE gene encoding serum amyloid protein and apolipoprotein E respectively. They showed that SAA1 α/α genotype was associated with a seven-fold increase of renal amyloidosis compared with other SAA1 genotypes. In their study the risk for male patients for developing renal amyloidosis was four folds higher than that for female patients. *Cazeneuve* strongly suggested that development of amyloidosis is influenced by at least two *MEFV* independent factors of genetic origin, SAA1 and sex-which act independently

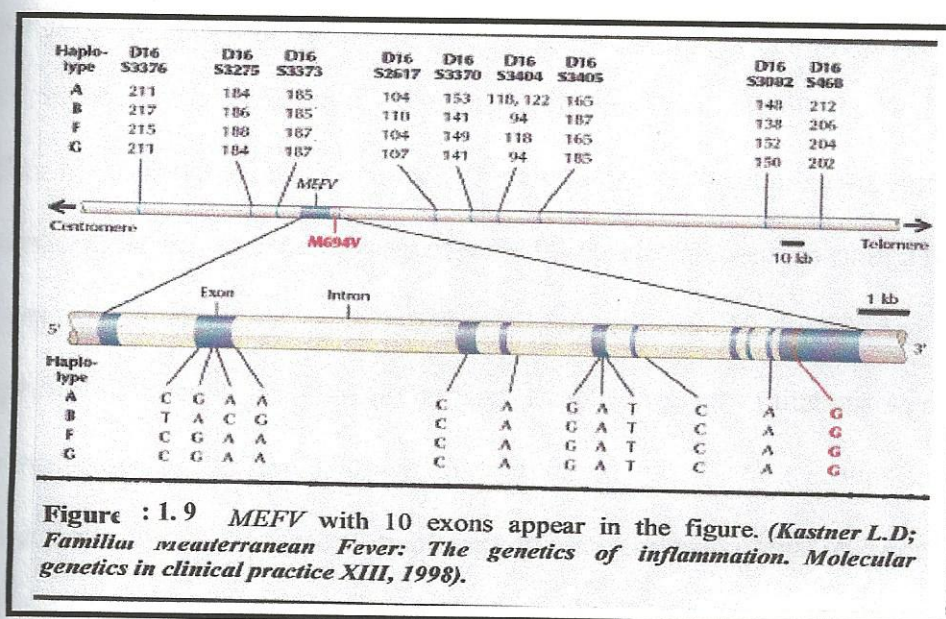
of each others. It is believed that the amyloidosis of FMF is transmitted independently from the disease itself (*Cazeneuve et al 2000*). *Melikoglu and his group* investigated the presence of phenotype II among the relatives of patients with FMF. They reported that phenotype II is uncommon among relatives of patients with FMF (*Melikoglu et al 2000*). Finally amyloidosis was linked to a certain mutation in the FMF gene, some authors stated that Met694Val mutation is associated with a higher incidence of systemic amyloidosis where as Val726Ala is not. While others disagree and show that there is no link between amyloidosis and any specific mutation. This topic will be discussed in detail in section (1.9.2).

1.8 Laboratory tests

There are no specific laboratory criteria for the precise diagnosis of FMF. Most abnormalities are due to the presence of an acute inflammatory process. During acute attacks, prominent leucocytosis (up to 3×10^4 per mm^3) is present, erythrocyte sedimentation rate and acute phase reactants are increased, and these values return to normal between attacks (*Ehrenfeld et al 1961*). Elevated plasma dopamine beta-hydroxylase level (which become normal during colchicine treatment) have been reported in-patients with FMF (*Kastner et al 1998*). Immunoglobulin measurement during FMF attacks showed a significant elevation in IgG, IgA, and IgM by 42%, 42%, and 55%, respectively (*Eliakim et al 1981*) as part of the body's response to the inflammation. Levels of complement components have been reported normal, reduced or elevated. These deviations appear to be non-specific consequences of the inflammation characteristic of the disease (*Matzner et al 1995*). With amyloidosis, laboratory abnormalities reflect the associated nephrotic syndrome and renal failure (*Eliakim et al 1981*).

1.9 MEFV

The *International FMF Consortium and the French FMF consortium (1997)* cloned a gene from a 115-kb FMF candidate interval on short arm of chromosome 16 (16p 13.3) (Fig 1.9) and identified 3 disease-related mutations. The novel gene encodes a 3.7-kb transcript that is expressed mainly in mature granulocytes. The predicted 781-amino acid protein, which they termed pyrin, is a member of a family of nuclear factors homologous to the Ro52 antigen. None of the mutations that they identified result in a truncated protein, and the periodic nature of inflammatory attacks in FMF is consistent with a protein that functions adequately at steady state but decompensates under stress. *The International*



FMF Consortium (1997) suggested that phenotypic differences might be related to different mutations. The Met694Val mutation is frequent in populations with a higher incidence of systemic amyloidosis whereas the Val726Ala mutation was found in a population in which amyloidosis is less common. *The French FMF Consortium (1997)*

identified transcriptional units in a critical *MEFV* interval of 60 kb on the basis of genomic sequence analysis and exon trapping. Four genes were identified, one of them encoding the protein that the investigators named marenostin from the Latin name of the Mediterranean Sea, mare nostrum. The partial predicted amino acid sequence is related to butyrophilin. The marenostin is the FMF disease associated gene, because scientist have demonstrated four sequence variations in it that correlated with disease in various ethnic groups. Initial studies showed that more than 80 percent of FMF chromosomes exhibit missense mutations located at one of three sites within a stretch of 46 amino acids encoded on exon 10 of *MEFV*. The mutations appear to confer only minor conformational changes in the structure of the peptide product as assessed by computer simulation. Only two of the three mutational sites, 694 and 726, are widely distributed.

Each of the 4 mutations that were first identified as the basis for FMF segregates with ancestral haplotype; all clustered in exon 10: Met694Val, Met694Ile, Val726Ala and Met680Ile. In a search for additional *MEFV* mutations in 120 apparently nonfounder FMF chromosomes, *Bernot and his group* observed 8 novel mutations, in exon 2 (Glu148Gln, Glu167Asp, and Thr267Ile), exon 5 (Phe479Leu), and exon 10 (Lys695Arg, Ala744Ser, and Arg761His). Except for Glu148Gln and Lys695Arg, all mutations were found in a single chromosome. Mutation Glu148Gln was found in all ethnic groups studied and in association with a novel ancestral haplotype in non-Ashkenazi Jews (*Bernot et al 1998*). Although FMF is rare among Bedouin families *Press and his colleagues* described two Bedouin families suffering from FMF. The *MEFV* mutations, Met694Ile, Val726Ala and Glu148Gln found in Bedouin patients are consistent with their Arab origin. The disease severity score showed mild to moderate severe disease. The unique nomadic life of

Bedouins may prove instrumental in unraveling the role of environmental factors in the course and the severity of FMF (*Press et al 2000*).

1.9.1 *MEFV* Mutations

To date, 28 *MEFV* mutations have been identified with higher frequency in eastern Mediterranean populations. Table (1.9.1) shows some of these mutations.

1.9.2 Genotype-phenotype Correlation

The first genotype – phenotype correlation study showed that the Met694Val homozygous genotype was associated with a more severe form of the disease, as judged by an earlier age of onset, higher prevalence of pleurisy, higher frequency of arthritis and most importantly in patients who did not have access to a regular colchicine therapy, a higher prevalence of renal amyloidosis (*Cazeneuve et al 1999, Shohat et al 1999, 1998, Dewalle et al 1998*). *Mimouni and his group* found a significant association between amyloidosis and Met694Val homozygous patients. Also the type and the severity of FMF inflammatory symptoms was found to be associated with both the genotype and the country of patient residence. Their finding suggests that factors other than *MEFV* mutation play a role in the determination of the severity of the inflammatory attacks in FMF. Based on these results, a clinical approach, assuming that the Met694Val mutation bears the worst prognosis for an FMF patient has been suggested, although most studies described FMF patients with amyloidosis bearing mutations other than Met694Val (*Mimouni et al 2000*).

Brik and his group conducted a phenotype genotype correlation study in a mixed population of Jewish and Arab children. They examined four mutations (Met694Val, Met680Ile, Val726Ala, Met694Ile) and then correlated disease severity and occurrence of

amyloidosis. All four mutations were identified among 94% of the Arab patients, but with no particular prevalence for any one of them. The homozygous Met694Val mutation was significantly associated with a more severe form of the disease (the clinical onset of the disease manifested at an earlier age, the number of attacks per months was higher). Only patients with Met694Val mutation had a family history of amyloidosis. No association was found between the type of mutation and the predominance of fever, abdominal pain, pleuritis skin eruption, or response to colchicine in the clinical picture. Met694Val mutation was less common among Arabs and when present, it occurred almost only in the heterozygous form. In Arab patients, the disease tended to run a milder course and seemed to bear a better prognosis (*Brik et al 1999*). *Yalcinkaya and his group* described the frequencies of seven *MEFV* mutations (Met694Val, Met680Ile, Val726Ala, Met694Ile, Lys695Arg, Arg761His, Glu148Gln) with the clinical findings in 20 Turkish FMF patients who had not developed amyloidosis by the age of 40 years in the absence of colchicine therapy and compared them with 27 Turkish patients with amyloidosis. Only one of 20 patients without amyloidosis did not carry the Met694Val mutation, and five were found to be homozygous for it, 8/27 patients with amyloidosis did not have the Met694Val mutation. These results suggest that the development of amyloidosis is not due to the presence of homozygosity of the Met694Val mutation only, indicating that mutation analysis does not predict the development of amyloidosis in a given patient (*Yalcinkaya et al 2000*). *Yalcinkaya and his colleagues* published another study to evaluate whether the phenotypic variation of FMF is associated with the existence of particular mutations in Turkish FMF patient living in Turkey. They published that the presence of the Met694Val mutation was not found to be associated with a severe form of the disease or the development of amyloidosis. Arthritis frequency was found to be lower in the patients with homozygous

Met680Ile mutation. They concluded that non of the four-missense mutations were associated with a severe disease or the development of amyloidosis in Turkish FMF patients living in Turkey. The influence of unknown environmental factors or the presence of other genetic changes is necessary to explain the phenotypic variation of the disease and the development of amyloidosis (*Yalcinkaya et al 2000*). *Tekin and his group* provided evidence that FMF patients without Met694Val mutation are also at risk for the development of amyloidosis, and concluded that particular mutations themselves do not appear to be sufficient to explain the occurrence of amyloidosis in all cases with FMF. They suggested that the withdrawal of colchicine treatment in individuals with *MEFV* mutations may result in a fatal complication, continuing colchicine treatment in all individuals with FMF regardless of the mutation results, appear to be the best approach until the uncertainties on the development of amyloidosis are resolved. The results of initial studies have led to the hypothesis that the occurrence of amyloidosis in FMF is attributable to the existence or absence of particular mutations. The Val726Ala mutation was found to be frequent in Ashkenazi Jews, Druze, and Armenians, among whom amyloidosis occurs at lower frequencies. This suggested that the Val726Ala mutation might be protective against amyloidosis. Recent case reports have shown that homozygotes or compound heterozygotes with the Val726Ala mutation may develop amyloidosis as well (*Tekin et al 2000*).

Kone Paut and his group described the clinical manifestations of (FMF) in 91 patients from 47 families. Phenotype genotype correlation showed a significant association of Met694Val homozygosity with earlier age of onset, fever >39°C, pleural crisis, splenomegaly and arthritis (*Kone Paut et al 2000*). *Akar and his group* study Ala138Gly mutation of the *MEFV* gene. They found that there was no significant difference between healthy controls and FMF patients for the Ala138Gly polymorphism but when FMF



amyloidosis patient were taken as another group, the differences was significant indicating that the carrier of 138 Gly are more prone to amyloidosis (*Akar et al 2001*). *Yael and his group* presented genotype phenotype for the 4 most common genotypes found among patients with FMF. Patients with Met694Val/Met694Val genotype were found to have an earlier age of onset, higher frequency of joint involvement, higher frequency of erysipelas like erythema and required higher doses of colchicine to control the disease compared to the other 3 genotypes studied. The Met694Val/Val726Ala genotype was found to be more severe than Met694Val/Glu148Gln and Val726Ala/Val726Ala. The variability in symptoms among patients with the Met694Val/Met694Val genotype suggests that enviromental factors and modifier genes may have an effect on the final phenotype (*Yael et al 2000*).

Table (1.9.1) *MEFV* Mutations table for single amino acid substitution

<u>cDNA Nucleotide Substitution</u>		<u>Genomic Nucleotide Substitution</u>	<u>Exon</u>		<u>Mutation</u>	<u>References</u>
442	G>C	1962	2	148	Glu>Gln	<i>Bernot et al 1998</i>
501	G>C	2019	2	167	Glu>Asp	<i>Bernot et al 1998</i>
800	C>T	2320	2	267	Thr>Ile	<i>Bernot et al 1998</i>
1105	C>T	7002	3	369	Pro>Ser	<i>Aksentjevich et al 1999</i>
1223	G>A	7120	3	408	Arg>Gln	<i>Cazeneuve et Al 1999</i>
1437	C>G	9422	5	479	Phe>Leu	<i>Bernot et al 1998</i>
2040	G>C	13141	10	680	Met>Ile	<i>International FMF Consortium 1997</i>
2040	G>A	13141	10	680	Met>Ile	<i>Aksentjevich et al 1999</i>
2080	A>G	13181	10	694	Met>Val	<i>International FMF Consortium 1997</i>
2082	G>A	13183	10	694	Met>Ile	<i>French FMF Consortium 1997</i>
2084	A>G	13185	10	695	Lys>Arg	<i>Bernot et al 1998</i>
2177	T>C	13278	10	726	Val>Ala	<i>International FMF Consortium 1997</i>
2230	G>T	13341	10	744	Ala>Ser	<i>Bernot et al 1998</i>
2282	G>A	13383	10	761	Arg>His	<i>Bernot et al 1998</i>

1.9.3 *MEFV* expression and regulation

MEFV is expressed mainly in neutrophils. *Centola and his group* reported the expression of *MEFV* mRNA in bone marrow leukocytes with differential expression observed among cells by *in situ* hybridization. CD34 hematopoietic stem cell cultures induced toward the granulocytic lineage expressed *MEFV* at the myelocyte stage. The promyelocytic cell line HL60 expressed *MEFV* only upon granulocytic and monocytic differentiation. *MEFV* was also expressed in two monocytic cell lines (THP-1, 937). Among peripheral blood leukocytes, *MEFV* expression was detected in neutrophils, eosinophils and to a varying degree in monocytes. *In vitro* stimulation of monocytes with proinflammatory agents IFN- γ , TNF- α and LPS induced the *MEFV* expression, while the antiinflammatory cytokines IL-4, IL-10 and TGF- β inhibited the *MEFV* expression. In granulocytes, *MEFV* was upregulated by IFN- γ and the combination of IFN- α and colchicine. No changes were noted upon stimulation of cells with C5a, IL-8 or TNF, indicating that *MEFV* upregulation is not a general property of activated cells. Upregulation by IFN- γ occurred rapidly and the IFN- γ mediated leukocyte activation functions primarily through the modulation of gene expression. The effect was not inhibited by cyclohexamide, showing that *MEFV* is an IFN- γ immediate early gene, suggesting that *MEFV* play a direct role for IFN- γ activation. *Centola* also examined the DNA sequences upstream of the start of *MEFV* transcription revealed DNA sequence that match the consensus Gamma Interferon Activation Sequence (GAS) site at position -731 and the NF-KB binding site at position 164 (*Centola et al 2000*). *Matzner and his group* reported the expression of *MEFV* in peritoneal and skin fibroblasts at a lower level than neutrophils, which could be further induced by PMA and IL-1 β (*Matzner et al 2000*). *Tidow and his group* also

reported the expression of *MEFV* mRNA in monocytes, CD19⁺ B cells and CD3⁺ T cells. In nonhematopoietic tissues, *MEFV* mRNA was detected predominantly in spleen, lung, muscle mRNA. *Tidow* suggested that the expression of *MEFV* in lung and muscle could, to some extent, result from infiltrating macrophages contaminating neutrophils. Also they found expression of *MEFV* predominantly in myeloid leukemic cell lines and in colon cancer cell lines. They demonstrated prominent induction of *MEFV* mRNA during granulocytic differentiation of HL-60 cells (*Tidow et al 2000*).

1.10 Pyrin/Marenostrin

Of the 3505 nucleotides in *MEFV*, about 2300 code for 781 amino acid protein weighing 86 kDa, with arginine and lysine constituting 13% of it (*Kastner et al 1998*). The resulting protein has two names, pyrin (named from the Greek word for fire and offered by the *International FMF consortium*) or marenostrin (The French name alludes to Mare Nortrusm (our sea), the ancient Latin name for Mediterranean). Protein sequence analysis revealed five different motifs: Motif one is a bZIP basic domain (a.a 266-270). Motif two is a B-box Zinc finger domain (a.a 375-407). Motif three is a coiled coil domain (a.a 408-594). Motif four is a B30.2 domain (a.a 598-774), a sequence have 40% to 60% homology to several nuclear proteins. Motif five are two overlapping potential nuclear localization signal (2NLS regions) (a.a 419-422) and (a.a 420-430), which permit the peptide to undergo transport through nuclear pores from the cell cytoplasm into its nucleoplasm (*Kastner et al 1998*). By homology the bZIP basic domain and the B-Box zinc finger were speculated to confer DNA binding and transcription modulation activity. The coiled domain was proposed to mediate protein-protein interaction including homo dimerization and the nuclear localization signals were postulated to localize the protein to

the cell nucleus. Based on such computational studies, pyrin was proposed to be a nuclear transcription factor that regulates inflammation. Its secondary structure composed of 16 alpha helices, 17- β sheet (*Kastner 1998*).

Dramatic accumulation of neutrophils at the symptomatic serosal sites is interpreted as the wild type gene acting as an upregulator of an anti-inflammatory molecule or as a down regulator of a pro-inflammatory molecule; Its role is to inhibit inflammation provoked by a minor insult and mutated pyrin is unable to inhibit unnecessary inflammatory events (*Kastner et al 1998*). In contrast to this prediction, recent in vitro transient expression of a fusion protein between marenostin and the green fluorescent protein (GFP) indicated that the protein encoded by *MEFV* is exclusively located in the cytoplasm (*Tidow et al 2000*, *Chen et al 2000*). *Papin and his group* described a splice variant of *MEFV* generated by an in-frame splice removal of exon 2. Thus transcript expressed in leuckocytes predicts a 570 residue protein designated marenostin-d2. They investigated the differences in subcellular localization between the full-length protein (marenostin fl) and truncated protein. The localization pattern of marenostin-d2 differs dramatically from that of marenostin-fl. Marenostin-fl is homogeneously distributed over the entire cytoplasm where as marenostin-d2 is concentrated in the nucleus. Deletion of the putative nuclear localization signals (NLS) does not alter the nuclear localization of marenostin-d2. The expression of *MEFV-d2* transcripts was also detected in polymorphonuclear cells as well as in the population of peripheral blood leuckocytes enriched for mononuclear cells; however, the *MEFV-d2: MEFV-fl* transcript ratio was found to be three folds higher in mononuclear cells than in polymorphonuclear leuckocytes. *Papin and his group* also showed *in vitro* stimulation of these two leuckocyte subpopulation with interferon IFN- γ for 2 hours resulted in increased levels of both *MEFV-fl* and *MEFV-d2* transcript, however in both leuckocyte

subpopulation, this treatment did not significantly alter the *MEFV-d2: MEFV-fl* transcript ratio. Several findings indicated the particular importance of the methionine residue at position 694 in the physiological role of pyrin. Recognition that *MEFV* mutations affecting only a single allele can give rise to FMF suggests that a 50% complement of normal pyrin activity is not sufficient to prevent disease susceptibility. Although pyrin has not yet been characterized, the *MEFV* mutations, which cause FMF presumably, disrupt its structure sufficiently to reduce its function and lead to neutrophil activation and migration in a situation that would not normally produce these effects (*Papin et al 2000*). *Chen and his group* excluded the transcription activation activity of pyrin and its ability to form homodimers. They identified p/M-IP1 (pyrine/marenostrin interacting protein 1). They have shown that P/M-IP1 colocalizes with pyrin in the perinuclear cytoplasm of cos-7 cells and that the interaction between these two proteins is impaired by FMF causing mutations in pyrin (*Chen et al 2000*). *Tidow and his group* showed that pyrin was localized in distinct patches in the cytoplasm, forming a perinuclear cap. They suggested that pyrin is expressed in the cytoplasm and therefore may evoke functions other than a transcription factor. Alternatively, the intracellular localization of pyrin might be translocated into the nucleus upon stimulation (*Tidow et al 2000*).

1.11 Diagnosis

It is crucial to establish the diagnosis of FMF since a confirmed diagnosis leads to daily lifetime use of colchicine, which is an efficient preventive treatment of both, the FMF attacks and amyloidosis. The diagnosis of FMF is based primarily on clinical presentation, history and physical examination and laboratory results obtained from patients experiencing attacks.

Tel-Hashomer hospital set a diagnostic criteria for FMF based on the presence of short-lived febrile episodes accompanied by inflammation of one of the serous membranes, the development of nephropathic amyloidosis, and the response to colchicine treatment (Langevitz et al 1999) (Table 1.11.1,1.11.2)

Cazeneuve and his group

evaluated the diagnostic and prognostic value of *MEFV* analysis, he found that the molecularly based FMF diagnosis is a clear-cut demonstration of both the incomplete penetrance and pseudo dominance of the disease phenotype (Cazeneuve et al 1999).

Table 1.11.1. Tel-Hashomer criteria for the diagnosis of FMF (Langevitz et al 1999).

Major criteria	Minor criteria
1.Recurrent febrile episodes accompanied by peritonitis, synovitis or pleuritis	1.Recurrent febrile episodes
2. Amyloidosis of AA-type without predisposing disease	2. Erysipelas-like erythema
3. Favorable response to continuous colchicine treatment	3. FMF in a first-degree relative
Definitive diagnosis: 2 major, or 1 major and 2 minor	
Probable diagnosis: 1 major and 1 minor	

Table 1.11.2. Tel-Hashomer key to FMF severity score. (Langevitz et al 1999).

1. Age of onset > 5 years 5-10 years 10-20 years >20 years	3 points 2 points 1 points 0 points
2. Frequency of attacks > 2 per month 1-2 per month <1 per month	3 points 2 points 1 point
3. Colchicine dosage to control attacks Non responder 2mg/day 1.5 mg/day 1 mg/day	4 points 3 points 2 points 1 point
4. Arthritis Protracted arthritis Presence of acute joints	3 points 2 points
5. Erysipelas-like erythema If present	2 points
6. Amyloidosis If present Phenotype II	3 points 4 points
Mild disease Moderate disease Severe disease	Total of 2-5 points 6-10 points > 10 points

Dode and his group investigated a large series of random and unrelated patients of various ethnic backgrounds with clinical suspicion of FMF. They determined the spectrum of mutations trying to confirm or invalidate the diagnosis of FMF. They concluded that the diagnosis of FMF should not be done only on the basis of clinical criteria and should include molecular studies, especially for the patients at the beginning of the disease or without family history (*Dode et al 2000*). *Eisenberg and her colleagues* described the ARMS method (Amplification Refractory Mutation System) as a rapid cost effective and accurate method for detecting three common mutations in Familial Mediterranean Fever (*Eisenberg et al 1998*). Patients of ethnic background (Jewish, Armenian, Arabs or Turk) may be helpful in raising suspicion of FMF, but since there is some American patients, suggests that FMF occurs in patients without a typical ethnic background. Duration of attacks is another important clue, in that FMF episodes tend to be shorter than those of either familial hibernian fever or hyper IgD syndrome (*Kastner et al 1998*). *Grateau and his group* evaluated the utility of molecular approach for FMF diagnosis in a large series of patients with various clinical presentations and different ethnic origin. Their results enable the confirmation of FMF in most typical cases of the disease and showed that the *MEFV* gene is implicated in atypical clinical presentation including patients who do not satisfy clinical criteria (*Grateau et al 2000*). *Nir-Paz and his group* evaluated the molecular diagnosis of FMF, and found that molecular analysis of FMF should complement the investigation of patients with fever of unknown origin. This test enables a definite diagnosis of the disease and may promote the diagnosis and treatment of patients with an unusual or incomplete clinical picture of FMF (*Nir-Paz et al 2000*). Cloning of *MEFV* allows a new and reliable diagnostic test for FMF. A set of polymerase chain reaction primers can be used to demonstrate the mutations responsible for the disease. This

important diagnostic test has become essential in the diagnosis of patients with FMF who have an atypical clinical course and manifestations and patients who do not fit clinical criteria.

1.12 Pathogenesis

The exact mechanism triggering the acute attacks in FMF is unclear, but several lines of evidence point to the neutrophil as the effector of the inflammatory response at serosal surfaces :a) There is neutrophil predominance in serosal fluids obtained from patients during acute attacks of FMF. b) Colchicine, which is effective in preventing attacks in FMF, is known to act by suppressing neutrophil phagocytosis and chemotaxis . c) Pyrin/marenostrin is expressed mainly in circulating neutrophils. So it may dampen the activation of neutrophils in response to mild proinflammatory perturbations in the nearby environment, thereby preventing further recruitment and enhancement of the inflammatory response (*Kastner et al 1998*).

Although many pathogenic explanations have been suggested for the acute inflammatory episodes of FMF, the etiology of this disease remains unknown. Extensive studies have failed to establish an infectious or allergic basis for the disease, and no good evidence exists to support suggestions that FMF represents a hormonal or psychosomatic disturbance.

An autoimmune etiology has been suggested but all laboratory tests for autoimmune etiology were reported to be nonspecific. Increased levels of circulating immune complexes were demonstrated in 27% of FMF patients, although Lupus Erythematosus (LE) cells,

antinuclear antibody, rheumatoid factor or antithyroid antibodies were not found. Measurement of serum immunoglobulin levels showed a diffuse increase in IgG and IgM consistent with the reduced level of T suppressor cell activity seen in FMF. However these deviations as well as the increase in serum amyloid A protein, appear to be nonspecific consequences of the general inflammatory response characteristic of this disease. Finally, autoimmune disease responds to lympholytic drugs such as corticosteroids and other immunosuppressive agents, where FMF patients respond exclusively to colchicine, an antineutrophilic drug (*Matzner et al 1995*).

Shohat and his group postulated that FMF patients are homozygous for a mutant allele for one of the lipocortin genes since these proteins are thought to control the biosynthesis of potent mediators of inflammation, prostaglandins and leukotrienes, by inhibiting release of their common precursor, arachidonic acid, a process that requires hydrolysis of phospholipids by phospholipase A2 (*Shohat et al 1989*).

Barakat and his group suggested that the disorder might be related to abnormal catecholamine metabolism since an attack could be provoked by metaraminol infusion and the levels of plasma dopamine beta-hydroxylase were found to be higher than normal in FMF patients. This was interpreted as metaraminol-induced attack that is not due to a primary defect in catecholamine metabolism but is secondary to the non-specific induction of inflammation that could not be aborted (*Barakat et al 1984*).

Deficiency of a specific complement inactivator may also be involved in the pathogenesis of FMF. A report in 1984 identified a chemotactic inhibitor in synovial and peritoneal fluid as a possible clue for the understanding of the pathogenesis of this disorder (Figure 1. 12). Normal peritoneal and synovial fluids contain an inhibitor of neutrophil chemotaxis that acts by antagonizing the complement-derived chemotactic anaphylatoxin

C5a. The inhibitor is a protein with molecular weight of 40 kDa (*Matzner et al 1984*). It was found that this inhibitor displayed reduced activity in serosal fluids from FMF patients (*Matzner and Brzezinski 1984, Matzner et al 1990*). It was produced by synovial and peritoneal fibroblast but not by skin fibroblast (*Matzner et al 1986*). *Ayesh and his colleagues* further characterized the 40-kDa inhibitor protein and showed that it is a serine protease (*Ayesh et al (1990)*). They expanded this work in 1993 and 1995 and showed that this serine protease inactivates C5a and IL-8. *Babior and Matzner* suggested that the pathogenesis of FMF be as follows: pyrin, or marenostin, is postulated to activate the biosynthesis of a chemotactic-factor inactivator, an enzyme that normally occurs in the serosal fluids. They suggested that a chemotactic factor (probably C5a) can be released by subclinical injury to the serosal tissue during normal activities, but the amounts released are small enough that they are cleared by the inactivating enzyme before they can provoke an inflammatory reaction. In FMF the inactivating enzyme is absent, allowing the chemotactic factors to survive long enough to call in neutrophils, which then release a variety of products, including an enzyme that generates more C5a. The end result is an upward spiral that culminates in a full-blown inflammatory reaction: an attack of FMF (*Babior and Matzner 1997*). However, the possible etiologic significance of these observations remains to be clarified and confirmed.

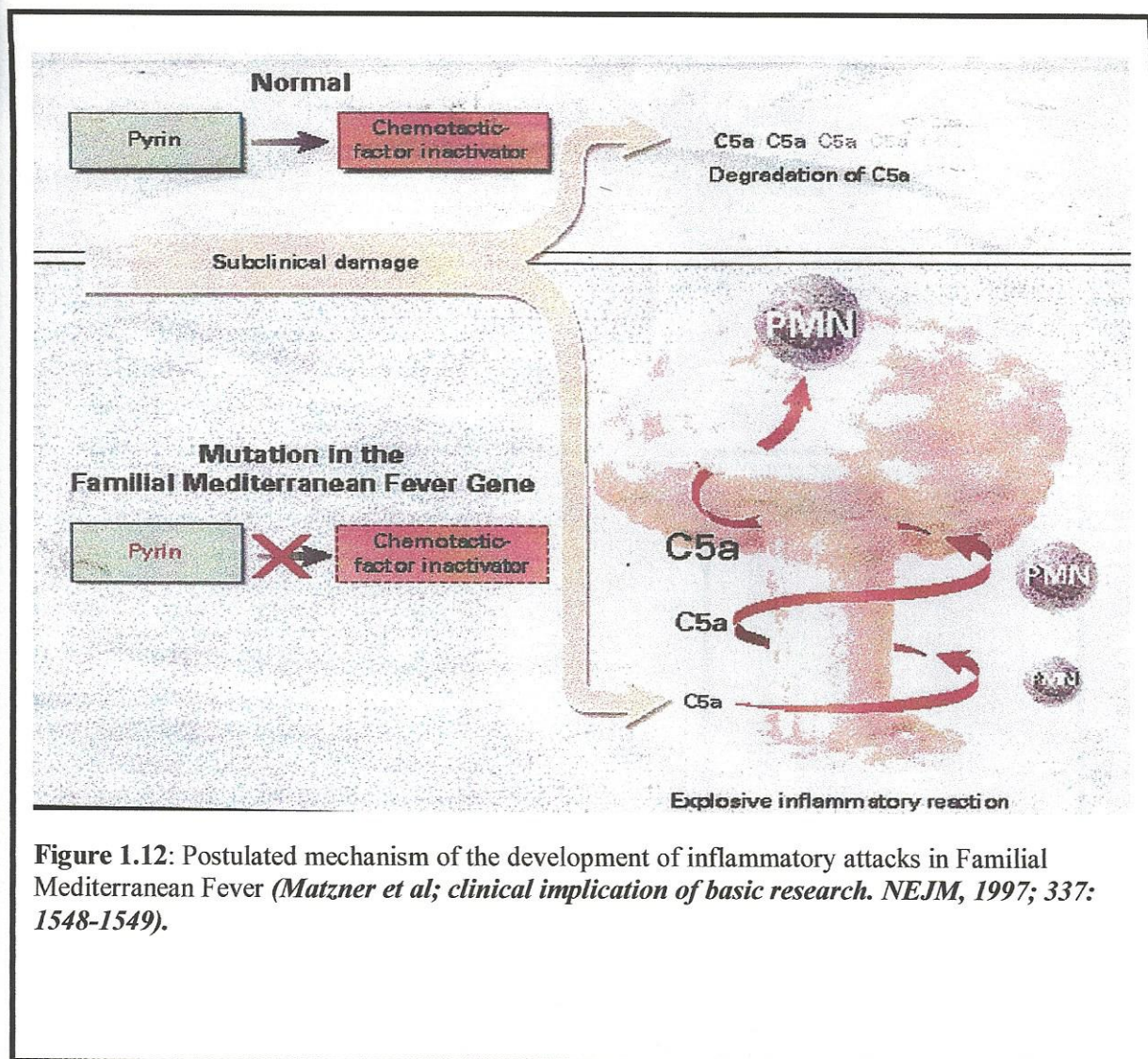


Figure 1.12: Postulated mechanism of the development of inflammatory attacks in Familial Mediterranean Fever (*Matzner et al; clinical implication of basic research. NEJM, 1997; 337: 1548-1549*).

The recent identification of the chromosomal location of the gene associated with FMF should assist in the eventual elucidation of the biochemical defect that underlies this disease and it has been proposed that FMF might be caused by a genetically determined defect in the normal regulation of acute inflammatory responses. *Matzner and his group* reported the activity of C5a inhibitor in synovial and peritoneal fibroblast that could be further induced with PMA and IL-1 β . Very low levels of chemotactic inhibitor were evident in skin fibroblast cultures or in peritoneal and skin fibroblast obtained from FMF

patients. They also showed that primary fibroblast cultures express *MEFV*, which can be further induced with PMA and IL-1 β (*Matzner et al 2000*). These results support the relation between C5a inhibitor activity and *MEFV* expression but more investigation is required.

1.13 Treatment

The major source of mortality from FMF is from the insidious development of secondary (AA) amyloidosis with eventual renal failure, thus the goals of therapy for FMF are two:

*Symptomatic relief from the acute attacks

*Prevention of amyloidosis



Early attempts to treat FMF with a variety of measures, including antibiotics, corticosteroids, salicylates, antihistamines, antimalarias, and low-fat diets, provided little benefit. It has now become clear that the disease can be managed successfully only by prophylactic treatment with colchicine (*Eliakim et al 1981*).

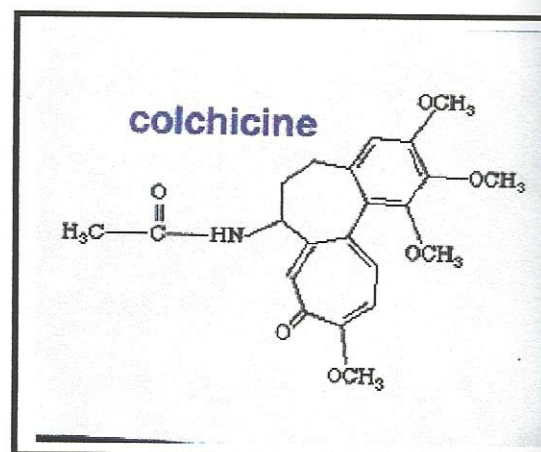
1.13.1 Colchicine

Colchicine is a water-soluble alkaloid extracted from the bulb of *colchicum autumnal* (Latin name for the meadow saffron) and *Glorosa superba* (Fig 1.13.1). Continuous colchicine treatment was introduced by *Goldfinger (1972)*. It is used orally but intravenous administration is available. The dose is 1mg/day and may be increased if indicated to 2 (in rare case 2.5) mg/day (*Ben-Chetrit et al 1998*).

Approximately 50% of circulatory colchicine are bound to plasma protein. It is excreted and undergoes extensive metabolism in the liver, mainly by deacylation followed by biliary excretion (*Ben-Chetrit et al 1998*).

1.13.1.1 Pharmacology

It is an alkaloid with the chemical formula N-(5,6,7,9, tetrahydro-1, 2,3,10,tetramethoxy-9 oxobenzo hep-tain-7-yl) acetamide. The drug is almost completely absorbed following its oral use. The exact site of absorption is unknown but the drug seems to reach the ileum since dysfunction of this portion of the bowel is common in chronic colchicine toxicity (*Ben-Chetrit et al 1998*).



1.13.1.2 Biological effects

Colchicine blocks or suppresses cell division by inhibiting mitosis. Specifically, it inhibits the development of spindles as the nuclei are dividing. It is most effective when used at the onset of symptoms. It inhibits collagen transport to the extracellular space, hence its use in the prevention or treatment of amyloidosis and scleroderma (*Ben-Chetrit et al 1998*).

1.13.1.3 Toxicity

Colchicine may exert numerous effects on the gastrointestinal tract. These include enzyme inhibition, histological mucosal change, and clinical symptoms ranging from nausea and vomiting to a cholera-like syndrome and malabsorption. Colchicine overdose may lead to a cholera-like syndrome associated with dehydration, shock and acute renal

failure. Death has been described after the injection of 10 mg within 11 days in one patient and after oral ingestion of 40 mg (*Ben-Chetrit et al 1998*).

1.13.1.4 Possible mechanism of colchicine in FMF

The mechanism of action of colchicine in preventing the attacks and amyloidosis of FMF has not been fully elucidated, However the pathophysiology of FMF appears to involve the recruitment and activation of neutrophils at serosal surfaces. Because colchicine is known to suppress neutrophil phagocytosis in gout, possibly due to its binding of tubulin and other intracellular proteins, it is probable that the drug's beneficial effect in FMF is related to the same mechanism. The ability of colchicine to cause mitotic arrest is unlikely to play a role in its therapeutic action, because the dosage required suppressing mitosis is in considerable excess of that used for FMF. Also it is suggested to be related to its action on leukocyte and monocyte functions. Colchicine has been shown to concentrate in leukocytes and to influence numerous leukocyte functions. *Ben-chetrit and his group* showed that colchicine accumulates in neutrophils in a higher concentration than in lymphocytes due to the absence of the P-glycoprotein (P-gly) efflux pump in the neutrophil. The relatively high affinity of colchicine to granulocytes may explain its beneficial effect in FMF and other colchicine-responsive inflammatory process (*Ben-chetrit et al 1998*). *Kiraz and his group* evaluated the basal levels of circulating cytokines and selectin before and after colchicine treatment. They found that all parameters except soluble P-selectin were significantly higher in FMF patients than in controls before colchicine treatment while after 2 months of treatment statistically significant decrease was observed in these parameters. Since cytokines reflect the presence of sustained inflammation in attack-free FMF patients, the effect of colchicine on these parameters may explain its therapeutic potential (*Kiraz et al 1998*).

1.13.1.5 Colchicine resistant patient

There is no known alternative drug for colchicine resistant cases, but there are various studies trying to investigate interferon alpha (IFN- α) as an alternative drug for colchicine resistant patients since IFN has strong anti-inflammatory properties antagonizing or suppressing IL-1 β and inhibiting granulocytes release from the bone marrow. These actions of IFN are reversible and of short duration. (*Tankurt et al 1996*). Another potential therapy is with free radical scavengers or antioxidants in particular during acute attacks when classical colchicine treatment is without effect since O₂⁻ production and other O₂ radicals levels are higher in patients with FMF compared with normal controls (*Sarkisian T et al 1997*).

1.14 Role of cytokines and chemotactic factors in inflammation

1.14.1 Inflammation

Inflammation, the response of tissue to injury, is characterized in the acute phase by increased blood flow and vascular permeability along with the accumulation of fluid, leukocytes, and inflammatory mediators such as cytokines. During both acute and chronic inflammatory processes, a variety of soluble factors are involved in leukocyte recruitment through increased expression of cellular adhesion molecules and chemoattraction. Many of these soluble mediators regulate the activation of the resident cells (such as fibroblasts, endothelial cells, tissue macrophages, and mast cells) and the newly recruited inflammatory cells (such as monocytes, lymphocytes, neutrophils, and eosinophils), and some of these mediators result in systemic response to the inflammatory process (e.g. fever, hypotension,

synthesis of acute phase proteins, leukocytosis). The soluble factors that mediate these responses fall into four main categories: Category one is, inflammatory lipid metabolites such as platelet activating factor (PAF) and the numerous derivatives of arachidonic acid (prostaglandins, leukotrienes, lipoxins), which are generated from cellular phospholipids. Category two is three cascades of soluble proteases/substrates (clotting, complement, and kinins), that generate numerous pro-inflammatory peptides. Category three is, nitric oxide, a potent endogenous vasodilator, whose role in the inflammatory process has only recently been described. Finally category four is, a group of cell-derived polypeptides, known as cytokines, which to a large extent orchestrate the inflammatory response, i.e. they are major determinants of the make-up of the cellular infiltrate, the state of cellular activation, and the systemic responses to inflammation. Most cytokines are multifunctional. They are pleiotropic molecules that elicit their effects locally or systemically in an autocrine or paracrine manner. Cytokines are involved in extensive networks that involve synergistic as well as antagonistic interactions and exhibit both negative and positive regulatory effects on various target cells (*Gallin et al 1992*).

1.14.2 Role of chemotactic factors in inflammation

Activation of complement generates several biologically active peptides, some of which are potent neutrophil and monocyte chemoattractants. The most clinically relevant complement-derived chemotactic factor appears to be C5a, a cleavage product of the fifth component of complement. In addition to having chemotactic activity, C5a can increase vascular permeability, cause smooth muscle contraction, and activate leukocytes to secrete lysosomal enzymes and reactive oxygen intermediates. Of potential importance to tissue leukocyte accumulation, C5a has been shown to directly induce the expression of P-selectin

by endothelial cells and indirectly augment intercellular adhesion molecule-1 in a model of inflammatory lung injury. Although chemotactic factors stimulate directional leukocyte movement, this alone is not sufficient to establish an inflammatory focus. Tissue leukocyte infiltration is a complex process that includes, but is not limited to, leukocyte chemotaxis. Briefly, the process of leukocyte migration from the intravascular space begins with leukocyte adherence to the endothelial surface. Initial "loose" contact between leukocyte and endothelium is mediated by the selectin family of adhesion molecules. Endothelial selectin expression is regulated by inflammatory stimuli such as interleukin-1 (IL-1), or tumor necrosis factor (TNF). Loose contact is followed by firm attachment through the interaction of integrins expressed by activated leukocytes and adhesion molecules expressed by activated endothelial cells. Proinflammatory mediators are required to enhance the adhesiveness of the integrins. Adherent leukocytes migrate between endothelial cells into the interstitial space along a chemoattractant concentration gradient. In addition to establishing chemoattractant gradients, chemotactic factors act early in the infiltration cascade by regulating leukocyte adherence to the endothelium. The activation of leukocytes by chemoattractants may directly or indirectly cause further production of chemotactic factors, providing a mechanism to amplify the inflammatory response by recruiting more white blood cells (WBCs) to a focus of inflammation. Finally, chemoattractants may also promote tissue leukocyte infiltration by enhancing the permeability of endothelium and extracellular matrix. C5a, a classic anaphylatoxin, mediates increased microvascular permeability, possibly through its actions on neutrophils (*Brad et al 1998*).

1.14.3 Cytokines

Cytokines are protein or glycoprotein molecules synthesized and secreted by cells. The interleukins, interferon, and colony stimulating factors were the first categories of cytokines discovered and have since been extensively studied. In general, induction of cytokines genes occurs in response to cellular injury or activation, although there are also examples of constitutive cytokine production. Cytokines exert their biologic effects by interaction with specific cytokine receptor molecules located on the surface of target cells. Cytokines as a group possess a broad spectrum of bioactivities and have been found to play a part in cell growth, repair, inflammation and the immune response. Cytokine molecules are both pleiotropic (with each having multiple functions) and redundant (with more than one cytokine possessing a given bioactivity). *In vivo*, these molecules do not exist in isolation, but rather as a network through which the expression of one cytokine may be influenced by other cytokines as well as by noncytokine mediators (*Gallin et al 1992*).

1.14.3.1 TNF- α (Tumor Necrosis Factor)

TNF, a product of stimulated monocytes and macrophages, is also produced by lymphocytes, endothelial cells, and keratinocytes. TNF- α trimerizes into a stable form; in addition, it is the trimer, rather than the monomer (17kD), that are biologically active. Although originally studied for its ability to kill tumor cells *in vitro*, as well as when injected in tumor bearing mice, the widespread biological effects of TNF on mesenchymal and other cells have been the focus of studies related to its inflammatory properties,

particularly in mediating synovial cell activity and cartilage and bone degradation. TNF activates B cells and T cells, including the expression of IL-2 receptors. TNF has many varied proinflammatory activators: it acts as a pyrogen, strongly activates polymorphonuclear leucocytes (PMN), effect endothelial permeability and adhesion properties, enhances major histocompatibility complex (MHC) expression and induces acute phase reactants and other mediators of inflammation, such as IL-1, IL-6, prostaglandins and leukotrienes. The effects of TNF can be either beneficial or deleterious to the host, according to the circumstances and the quantity of its production (*Gallin et al 1992*).

1.14.3.2 IL-1 β (Interleukin-1 β)

There is a dramatic increase in IL-1 production by a variety of cells in response to infection, microbial toxins, inflammatory agents, products of activated lymphocytes, complement, and clotting components. IL-1 is considered to be a mediator in disease and in production of systemic "acute-phase" responses. IL-1 β was cloned from human blood monocytes. The human IL-1 β gene is 7.8 kb, which located on chromosome 2. Within the various animal species, the sequence of amino acids of IL-1 β , is conserved in the range of 75-78% (*Gallin et al 1992*).

1.14.3.3 Interferons

IFNs are part of our immune system proteins. They are synthesized and secreted by specialized cells upon induction by viral or other challenges. They function by binding to highly specific receptors on their target cells and these specific interactions with their receptors cause signal transduction to the nucleus and thus regulates the production of about 50 different genes.

The first bioactivity of IFNs to be discovered became the basis for naming them: thus they were named "interferon" because of their interference with the replication of viruses. Through their receptors binding interferons act pleiotropic, meaning they mediate various biological activities like, antiviral, antiinfectious, antiproliferative, cytotoxic and immunomodulating. All these activities are of high pharmaceutical value as shown by several clinical applications in cancer and viral infection treatment. There are three classes of Interferons: 16 subgroups of IFN-alpha, one IFN-beta and one IFN-gamma. The Interferons are relatively small proteins with 143 to 172 aminoacids ranging from 19 to 45 kDa. They are modified by glycosylation. Interferon-gamma is a homodimer while the others are monomers (*Gallin et al 1992*).

1.14.3.3.1 IFN- γ (*interferon gamma*)

Interferon- γ is a homodimeric glycoprotein with 143 aminoacids and it is less antiviral than the type I Interferons, with much higher antiproliferative and immunomodulatory activities. IFN- γ is expressed from a single gene that contains three introns and is located on chromosome 12. IFN- γ is one of the most common products of both helper and suppressor/cytotoxic phenotype T cell clones and stimulates its own production. Thus, during a physiologic immune response involving T cells, it can be anticipated that IFN- γ usually will be released along with other cytokines. Within 15 minutes of binding IFN- γ to the human IFN- γ receptor, there is a two to five fold increase in the basal level of phosphorylation of the receptor on serine and threonine residues. Although activation of protein kinase C leads to phosphorylation of the IFN- γ receptor, the kinase that phosphorylates the receptor in response to IFN- γ is apparently distinct from protein kinase C (*Gallin et al 1992*).

1.14.3.3.2 IFN- α (Interferon-alpha)

IFN- α has antiviral, antiproliferative, and immunomodulatory functions. IFN- α is produced in the course of an immune response, especially during viral infections. Macrophages and other antigen-presenting cells are the main cellular sources of IFN- α . Immunomodulatory functions of IFN- α include the enhancement of natural killer (NK) cell and T-cell cytotoxicity. IFN- α induces the expression of IFN-sensitive genes (ISGs). The human IFN-alpha gene lack introns. It lies on chromosome 9. IFN- α appears to play a role in maintaining the corpus luteum (*Gallin et al 1992*).

Objectives

- ◆ To investigate the expression of *MEFV* in primary cultures of human peritoneal fibroblast.
- ◆ To investigate the effects of colchicine (the main therapeutic agent for FMF patients) and certain inflammatory cytokines (IL-1 β , TNF- α , IFN- α , IFN- γ) on *MEFV* expression in neutrophils and primary peritoneal fibroblast cultures.
- ◆ To examine the expression pattern of induced *MEFV* in normal human primary fibroblast cultures with the inflammatory cytokines and colchicine and its correlation with C5a inhibitor activity in these cultures.
- ◆ To investigate the difference of *MEFV* expression level in normal human peritoneal fibroblast compared to *MEFV* expression in human neutrophils.

Materials and Methods

2.1 Materials

Recombinant C5a (rC5a) was purchased from Sigma (St. Louis, MO, USA) and was dissolved in distilled-H₂O containing 2.5mg/ml of bovine serum albumin (BSA). Dulbecco's phosphate-buffered saline (PBS), Hank's balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), F-10 medium (Beit Haemek, Israel) which used for fibroblast cultures, Trypsin-EDTA solution B, L-Glutamine solution, Antibiotic (Pen-Strep solution) were obtained from biological industries (Beit Haemek, Israel), and fetal calf serum (FCS) from Gibco, (Grand Island, NY, USA). Interleukin-1 β (IL-1 β), Interferon- γ (IFN- γ), Interferon- α (IFN- α) and Tumor Necrosis Factor- α (TNF- α) were purchased from (Pepro Tech Inc., Rocky Hill, NJ, USA). Colchicine was purchased from (Sigma; St. Louis, MO, USA) and was dissolved in distilled water. Phorbol 12-myristate β -acetate (PMA), Pottasium chloride (KCl), O-phenylene diamine dihydrochloride tablets, Cytochalasin B, Hydrogen peroxide (H₂O₂), Hydrogen chloride, Tris-base, Ethylene Diamine Tetraacetic Acid (EDTA), Puc-marker were purchased from (Sigma; St. Louis, MO, USA). Ficoll-hypaque dextran 70 (Macrodex 6% in 0.9% NaCl) were obtained from (Pharmacia Piscataway, NJ, USA). RNase H reverse transcriptase (Superscript II), dNTPs mix (dATP, dGTP, dCTP, dTTP) were purchased from (Gibco BRL, Gaithersburg, MD, USA). Hexamer primer, RNase inhibitor, was obtained from (Amersham Pharmacia biotech). RNA isolation kit was purchased from (Boehringer Mannheim, Indianapolis, IN, USA). Supertherm DNA polymerase was purchased from Hoffmann-La-Roche (SRP-801), Agarose gel was purchased from BioLab. *MEFV* primers designed from exon 8 and 10 or 9 and 10 were

purchased from (Sigma St. Louis, MO, USA). All other chemicals were of molecular grade and were purchased from Sigma.

2.2 Cell Cultures

M9K and JMN (mesothelioma cell lines) were generously provided by Dr. Brenda Gerwin, National Institutes of Health, Bethesda, MD, USA. Peritoneal biopsies were obtained from patients undergoing surgical procedures because of hernia or suspected appendicitis. Skin biopsies were obtained from FMF patients and healthy volunteers. Primary cultures were established and maintained by a modification of the method of *Matzner et al (1986)*. Briefly, the biopsy material was incubated in 0.025% EDTA/ 0.125% trypsin solution at 4°C over night and then transferred to new, identical solution, incubated at 37°C for 20 min and teased apart. The monolayers thus obtained were identified as fibroblasts by their morphology. Freshly passaged fibroblasts were prepared for an experiment by growing in F-10 medium containing 20nM L-glutamine, 100 units/ml penicillin, 100mg/ml streptomycin and 20% FCS and passaged after confluence. In all experiments, the numbers of cells were 0.5×10^6 cells/T25 flask. After 4 days, the supernatant and non-adherent cells were removed, the monolayers were washed, and fresh F10 medium supplemented with FCS was added. Fibroblasts were incubated without (control) or with IL-1 β (10ng/ml), TNF- α (10ng/ml), IFN- α (1ng/ml), IFN- γ (10ng/ml), PMA (100nmol/L) or colchicine (0.02, 0.2, 2, 10, 50 μ M). Fibroblasts were also incubated with a combination of either one of the inflammatory cytokines [TNF- α (10ng/ml), IFN- α (1ng/ml) or IL-1 β (10ng/ml)] with 10 or 50 μ M colchicine, to investigate the possibility of synergistic or antagonistic effect on *MEFV* expression. Experiments assaying concomitantly, *MEFV* expression and C5a-inhibitor activity, were carried out in two sets: One set was harvested after one day of incubation for RNA extraction and RT-PCR analysis. The cells incubated with PMA were

harvested also after 1 hour. For the second set, the medium was replaced with serum-free medium for an additional 24 hours; supernatants were then harvested for analysis of C5a-induced myeloperoxidase release.

2.3 Neutrophils

Blood was collected from healthy volunteers in citrated tubes mixed with 6% dextran at a ratio of 2:1, left in the syringe at upright position for 60 minutes at room temperature for sedimentation, then the upper layer (rich with leuckocytes) was transferred into another tube and centrifuged for 12 minutes at 1000 rpm, 4°C. The pellet was washed twice with 1ml ice cold 0.6M KCl and 3 ml ice cold water for hypotonic lysis, the cells were resuspended in PBS buffer, then the ficoll hypaque was added slowly, followed by centrifugation for 25-30 minutes at 4 °C, 1000rpm, the pellet was washed with PBS, the cells (>90% neutrophils were suspended at 10⁶ cells/ml) were resuspended in F10 media containing 20% FCS. Neutrophils were incubated without (control) or with IL-1 β (10ng/ml), TNF- α (10ng/ml), IFN- α (1ng/ml), IFN- γ (10ng/ml), or colchicine (10 μ M). The cells were harvested after 3 hours of incubation for RNA extraction and RT-PCR analysis.

2.4 RNA preparation

Fibroblast cells were resuspended in 200- μ l PBS buffer; cell lysis was accomplished by incubation of the sample in a lysis/binding buffer. RNase was inactivated at the same time. The RNA was prepared and purified according to kit protocol. The bound RNA was purified from salt, proteins and other cellular impurities by several washes with buffer and then eluted in 50 μ l H₂O. (High pure RNA isolation

kit, Boehringer Mannheim). The RNA integrity was examined by agarose gel electrophoresis.

2.5 Reverse Transcription -PCR

Total RNA from Neutrophils was prepared using Tri ReagentTM (Molecular Research Center, Cincinnati, OH). Total RNA from the various cultured fibroblasts was prepared using High pure RNA isolation kit, (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer protocol. All samples included the same amount of RNA (4µg RNA/sample) in the RT reaction. RNA concentration indicated by multiplying the RNA absorbance at 260nm by 40µg/ml and the ratio of purity calculated ($O.D_{260}/O.D_{280}$) was (1.8-2). RNA absorbance was indicated using (Uvikon 930, Kontron Instrument). The cDNA was synthesized using hexamer primer and the superscript II RNase H reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA). The cDNA was amplified with Supertherm DNA polymerase, (SRP-801, Roche Diagnostics GMBH, Mannheim, Germany) and primers designed previously by the lab from exon 8 and 10 or 9 and 10 of *MEFV*. The primer sequences from exons 8 and 10 were forward 5' -TTCAATGTTCCAGAGCTG-3'; Reverse 5' -TGTAGTCCACGAAGATGC-3', respectively. The primers from exons 9 and 10 were: Forward 5'-GATTGGCGCTCAGGCACATGCTGTTA-3'; Reverse 5'-GTCGGGGGAA CGCTGGACGCCTGGTA-3', respectively. PCR mixtures (each reaction include 2.5µl dNTP (2mM), 2.5µl (10x buffer), 0.1µl (Supertherm DNA polymerase), 1µl primer (β -actin\ *MEFV*), 1µl cDNA and the volume was completed to 25µl with sterile distilled water) were incubated in a thermocycler (MJ Research Inc., Watertown, MA, USA) under the following conditions: 1 cycle of 2 minutes at 95⁰C followed by 35 cycles each consisting of 30 sec. at 95⁰C, 30 sec. at 55⁰C, and 30 sec. at 72⁰C, and, at the end,

followed by 1 cycle of 10 min. at 72°C. The amplified products were separated by electrophoresis on a 2% agarose gel. Ethidium bromide staining of the agarose gel was used to detect the amplified fragments (420-bp and 351-bp, respectively). Amplification of a fragment of the housekeeping gene β actin (220-bp fragment) was used as a control for successful amplification of the cDNA. β -actin primers were 5'-AAGTGTGACGTTGACATCCG-3' and 5'-GATCCACATCTGCTGGAAGG-3'. Negative control included replacement of the cDNA with H₂O in the PCR reaction.

2.6 Myeloperoxidase (MPO) Release

C5a-induced MPO release from neutrophils was used to measure the presence of C5a inhibitor activity in conditioned media of human primary fibroblast cultures, as described by *Matzner et al (1990)*. Briefly, 50 μ l of 5nmol/L rC5a and 50 μ l of the conditioned media derived from T₂₅ flasks after 24 hours starvation for C5a induced myeloperoxidase release analysis were each loaded into 3 wells in a 96-well microtiter plate and incubated for 20 min at 37°C. Twenty-five μ l of freshly prepared human neutrophils (4X10⁶ cells/ml in HBSS/25mM Hepes/0.25% BSA) that had been incubated with 5 μ g/ml cytochalasin B for 10 min at 37°C, were added to each well. Degranulation was allowed to proceed for 10 min at 37°C and MPO release was then measured using ELISA reader (Dynatech MR 5000). The results were corrected for MPO release in the absence of rC5a and compared with those obtained in wells containing rC5a in the absence of a putative source of C5a-inhibitor.

2.7. Calculations

MPO release was corrected for enzyme release in the absence of rC5a. Inhibition of MPO release was calculated in comparison with its release in wells containing rC5a in the absence of a putative source of C5a inhibitor ("culture") as follows:

$$\text{Percent inhibition} = 100 \times \left[1 - \frac{\text{MPO release with a putative source of C5a inhibitor}}{\text{MPO release without a putative source of C5a inhibitor}} \right]$$

Induced culture medium was always assayed in comparison with culture medium in the absence of an inducer. Results are expressed as mean \pm SE. Significance was determined by the paired t-test.

2.8. Transfection experiment

MEFV were introduced into 293 cell line (Human embryonic kidney cell line) by calcium phosphate transfection experiment. Briefly 293 cell line was cultured in 5mm plate with DMEM media, each plate contain 0.8×10^6 cells/5ml DMEM media and incubated for 24 hours at 37 C°, 5% CO₂. All plates were washed with PBS, supplied with fresh DMEM medium. A mixture of 125 μ l (2x HEPES), 12.5 μ l (2.5M CaCl₂), 112.5 μ l (Tris-EDTA, pH 8) and 2 μ g PC3-DNA (recombinant plasmid DNA) was added to each plate of 293 cells for 48 hour. Three types of DNA were used : PC3-*MEFV*, PC3-GFP, PC3- β -GAL. Plasmid DNA was introduced to monolayer cell cultures via precipitate that adheres to the cell surface and takes up the DNA precipitate through an as yet undetermined mechanism. The efficiency of transfection was checked under the florescence microscope. All the plates were washed with colorless DMEM and supplemented with 2.5 ml of colorless DMEM without fetal calf serum for 24-hour starvation. The media was collected for MPO assay and the cells were harvested for RT-PCR reaction.