BIRZEIT UNIVERSITY

Faculty of Graduate Studies: MCLS Program

Molecular Analysis of MEFV Gene and Factor V Leiden Mutations among Palestinian Patients with Behçet's Disease

By

Hilal Abu Rmaileh

Supervised by
Dr. Suhail Ayesh
Dr. Tamer Essawi

Thesis
Submitted in Partial Fulfillment of the Requirements for the Masters Degree in Clinical Laboratory Sciences from the Graduate Faculty at Birzeit University
Palestine

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This Thesis is Dedicated to:

My Mother and Father,
My Sisters and Brothers,
My Friends (Aysha and Nashwa),
And Mr. Iyad Barghouti.

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Hilal Abu Rmaileh

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LIST OF ABBREVIATIONS

BD	Behçet's Disease
ISG	International Study Group
DVT	Deep Venous Thrombosis
TNF	Tumor Necrosis Factor
IL	Interleukin
HSP	Heat Shock Proteins
HSV	Herpes Simplex Virus
MHC	Major Histocompatability Complex
λs	Sibling recurrence risk ratio
OR	Odds Ratio
MICA	MHC class I chain related A
APCR	Activated Protein C Resistance
ICAM-1	Intercellular Adhesion Molecules-1
sICAM-1	Soluble ICAM-1
LPS	Lipopolysaccharide
ESR	Erythrocyte Sedimentation Rate
CRP	C-Reactive Protein

FMF	Familial Mediterranean Fever
MEFV	MEditerranean FeVer
aa	Amino Acids
rfp	Ret Finger Protein
DDs	Death Domains
DEDs	Death Effector Domains
CARDs	Caspase Recruitment Domains
ASC	Apoptosis associated Speck-like protein with a CARD
ICE	IL-1β Converting Enzyme
NF-κB	Nuclear Factor-κB
IKK	IκB Kinase
PSTPIP1	Proline Serine Threonine Phosphatase-Interacting Protein 1
PAPA	Pyogenic Arthritis Pyoderma gangrenous and Acne
SNP	Single Nucleotide Polymorphisms
NAJ	Non-Ashkenazi Jews
OD	Optical Density
PCR	Polymerase Chain Reaction
ARMS	Amplification Refractory Mutation System
ASP	Allele-Specific PCR
PASA	PCR Amplification of Specific Alleles
ASA	Allele-Specific Amplification
TAE	Tris-Acetate EDTA

χ^2	Chi-square
RAS	Recurrent Aphthous Stomatitis

AIMS AND OBJECTIVES

(*i*) Determine the prevalence and significance of MEFV mutations and Factor V Leiden mutation among Palestinian patients with Behçet's disease.

(*ii*) Assess the MEFV mutations profile in Palestinian Behçet's disease patients.

(*iii*) Determine whether the clinical severity of Behçet's disease phenotype correlates with the nature of MEFV mutation.

(*iv*) Evaluate the significance of MEFV gene analysis for Behçet's disease diagnosis.

ABSTRACT

Keywords: Behçet's disease**,** Familial Mediterranean fever, MEFV, Pyrin, Factor V Leiden.

Behçet's disease (BD) and familial Mediterranean fever (FMF), both inflammatory diseases, are highly prevalent in the Middle Eastern and Mediterranean populations. BD is a multisystemic disorder with major manifestations of recurrent orogenital ulcerations, skin lesions, and uveitis, in which vasculitis plays an important pathogenic role. BD is a polyfactorial disease closely associated with HLA-B51. On the other hand, FMF is characterized by recurrent, short, self-limited episodes of fever, serosal inflammation manifested mainly by sterile peritonitis, pleurisy, and arthritis, and gradual development of nephropathic amyloidosis. FMF is an autosomal recessive disease linked to a gene located on the short arm of chromosome 16, designated "MEFV". It encodes a novel protein called "pyrin" or "marenostrin" that is expressed predominantly in neutrophils and monocytes and believed to play a role in inflammation and apoptosis.

Considering the phenotypic and epidemiological similarities between FMF and BD, the inflammatory reactions that underlie the genesis of both diseases, and that factor V Leiden mutation may play a role in the hypercoagulable state in BD, we identified MEFV and factor V Leiden mutations in 44 Palestinian patients with Behçet's disease who did not meet the clinical criteria for FMF. We screened for 34 MEFV mutations, 9 MEFV polymorphisms, and factor V Leiden mutation in 88 chromosomes from 28 definite BD patients (meeting the criteria of the International Study Group for BD), 12 probable BD patients (meeting at least two of these criteria), and 4 suspected BD patients (meeting at least one of these criteria).

Four patients carried two mutated MEFV alleles, and fourteen had one mutated MEFV allele. Altogether, 22 of the 88 chromosomes studied bore MEFV mutations/polymorphisms. Furthermore, 18 BD patients (40.9%)

had nine different MEFV mutations and two polymorphisms, which represents the sensitivity of testing these mutations in our samples. V726A, M694V, M694I, and E148Q mutations and P706 polymorphism have been previously described in BD patients, whereas F479L, P369S, R408Q, and A744S mutations and F721 polymorphism are reported in BD patients for the first time in this study. Interestingly, we identified a novel MEFV mutation that have not been reported before in either FMF or BD patients. This mutation encodes a substitution of methionine for valine at position 722 (V722M). In addition, factor V Leiden mutation was detected in only five of the forty four (11.4%) studied BD patients.

This study is the first genetic analysis of the MEFV and factor V Leiden mutations among Palestinian BD patients. It reflects the mutations profile in our BD patients. Our results were in concordance with other studies in providing further data that MEFV gene mutations are an additional genetic susceptibility factor in BD, and therefore, the genetic testing for MEFV mutations may be advisable in the investigation of BD among patients of Mediterranean origin. The population comprising this study may not be enough to role out the correlation between MEFV mutations and the clinical manifestations of BD on one hand, and factor V Leiden and venous thrombosis on the other hand. Therefore, studies in large number of BD series are needed.

الملخص

يعتبر كل من مرض البهجت و حمى البحر الأبيض المتوسط العائلية من الأمراض المنتشرة في منطقة حوض البحر الأبيض المتوسط. مرض البهجت هو التهاب أوعية معمم يتميز بتقرحات فموية متكررة، وتقرحات تناسلية، وآفات جلدية، وإصابة عينية، بالإضافة إلى إصابة مفصلية وعصبية وهضمية. بينما حمى البحر الأبيض المتوسط العائلية هي مرض وراثي يتميز بنوبات متكررة من ارتفاع درجة حرارة الجسم مصاحبة لآلام بالبطن والصدر والمفاصل نتيجة التهاب الأغشية المغلفة لها. أهم مشاكل هذا المرض التي تظهر نتيجة لعدم العلاج هو الترسيب البروتيني (أميلويدوزيز). في عام 1997م توصل الباحثون إلى معرفة الجين المرتبط بحمى البحر الأبيض المتوسط العائلية الموجود على الكروموسوم رقم 16 والذي أطلق عليه اسم "MEFV". هذا الجين ينتج بروتين يدعى "بايرين" يلعب دورا في تقليل حدة الالتهاب، إذا حدثت طفرة في هذا الجين فإن حدة الالتهاب لا تكون منضبطة بشكل طبيعي مما يسبب نوبات الحمى للمرض.

نظرا للتشابه القائم بين مرض البهجت وحمى البحر الأبيض المتوسط العائلية في الظواهر الإكلينيكية والتوزيع الجغرافي، إلى جانب دور بروتين البايرين في التفاعلات الالتهابية، بالإضافة إلى أن طفرة لايدن العامل الخامس وجد بأنها تلعب دورا في حالة التخثر الزائدة التي تظهر في مرض البهجت، قمنا بفحص كل من طفرات ال MEFV وطفرة لايدن في 44 مريض فلسطيني مصابين بمرض البهجت. يجب الإشارة هنا إلى أن هؤلاء المرضى والتي شملتهم هذه الدراسة لا تنطبق عليهم المعايير الإكلينيكية الخاصة بتشخيص مرض حمى البحر الأبيض المتوسط العائلية. في هذا البحث قمنا بمسح جيني ل 34 طفرة MEFV و 9 من المتعددات الشكلية في جين ال MEFV و طفرة لايدن ل 88 كروموسوما من 28 مريض مؤكد أنهم مصابين بمرض البهجت (تنطبق عليهم معايير مجموعة الدراسة العالمية لمرض البهجت)، و 12 مريض محتمل أنهم مصابين بمرض البهجت (ينطبق عليهم على الأقل معيارين من هذه المعايير)، و 4 مرضى متوقع أنهم يعانون من مرض البهجت (ينطبق عليهم معيار واحد من هذه المعايير).

بعد التحليل الجيني وجد أن 22 كروموسوما من أصل ال 88 التي تمت دراستها احتوت على طفرات أو متعددات شكلية لجين ال MEFV. 4 مرضى حملوا كروموسومين ذي صفة طفرية بينما 14 مريضا آخرين حملوا كروموسوما واحدا ذي صفة طفرية. علاوة على ذلك، 9 طفرات مختلفة واثنتان من المتعددات الشكلية لجين ال MEFV تم تشخيصها في 40.9 % من مرضى البهجت، هذه النتيجة تمثل حساسية فحص طفرات ال MEFV في المرضى الفلسطينيين الذين يعانون من مرض البهجت. عدد من الطفرات والمتعددات الشكلية التي تم تشخيصها في هذه الدراسة قد تم وصفها في مرضى البهجت في دراسات سابقة مثل: V726Aو M694V و M694Iو E148Q و P706. بينما عددا آخر مثل: F479L و P369S و R408Q و A744Sو F721 تم تسجيلها في مرضى البهجت للمرة الأولى في هذا البحث. من الجدير بالاهتمام، أنه تم الكشف عن طفرة MEFV جديدة تمثل استبدال الحامض الأميني من مثيونين إلى

فالين على كودون رقم 722 (V722M)، هذه الطفرة لم يتم تسجيلها مسبقا في كل من مرضى حمى البحر الأبيض المتوسط أو مرضى البهجت. أضف إلى ذلك، أنه تم تشخيص طفرة لايدن في خمسة مرضى فقط (11.4%) من أصل ال 44 مريضا الذين شملتهم هذه الدراسة.

تعتبر هذه الدراسة التحليل الجيني الأول لطفرات ال MEFV وطفرة لايدن بين المرضى الفلسطينيين المصابين بمرض البهجت, بالإضافة إلى أنها تعكس صورة هذه الطفرات في مرضانا من ناحية أخرى. نتائج هذه الدراسة تتفق مع نتائج الدراسات الأخرى بكونها تزود معطيات إضافية أن طفرات ال MEFV هي عبارة عن عامل جيني إضافي مؤثر في مرض البهجت، ولذلك، فإن التحليل الجيني لطفرات ال MEFV يمكن أن يكون ملائما لفحص مرضى البهجت الذين تنحدر أصولهم من منطقة البحر الأبيض المتوسط. قد يكون عدد المرضى الذين شملتهم هذه الدراسة غير كاف لاستبعاد الصلة بين طفرات ال MEFV وبين الظواهر الإكلينيكية لمرض البهجت من جهة، والصلة بين طفرة لايدن وبين حالة التجلط في هذا المرض من جهة أخرى. ولذلك، تقتضي الحاجة إلى دراسات على عدد أكبر من مرضى البهجت.

CHAPTER I

INTRODUCTION

CHAPTER I

INTRODUCTION

I.1 BEHÇET'S DISEASE

Behçet's disease (BD) is a multisystemic inflammatory disease characterized by recurrent oral and genital ulcers, uveitis and skin lesions, as well as articular, vascular, neurological, intestinal and pulmonary manifestations [Sakane *et al.*, 1999].

I.1.1 Historical Background

One of the earliest descriptions of an illness, which bears similarities to BD, is found in Hippocrates writing in 450 B.C. from ancient Greece. Many of the features described are recognized in the various disease criteria in use today:

"But there were also other fevers – Many had their mouth affected with aphthous ulcerations. There were also many defluxions about the genital parts, and ulcerations, boils externally and internally, about the groins. Watery ophthalmies of chronic character, with pains; fungous excretions of the eyelids, externally and internally, called fici, which destroyed the sight of many persons. There were also attacks of carbuncle

through the summer – and large ecthymata and herpetes (rash) in many instances" [Verity *et al.*, 1999a].

An earlier reference to this collection of clinical signs is reported from the writings of the Chinese physician Zhong-Jing Zhang in about 200 A.D., but no further description is encountered in the medical literature for 1,700 years. Not until the early of 1900s, were individual features such as recurrent relapsing hypopyon uveitis, oral aphthae and combined orogenital ulcerations were described by Blüthe in 1908, Planner and Remenovsky in 1922, Shigeta in Japan in 1924, Gilbert in 1925, Dascalopoulos in 1932, and Whitwell in Great Britain in 1934 [Verity *et al.*, 1999a; McCarty, 1989].

Behçet's disease owes its name to the Turkish physician professor Hûlusi Behçet, who, in 1937, described the classic trisymptom complex of hypopyon, iritis, and orogenital aphthosis [Behçet, 1937]. Behçet first saw a patient in 1924/25 who had oral and genital aphthae, eye involvement, and nodular lesions in his legs. Syphilis, tuberculosis, staphylococci, or streptococci were interpreted in the etiology by ophthalmologists who followed the relapsing and remitting uveitis. The patient, who underwent several iridectomies, completely lost his vision. The other attacks continued with wider intervals. Behçet closely followed this patient and carefully evaluated his attacks. The second patient was a woman who emerged in 1930s with several-year history of oral aphthae, genital ulcers, and

hemorrhage in the eye. Syphilis, tuberculosis, and fungal tests were performed in addition to biopsies and laboratory examinations. The results were not compatible with a known disease. A male patient who had pemphigus-like lesions in his mouth was referred in 1936 from a dental clinic. He had acneiform lesions in the back, ulcers in the scrotum, hemorrhage and loss of vision in the eye, and fevers and myalgias were observed mainly at night. Behçet proposed that these signs belong to a specific disease entity and suggested a virus as a causative agent. Eventually, in 1947, he gained widespread international recognition for this finding [Saylan *et al.*, 1999]. The Greek physician Benediktos Adamantiades reported the disease in 1931, accounting for the alternative eponym Adamantiades-Behçet disease [Adamantiades, 1931]. Adamantiades defined thrombophlebitis as the fourth cardinal sign of the disease and pointed out that the disease can occur for years as a monosymptomatic disorder and that eye involvement and severe prognosis are more common in men than in women [Zouboulis and Kaklamanis, 2003].

I.1.2 Epidemiology

Cases of BD cluster in countries spanning the Mediterranean basin and the Far East. This led to the synonym of Silk Road disease. These regions

lie between latitudes 30º and 45º north. One attractive hypothesis is that BD spread through Asian and Eurasian populations from Japan to the Middle East, together with its associated HLA allele, HLA-B51, as a result of the movement of nomadic or Turkish tribes traveling the ancient trading Silk Route [Verity *et al.*, 1999a].

The prevalence of BD varies in different parts of the world. Turkey has the highest prevalence, where two epidemiological studies were done. A prevalence of 80 per 100,000 populations were reported in nine villages near Istanbul [Tüzün *et al.*, 1996], and a higher prevalence of 370 per 100,000 in a rural area in northern Turkey [Yurdakul *et al.*, 1988]. The prevalence in Japan, Korea, China, Iran, and Saudi Arabia ranges between 13.5 to 20 cases per 100,000, whereas it is lower in Western countries ranging between 0.64 per 100,000 in the United Kingdom and 0.12 to 0.33 per 100,000 in the United States, 21 per 100,000 among citizens of Turkish origin in Germany and 0.42 to 0.55 per 100,000 among German natives [Sakane *et al.*, 1999; Saylan *et al.*, 1999]. A prevalence of 120 per 100,000 was reported in an Arab community in Israel [Jaber *et al.*, 2002].

Behçet's disease is somewhat more common among females in Japan, Korea, United States and Germany, whereas males are more frequently affected in Middle Eastern countries [Sakane *et al.*, 1999]. The male to female ratio is 3:2; however, males often have more severe disease [Önder and Gürer, 1999]. The onset of BD occurs typically in the third or fourth

decade of life, and the frequency within families is 2% to 5% in Europe, while in Middle Eastern countries, it is 10% to 15% [Sakane *et al.*, 1999].

I.1.3 Diagnosis

In the absence of a diagnostic laboratory test, BD is identified by clinical diagnosis, that is based on a proposed criteria by the International Study Group (ISG) for BD in 1990, as summarized in Table I.1 [International Study Group for Behçet's Disease, 1990]. The ISG criteria are widely accepted in Middle Eastern countries, whilst Japan, Korea, and other countries in the Far East use the revised Japanese criteria that consist of four major symptoms and a number of minor features [Saylan *et al.*, 1999]. The major symptoms include oral and genital aphthae, skin lesions, and ocular disease. Minor features include arthritis without deformity, neurological symptoms, gastrointestinal lesions, epididymitis, and vascular lesions [Behçet's Disease Research Committee of Japan, 1974].

Inclusion of recurrent oral ulceration as a non-optional criterion may exclude approximately 3% of patients considered to have definite BD, in whom the disease exists without a history of oral ulceration [International Study Group for Behçet's Disease, 1990]. Besides, the ISG criteria omit the minor symptoms or signs that may indicate a possible diagnosis of BD, such as arthralgias and arthritis; subcutaneous thrombophlebitis, deep vein

thrombosis, gastrointestinal ulcerations; and central nervous system disease involvement [Önder and Gürer, 1999].

The pathergy test is useful for evaluating skin irritability and is considered a diagnostic criterion [International Study Group for Behçet's Disease, 1990]. The test consists of pricking a sterile needle into the patient's forearm. The results are judged to be positive by a physician, when the puncture causes an aseptic erythematous nodule or pustule that is more than 2 mm in diameter at 24 to 48 hours [Özarmagan *et al.*, 1991; Akmaz *et al.*, 2000]. Positive pathergy test is an important parameter in the diagnosis of BD in Middle Eastern countries [Saylan *et al.*, 1999].

The differential diagnosis of BD includes chronic oral aphthosis, herpes simplex virus infection especially with recurrent aseptic meningitis, Sweet's syndrome, Stevens-Johnson syndrome, HLA-B27–related syndromes such as ankylosing spondylitis and Reiter's syndrome, and inflammatory bowel disease [McCarty, 1989; Sakane *et al.*, 1999].

I.1.4 Clinical Manifestations

The widespread organ involvement in BD is well recognized as illustrated in Figure I.1. Remissions and exacerbations characterize the course of the disease [Verity *et al.*, 2003].

I.1.4.1 Oral Ulcerations

The hallmark of BD is recurrent multiple oral ulcerations, that sometimes precedes other manifestations by a number of years [Bang *et al.*, 1995]. The frequency of this finding is 96% to 100 % in Middle Eastern countries and Japan [Saylan *et al.*, 1999; Sakane *et al.*, 1999]. Painful oral ulcers appear in the gingiva, tongue, buccal and labial mucosal membranes. The typical ulcer appears round, with a sharp, erythematous border, and the surface is covered with a yellowish pseudomembrane as shown in Figure I.2-A [Kelley *et al.*, 1993]. Oral ulcers are typically the first to come and last to leave in the course of the disease and they may become less common after about 20 years of the disease onset [Bang *et al.*, 1995].

There are three types of aphthous lesions in BD: minor, major, and herpetiform [Main and Chamberlain, 1992]. Minor ulcers are superficial with a diameter of 2 to 6 mm and appear as multiple lesions, developing within 1 to 2 days. They heal without scaring within 7 to 10 days and recur at various frequencies. Major ulcers are seen less frequently. They are deeper and painful, have a diameter of more than 10 mm with a grayish white ulcer base, and heal in 2 to 6 weeks leaving scars. The ulcers that are observed the least consist of the herpetiform variety. They are numerous, hundred in number and grouped as small ulcers with a diameter of 2 to 3

mm, and heal in a short time [Main and Chamberlain, 1992; Önder and Gürer, 2001].

I.1.4.2 Genital Ulcers

Genital ulcers consist one of the cardinal manifestations of BD that occur in about 75% of patients. They are most commonly found on the scrotum and penis in men and on the vulva in women as shown in Figure I.2-B. Genital ulcers are painful and morphologically similar to the oral ulcers but they are usually larger and deeper with an irregular margin, which recur in 3 weeks and usually leave scars. Recurrence is typically less frequent than oral ulcerations [Saylan *et al.*, 1999; Kelley *et al.*, 1993].

I.1.4.3 Cutaneous Manifestations

Cutaneous lesions occur in over one-half of patients with BD. The most frequent cutaneous findings consist of papulopustular manifestations and erythema nodosum-like signs [McCarty, 1989]. Erythema nodosum-like lesions are common in female patients and usually occur on the lower extremities, but can also be seen on the arms, neck and face. The areas of erythema are slightly elevated and characterized by painful livid, purple-colored nodules surrounded by a peripheral halo as shown in Figure I.2-D. Their numbers can be restricted to three to four or they may be more numerous. These lesions are usually resolved spontaneously, leaving a

deeply pigmented area, but they sometimes ulcerate [Sakane *et al.*, 1999; Önder and Gürer, 1999].

Papulopustular eruptions are a neutrophil-induced, vessel-based reaction, referred to as "pseudofolliculitis" lesions that are common in male patients and are distributed on the back, face, and neck, especially along the hairline. The other types of papules are observed in the extremities [Saylan *et al.*, 1999; Önder and Gürer, 2001].

I.1.4.4 Ocular Lesions

Inflammation of the eye is the main cause of morbidity in patients with BD, and affects 50% to 70% of men and 20% to 30% of women [Yazici, 2002]. Although it typically occurs after the onset of oral aphthosis, the delay between the two may be as long as 14 years. Conversely, intraocular inflammation is the presenting feature in over 10% of patients, and in rare cases oral aphthosis may not occur at all [Verity *et al.*, 2003].

Ocular involvement typically consists of a relapsing pan uveitis that affects both the anterior and posterior chambers and of episodes of retinal vasculitis [Nussenblatt, 1997]. The majority of ocular cases eventually suffer bilateral disease, but in 6% of cases, the disease remains uniocular. Patients with anterior ocular problems have better visual prognosis than posterior uveitis, but the later is more common. The cumulative destruction caused by the attacks and the vasculitic involvement of the optic nerve and

retinal vessels result in permanent visual loss [Önder and Gürer, 1999; Nussenblatt, 1997].

Hypopyon, a visible layer of pus in the anterior ocular chamber, is characteristic of BD, observed in 6–30% of the patients as shown in Figure I.2-C [Sakane *et al.*, 1999].

I.1.4.5 Articular Manifestations

Articular findings are observed in about 50% of the patients with BD and constitute one of the major manifestations of the disease. It can be seen as arthralgias or arthritis. The pattern is monoarticular or oligoarticular, although a symmetric or asymmetric polyarthropathy may also occur. Arthralgias are always more frequent than true arthritis, but arthritis itself develops in more than half of the patients. The knee is most commonly involved, followed by the ankle, elbow, and wrist; small joints are less commonly involved [McCarty, 1989; Yurdakul, 1983].

Onset of arthritis tends to be insidious and duration variable, ranging from few weeks to several years. The intervals between arthritic attacks tend to be longer than those between attacks of extra-articular manifestations. Morning stiffness is present, while permanent changes and disability of the joints are rare [Önder and Gürer, 1999; Yurdakul, 1983].

I.1.4.6 Vascular Manifestations

The vasculopathy of BD is unique, and although involvement of blood vessels of all types and sizes is a prominent feature of the disease, veins are affected more often than arteries. The vascular involvement which occurs in approximately 25% of the patients with BD includes both venous and arterial thrombosis, small vessel vasculitis, and arterial aneurysms [Koç *et al.*, 1992; Kiraz *et al.*, 2002]. It is usually seen in the first 5 years and can be the initial symptom, besides, it is more frequently and severe in males. Superficial thrombophlebitis is the most frequent form of venous involvement seen in a frequency of 7.3% as an initial finding whereas it occurs in 38% of the cases during the course of the disease, followed by deep venous thrombosis (DVT) especially in the lower extremities [Koç *et al.*; Saylan *et al.*, 1999].

Occlusion in large vessels especially that of superior and inferior vena cava and the hepatic vein is common. Arterial involvement is more rare compared to venous involvement (12% compared to 20-40% of all vascular involvement) but has a greater morbidity. Multiple spindle-shaped aneurysms are the most common lesions and predominantly affect the aorta, renal arteries, and popliteal arteries. Vascular lesions in the lung, cause recurrent episodes of dyspnea, cough, chest pain, and hemoptysis. [Hassikou *et al.*, 2002; Kiraz *et al.*, 2002].

I.1.4.7 Neuro - Behçet's Disease

Neurological involvement is noted in 5% to 10% of patients, particularly males in whom the disease began at an early age. It appears mainly in the parenchymal form (80%) or as dural sinus thrombi (20%). The parenchymal form has a more serious prognosis that is usually associated with brain stem involvement; whereas the dural sinus thrombi are associated with headache and intracranial pressure [Akman-Demir *et al.*, 1999; Yazici, 2002].

I.1.4.8 Gastrointestinal Involvement

Gastrointestinal involvement in BD can present as ulcerative lesions, most frequently occurring in the ileocaecal regions, but ulcers have been described in other parts of the small and large bowel as well as the esophagus and stomach. Gastrointestinal involvement causes abdominal pain, diarrhea, melena, and sometimes perforation. These findings are more common in Japan (10% to 40%) and rare in Mediterranean countries (for example 1% in Turkey) [Chung *et al.*, 2000].

I.1.4.9 Other Rare Findings

Renal Behçet's disease is most often mild in nature. Glomerulonephritis, amyloidosis, renal vascular involvement, interstitial nephritis, and other problems, such as complications of drug therapy or genitourinary system abnormalities have been associated with BD. The

frequency of renal problems among BD patients has been reported to vary between 0% to 55%. Male gender is a risk factor for all types of renal BD [Akpolat *et al.*, 2002a]. Cardiac complications in BD have been reported relatively rarely. Pancarditis, acute myocardial infarction, conduction system disturbances, and valvular disease have been described. Intracardiac thrombus formation is very uncommon [Türsen *et al.*, 2002]. Muscular involvement in BD has been disclosed in only few cases. It presents as a predominantly myositic lesion, localized mostly in the lower extremities, although in few cases, other localizations may occur such as right arm and generalized weakness [Worthmann *et al.*, 1996]. Epididymitis, fever, hearing disturbances and headache may also occur in patients with this disorder [McCarty, 1989].

I.1.5 Pathology

Behçet's disease is a systemic perivasculitis, in which early neutrophil infiltration, endothelial cell swelling, and fibrinoid necrosis are well described [Ergun *et al.*, 1998]. Significant neutrophil infiltration is seen in all early lesions, including mucocutaneous aphthae, pathergy reaction, nodular cutaneous lesions, and ocular lesions [Hegab and Al-Mutawa, 2000]. Neutrophils from patients with BD have increased superoxide production, enhanced chemotaxis, and excessive production of lysosomal

enzymes, indicating that the neutrophils are overactive, which leads to tissue injury [Fordham *et al.*, 1982; Takeuchi *et al.*, 1981; Efthimiou *et al.*, 1989; Freitas *et al.*, 1998]. In addition, serum levels of neutrophil priming cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-8 have been reported to be elevated [Mege *et al.*, 1993].

I.1.6 Etiopathogenesis

Behçet's disease is a polyfactorial disease, associated with multiple hereditary and environmental risk factors, as illustrated in Figure I.3 [Verity *et al.*, 2003]:

I.1.6.1 Behçet's Disease-An Infective Etiology

Two areas of research now support a microbial role in the BD. First; skin and peripheral blood monocytes have been shown to exhibit a hypersensitivity response to certain *Streptococcus sanguis* antigens in patients but not in healthy controls [Verity *et al.*, 2003]. These bacterial antigens share amino acid sequences in common with one class of cell membrane proteins called heat shock proteins (HSP), which are expressed on cell membranes in response to physiological shock and microbial challenge. Several groups have demonstrated that both *Streptococcus sanguis* and the human HSP (especially the 60/65 kDa HSP) activate γδT

cells in patients but not controls. This observation has led to the suggestion that, following bacterial challenge, oral mucosal cells express HSP which are antigenic and lead to antimucosal T cell cross reactivity in susceptible individuals [Direskeneli, 2001; Freysdottir *et al.*, 1999]. On the other hand, serum antibodies to herpes simplex virus (HSV)-1 and circulating immune complexes with HSV-1 are both reported to be raised in BD patients [Direskeneli, 2001]. Moreover, HSV DNA has been demonstrated in the genital and intestinal ulcers of patients with BD, but not in oral ulcers [Lee *et al.*, 1996]. Second, an animal model for BD was described in 1998, in which 30% of HSV-1 inoculated mice were reported to exhibit BD-like symptoms, which included skin, tongue, gastrointestinal and genital ulcers, ocular inflammation and arthritis [Sohn *et al.*, 1998].

I.1.6.2 Heritable Risk Factors for Behçet's Disease

Family Studies

Behçet's disease is not a monogenic disorder, and no clear Mendelian pattern of inheritance has yet emerged. Nevertheless, a number of features point to a genetic etiology. The unusual geographical distribution of disease and its close association with the major histocompatability complex (MHC) allele HLA-B51 are perhaps the strongest indicators that certain gene(s) are either directly responsible for disease, or promote indirectly the

characteristic underlying inflammatory changes [Verity *et al.*, 1999a]. Furthermore, familial aggregation in BD is well recognized [Koné-Paut *et al.*, 1999]. Recently, the sibling recurrence risk ratio (λs) among patients in Turkey is reported to be 11.4 – 52.5 that support a hereditary basis for BD [Verity *et al.*, 2003]. Further evidence for hereditary risk factors is the finding of genetic anticipation in BD, in which earlier disease onset is encountered in children of affected parents [Fresko *et al.*, 1998].

HLA-B51

A heritable risk factor for BD was first identified in 1982, when an association with HLA-B5 in the human MHC was first reported [Ohno *et al.*, 1982]. In the great majority of racial groups particularly those from the Middle to Far East, the greatest heritable risk factor for BD, and indeed for ocular disease or progressive central nervous system disease severity, is HLA-B51 [Mizuki *et al.*, 2000; Mizuki *et al.*, 2001; Verity *et al.*, 1999b]. HLA-B51 is relatively frequent, ranging from 50% to 80% in patients in many ethnic groups, as compared with 10% to 20% in healthy individuals, with relative risk ranging from 1.38 to 20.70 [Sano *et al.*, 2001; Cohen *et al.*, 2002]. Recent data indicate that while at least 21 different alleles may exist at the HLA-B*51 locus, both HLA-B*5101 and HLA-B*5108 are associated with BD [Paul *et al.*, 2001; Mizuki *et al.*, 2002]. Where HLA-B*51 is common, BD is encountered, and where this allele is rare, so too is

disease. However, HLA-B*51 cannot be the sole causative factor for the simple reason that roughly a third group of patients, even in countries with high disease prevalence, do not possess this gene [Verity *et al.*, 2003]. Furthermore, the odds ratio (OR) of BD in an individuals positive for HLA-B*51 shows a variation from 1.5 to 16 in different series [Verity *et al.*, 1999a].

Tumor Necrosis Factor

An association between BD and alleles in the TNF promoter region is reported in Japanese and Middle Eastern patients [Mizuki *et al.*, 1992; Verity *et al.*, 1999b]. Two possible alleles may exist at the promoter site, TNFB*1 and TNFB*2 [Pociot *et al.*, 1993; Wilson *et al.*, 1997]. The latter is associated with higher TNF production by stimulated monocytes, and might therefore lead to a more severe and prolonged inflammatory response than the former and is not only more prevalent among patients with BD but is also weakly associated with a poor visual outcome. However, the TNFB*2 allele is also in linkage with HLA-B51, and these two alleles may therefore be co-inherited, each contributing both to disease risk and to severity of organ involvement [Verity *et al.*, 1999b; Verity *et al.*, 2003].

MHC Class I Chain Related Gene

The MHC class I chain related A (MICA) gene appeared to be a strong candidate as the gene responsible for susceptibility to BD based on its

chromosomal localization [Bahram *et al.*, 1994], its restricted and heat shock-induced expression in epithelial cells [Groh *et al.*, 1996], its predicted immunologic function as a ligand of γδ T cells [Groh *et al.*, 1998], and a strong associations between certain alleles of the MICA gene (MICA-A6 and MIC-A009) and BD [Mizuki *et al.*, 1997; Wallace *et al.*, 1999]. Subsequent analysis revealed a tight linkage between these alleles and HLA-B51, which itself remained the strongest genetic risk factor for BD [Mizuki *et al.*, 2000].

Factor V Leiden Mutation

In 1994, the molecular basis of activated protein C resistance (APCR) was discovered in Leiden Holland, and has been shown to be a single point mutation in the coagulation factor V gene, which results from a G→A transition (CGA→CAA) at nucleotide position 1691, leading to the substitution of arginine by glutamine at amino acid position 506 [Bertina *et al.*, 1994]. Factor V Leiden was found in ~20–40 % of patients with idiopathic DVT and it is also suggested that this mutation increases the risk of DVT in BD [Gül *et al.*, 1996; Koşar *et al.*, 2002; Kiraz *et al.*, 2002]. Furthermore, Factor V Leiden mutation is found to be associated with ocular BD [Verity *et al.*, 1999c].

ICAM-1 Gene Polymorphisms

The intercellular adhesion molecules-1 (ICAM-1) gene is known to contain at least two polymorphic sites, situated at codon 241 (GGG→AGG; Gly→Arg) and at codon 469 (AAG→GAG; Lys→Glu) [Vora *et al.*, 1994]. The gene products of these two regions are thought to correspond to structural domains in the ICAM-1 molecule which are involved in ICAM-1 binding to the Mac-1 leukocyte ligand (codon 241) and leukocyte LFA-1 (codon 469), and it has been hypothesized that the two mutations, G241R and K469E, result in more effective binding of ICAM-1 to Mac-1 and LFA-1, thereby enhancing the inflammatory response [Vora *et al.*, 1994; Joling et al., 1994]. Evidence that ICAM-1 is involved in the pathogenesis of BD comes from histological studies of BD lesions in which ICAM-1 is expressed and from reports that levels of soluble ICAM-1 (sICAM-1) are not only increased in the disease, but are also associated with disease activity [George *et al.*, 1997; Verity *et al.*, 2000]. While the pathogenic role of these polymorphisms is unknown, studies show that the ICAM-1 mutations are associated with BD susceptibility in different populations [Verity *et al.*, 2000; Kim *et al.*, 2003].

IL-1 Gene Polymorphisms

Serum levels of pro-inflammatory and Th1-type cytokines, including IL-1, have been reported to be elevated in BD [Yosipovitch *et al.*, 1995]. In

addition, peripheral blood monocytes from patients with BD produce significantly more IL-1 when stimulated with bacterial lipopolysaccharide (LPS) than monocytes from normal controls or from patients with other inflammatory diseases [Mege *et al.*, 1993]. Given the involvement of IL-1 in BD, IL-1 gene cluster polymorphisms was investigated and suggested to be associated with an increased risk for BD by contributing to the enhanced inflammatory reactivity observed in these patients [Karasneh *et al.*, 2003]. Susceptibility to BD is increased in individuals carrying both the IL-1A –889C and IL-1B +5887T haplotypes. Individuals who are both homozygous CC at IL-1A –889 and TT at IL-1B +5887 appear to have twice the risk of developing BD as individuals having other IL-1A –889/IL-1B +5887 genotypes [Karasneh *et al.*, 2003].

I.1.7 Laboratory Findings

Laboratory findings are mainly nonspecific indices of inflammation. A mild anemia and leukocytosis is observed in 15% of chronic patients [Saylan *et al.*, 1999]. Patients with active disease may generate an acute phase response leading to a raised erythrocyte sedimentation rate (ESR), increased serum levels of C-reactive protein (CRP), and elevated plasma complement component such as C3, C4, C9, and Factor B [McCarty, 1989].

Elevated IgG, IgA, IgM, and α2-globulin are often seen in disease exacerbations [Önder and Gürer, 1999].

I.1.8 Treatment

The main objectives of BD treatment are to relieve symptoms associated with mucocutaneous lesions and arthritis, control inflammation, minimize functional disability, and prevent recurrences. An outline of BD management is presented in Table I.2. The choice of treatment is empirical that depends on the clinical presentation, the site, and the severity of the manifestations. Priority is given to the treatment of gastrointestinal symptoms, central nervous system involvement, and large-vessel lesions, which require high-dose corticosteroids, immunosuppressants, or both and, in some cases, surgical intervention. Treatment of ocular lesions requires more careful consideration than the treatment of mucocutaneous symptoms [Kaklamani and Kaklamanis., 2001; Sakane *et al.*, 1999].

I.1.9 Prognosis

Clinical course of BD is variable, even in an early stage, making it difficult to determine the long-term prognosis. Men appear to have poorer prognosis. The disease usually runs a protracted course with attacks generally lasting for several weeks and recurring more frequently early in

the disease [Önder and Gürer, 2001]. Mucocutaneous and arthritis involvements usually occur early. Chronic morbidity is usual; the leading cause is ophthalmic involvement, which can result in blindness. The effects of the disease may be cumulative, especially for neurologic, vascular, and ocular involvement. Mortality is low, but can occur from neurologic involvement, vascular disease, bowel perforation, cardiopulmonary disease, or as a complication of immunosuppressive therapy [McCarty, 1989].

I.2 FAMILIAL MEDITERRANEAN FEVER

Familial Mediterranean fever (FMF) is an autosomal recessive disease, occurs predominantly in populations inhabiting or originating from Mediterranean basin, namely Jews, Arabs, Armenians and Turks [Eliakim *et al.*, 1981]. FMF is characterized by recurrent episodes of fever and serosal inflammation manifested by sterile peritonitis, pleuritis and arthritis [Langevitz *et al.*, 1999].

I.2.1 MEFV Gene and Pyrin

The gene responsible for FMF, denoted MEFV (short for **ME**diterranean **F**e**V**er) is located on the short arm of chromosome 16 and is made up of 10 exons [The International FMF Consortium, 1997; The French FMF Consortium, 1997]. MEFV encodes a 781 amino acids (aa)

protein called "pyrin" for its role in the regulation of pyrexia by the International consortium [The International FMF Consortium, 1997], and "marenostrin" in allusion to mare nostrum, the ancient Latin name for the Mediterranean Sea by the French consortium [The French FMF Consortium, 1997], that is predominantly expressed in neutrophils and cytokine–activated monocytes [Tidow *et al.*, 2000]. Pyrin consists of six functional domains as illustrated in Figure I.4:

(i) a PYRIN domain (aa: 1 – 95) [Martinon *et al.*, 2001],

(ii) a bZIP basic domain (aa: 266 – 280) [Shuman *et al.*, 1990],

(iii) a B-box zinc finger domain (aa: 375 – 407) [Reddy *et al.*, 1992],

(iv) two overlapping nuclear localization signals (aa: 420 – 437) [Robbins *et al.*, 1991],

(v) an α-helical or coiled – coil domain (aa: 408 – 594) [Lupas, 1997],

(vi) a B30.2 or ret finger protein (rfp) (aa: 598 – 774) [Henry *et al.*, 1997].

Although the C-terminal half of pyrin shares homology with several transcription factors, transfected pyrin localizes in the cytoplasm and interacts with microtubules via its N-terminal half [Mansfield *et al.*, 2001].

A hypothesis postulates that the normal function of pyrin is to downregulate granulocyte-mediated inflammation, either by

downregulating inflammatory mediators, microtubules, or adhesion molecules, or by transcriptional upregulating anti-inflammatory mediators [Samuels *et al.*, 1998]. One of the most important advances in the past years has been the demonstration that the PYRIN domain is shared by a number of proteins involved in apoptosis and inflammation and is the fourth member of the six-helix bundle death domain-fold superfamily that includes death domains (DDs), death effector domains (DEDs), and caspase recruitment domains (CARDs) [Fairbrother *et al.*, 2001]. Similar to other members of this superfamily, the PYRIN domain appears to allow for the interaction, or assembly, of macromolecular complexes by PYRIN–PYRIN interactions [Liu *et al.*, 2003]. Consistent with this, in vitro studies have demonstrated pyrin to interact specifically with another PYRIN domain protein denoted apoptosis-associated speck-like protein with a CARD (ASC) [Masumoto *et al.*, 2001; Richards *et al.*, 2001]. In addition to ASC N-terminal PYRIN domain, it contains a C-terminal CARD that specifically binds the CARD of procaspase-1 [also referred to as IL-1β converting enzyme (ICE)], leading to its aggregation and autoactivation. Activated caspase-1 then processes pro- IL-1β to IL-1β, which is secreted and interacts with the IL-1 receptor to mediate inflammation [Srinivasula *et al.*, 2002; Stehlik *et al.*, 2003]. Recently, in vivo significant of the interaction between pyrin and ASC was established by demonstrating increased IL-1β processing and defective LPS and IL-4-induced apoptosis in peritoneal

macrophages from pyrin knockout mice. In this study, a proposed model of pyrin in regulating ASC-mediated caspase-1 oligomerization and IL-1β processing was defined as illustrated in Figure I.5 [Chae *et al.*, 2003]. Pyrin also inhibits ASC-mediated apoptosis and nuclear factor-κB (NF-κB) activation in transfected cells by disrupting: the interaction between ASC and cryopyrin [Dowds *et al.*, 2003], and the interaction between ASC and Ipaf, thus inhibits NF-κB activation and apoptosis mediated by IκB kinase (IKK) and Caspase-8, respectively [Masumoto *et al.*, 2003].

The anti-inflammatory role of pyrin has been further evidenced by the identification of mutations in proline serine threonine phosphatase-interacting protein 1 (PSTPIP1), a protein that have been found to interact with pyrin in vitro, through pyrin's B-box zinc finger domain. The mutations in PSTPIP1 underlying pyogenic arthritis, pyoderma gangrenous, and acne (PAPA) syndrome lead to hyperphosphorylation and a marked increase in the strength of the interaction with pyrin. Increased pyrin-PSTPIP1 binding may, in turn, modulates pyrin's normal immunoregulatory function by preventing its interaction with other proteins such as ASC, thus sequesters pyrin, resulting in elevated IL-1β synthesis [Shoham *et al.*, 2003].

I.2.2 MEFV Mutations

To date, about 53 MEFV mutations have been identified. 47 are missense mutations, one is nonsense mutation, two are insertion mutations, and three are small deletions as presented in Table I.3.

Mutations have been found in exons 1, 2, 3, 5, 7, 9 and 10 of the MEFV gene as shown in Figure I.6 [Hull *et al.,* 2003]. There are two apparent mutational "hot spots": one in exon 10 and one in exon 2. Remarkably, two hot spots are also contained within exon 10, one at codon 680 [Dodé *et al.*, 2000] and one at codon 694 [Bernot *et al.*, 1998]. Three mutations have been identified at codon 680, and four mutations at codon 694. Interestingly Y688X [Notarnicola *et al.*, 2000], the sole premature stop codon found to date in FMF, is located between these two codons. In exon 2, two mutations have been found at codon 148 [Bernot *et al.*, 1998; Touitou, 2001]. In addition, a number of MEFV single nucleotide polymorphisms (SNP) corresponding to silent substitutions have also been identified as shown in Table I.4.

In general, most missense mutations in MEFV gene resulted in a conservative change of a hydrophobic amino acid. Although this kind of amino-acid change often has little or no phenotypic effect, its impact can be much more dramatic [Touitou, 2001]. Several mechanisms – such as modification of conformation or stability, alteration of a binding site or

other sites of interaction – could account for phenotypic effects of such mutations [The French FMF Consortium, 1997].

I.3 ASSOCIATION BETWEEN BD AND FMF

Behçet's disease and Familial Mediterranean fever, have for centuries been highly prevalent in the Middle Eastern and Mediterranean populations, namely in Turks, Arabs, and Non-Ashkenazi Jews (NAJ), and have been recognized as distinct entities on the basis of a panel of clinical features in the absence of a specific laboratory test [International Study Group for Behçet's Disease, 1990; Samuels *et al.*, 1998]. Both diseases are inflammatory, periodic, multisystemic, and share some common symptoms, such as: sterile inflammation, recurrent febrile attacks, abdominal and arthritic signs due to serositis, cutaneous features and acute scrotum [Drenth *et al.*, 2001; Sakane *et al.*, 1999]. It is interesting to note that, in patients of Mediterranean origin; these periodic conditions are sometimes difficult to distinguish because their main symptoms are not specific and may be associated in a same family and in a same patient. For example, recurrent fever, a key symptom in FMF, is also observed in pediatric BD patients [Touitou *et al.*, 2000]. Furthermore, colchicine, a drug known to inhibit neutrophil chemotaxis, is extremely effective in preventing attacks of FMF and ameliorating some of the clinical manifestations of BD [Ben-Chetrit *et*

al., 1998]. In addition, the most severe complication of FMF is progressive amyloidosis of the AA type; BD may be complicated with amyloidosis of this type; which occurs to a lesser extent in BD patients than in FMF [Akpolat *et al.*, 2002a; Grateau, 2000]. Vasculitis is usually considered to be the central pathological feature in BD, and although a possible autoimmune cause of FMF has not been confirmed, several vasculitis-associated clinical entities among FMF patients, such as Henoch-Schönlein purpura, polyarteritis nodosa (approximately 5% and 1% respectively), and protracted febrile myalgia have been reported more commonly than in the general population [Ozen, 1999; Tekin *et al.*, 2000]. As with FMF, the pathophysiology of BD is still unknown [Direskeneli, 2001; Ozen, 2003]. In both diseases, increased chemotaxis of neutrophils [Fordham *et al.*, 1982; Territo *et al.*, 1976] and high serum levels of IL-1β, IL-8, IL-6, and TNF-α have been described [Direskeneli *et al.*, 1999; Mege *et al.*, 1993; Baykal *et al.*, 2003].

Recently, the possible association between FMF and BD has been of interest. A number of patients with FMF and BD (FMF-BD) have been described [Birlik *et al.*, 1998; Schwartz *et al.*, 2000; Ben-Chetrit *et al.*, 2002], and it was postulated that BD coexistence with FMF is higher than expected [Schwartz *et al.*, 2000]. Furthermore, MEFV mutations have been associated with an increased risk of BD [Touitou *et al.*, 2000] and, conversely, the presence of BD might increase the likelihood of a single

MEFV mutation causing FMF [Livneh *et al.*, 2001]. Besides, Schwartz *et al.* has suggested that BD should be included among vasculitidis complicating FMF [Schwartz *et al.*, 2000]. Moreover, a BD patient complicated with amyloidosis was reported to have M680I/M680I mutation, suggesting that the relationship between FMF and BD may be more complex than implied by the description of BD as a vasculitis complicating FMF [Akpolat *et al.*, 2002b].

CHAPTER II

MATERIALS AND METHODS

CHAPTER II

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II.1 STUDY GROUP

II.1.1 Patients

A total of forty four BD patients from different areas of the West Bank and Jerusalem were investigated in the present study, representing a total of 88 independent alleles. All the patients were from Arab Palestinian descent, recruited from Makassed Islamic Charitable Hospital, Augusta Victoria Hospital, and private clinics.

II.1.2 Diagnostic Criteria

Behçet's disease patients were diagnosed by rheumatologists and ophthalmologists. On the basis of the criteria established by the ISG for BD in 1991 [International Study Group for Behçet's Disease, 1990], the selected patients were divided into three groups:

- **Definite type [D]:** patients who fulfilled the ISG criteria for BD; that are, oral aphthous ulcers plus at least two of the followings: genital ulcers, eye lesions, and skin lesions (n = 28).

- **Probable type [P]:** patients who manifested at least two of the International criteria - oral ulcers plus one of the followings: genital ulcers, eye lesions, and skin lesions (n = 12).
- **Suspected type [S]:** patients with recurrent oral ulcers only (n = 4).

Pathergy test was not performed due to difficulties in following up BD patients and interpretation of its results after 24–48 hours. Personal, clinical, drug therapy, family history, and specimen information of the studied patients were obtained through patients' interview, recorded on a standard clinical form. The age of onset was determined as the time of presenting manifestation attributable to BD, even if it is only aphthosis.

To assess the effect of MEFV mutations on BD expression, BD patients were divided into 2 groups, one with MEFV mutations and one without MEFV mutations. Then, both groups were compared for their varying demographic features and clinical manifestations of BD, such as age of onset, and the number of organs involved. Since there is no accepted clinical severity index for BD, Table II.1 represents the severity scoring system utilized in this study, which was proposed by experienced rheumatologists at Makassed hospital. The total score represented an estimation of the disease severity and spread.

II.2 SAMPLES PROCESSING

3 – 4 ml of blood was drawn from all participants into an EDTA Vacutainer tubes.

II.2.1 Buffy Coat DNA Purification Protocol

Genomic DNA was extracted from the buffy coat of blood samples by using a commercially available kit "MasterPure™ Genomic DNA Purification Kit for Blood Use" (Epicentre Technologies Co., U.S.A.), according to the following procedure:

- Blood samples were centrifugated at 1,000 rpm for 15 min.. Carefully, 600 µl of buffy coat were transferred into two 1.5 ml microcentrifuge tubes, each containing 300 µl and mixed thoroughly by vortexing.
- To lyse red blood cells, 1200 µl of Lysis Buffer 1 were added to each tube, inverted 3 times to be mixed and the tube bottom was flicked to suspend any remaining materials. The tubes were incubated for 5 min. at room temperature, then inverted 3 times and flicked as outlined above. After continuing incubation for an additional 5 min., again the tubes was inverted 3 times and flicked.
- The tubes were centrifugated at ≥ 15,000 rpm for 25 sec. in a microcentrifuge (Hettich, Germany).

- The supernatant was discarded leaving approximately 25 µl of liquid. To white blood cells pellet so obtained 600 µl of Lysis Buffer 2 were added and pipetted up and down 5-7 times.
- 250 µl of Precipitation Solution were added and vortexed vigorously for at least 30 sec. leading to protein denaturation, then centrifuged for 10 min. at ≥ 15,000 rpm.
- The supernatant was poured into clean microcentrifuge tubes, and DNA was precipitated by adding 700 µl of cold isopropanol. Thereafter, the tubes were inverted several (30-40) times until a stringy DNA precipitate was visible. The DNA was pelleted by centrifugation at ≥ 15,000 rpm for 5 min..
- Carefully, the supernatant was decanted without dislodging the DNA pellet, which then was washed twice with cold 75% ethanol, air dried at room temperature for a few min., dissolved in 70 µl of TE-Buffer and incubated overnight at room temperature.

II.2.2 Genomic DNA Quantification

The prepared DNA samples were diluted in TE-buffer in a ratio equals 1:100. The optical density (OD) of this DNA dilution was measured at 260 nm and 280 nm against plain TE-buffer as a blank using a spectrophotometer "GENE QUANT II" (Pharmacia Biotech, England). The

OD reading at 260 nm allowed calculation of the nucleic acid concentration in the sample. An OD of 1.0 corresponds to approximately 50µg/ml of dsDNA. The concentration of the prepared DNA samples ranged from 0.219–1.258 µg/µl with a protein concentration ranged from 0.1–2.5 mg/ml. The ratio of the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provided an estimate of the purity of the DNA. Pure preparations of DNA have OD_{260}/OD_{280} value of 1.8. The ratio OD_{260}/OD_{280} of the DNA samples obtained ranged from 1.7–2.0. The OD at 320 nm was used for background compensation, whereas, OD at 230 nm was used as a guide for protein determination using the peptide bond absorbance. Finally, DNA samples were stored at –20ºC till further use.

II.3 MUTATIONS ANALYSIS

II.3.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is an in vitro technique which allows the amplification of a specific DNA fragment that lies between two known DNA sequences. The basic components of a typical PCR mixture are: template DNA, buffer, dNTPs, primers, and the thermostable enzyme DNA polymerase. Initial denaturation of template DNA at 94-98ºC is sufficient to completely denature complex genomic DNA so that the primers can anneal

after cooling. The primer annealing step is an important parameter in optimizing the specificity of a PCR, and the calculated annealing temperature is used as a starting point for experimental work; however, the annealing temperature should also be optimized empirically. Primer extension is performed at 72ºC, which is the optimum temperature for Taq DNA polymerase [Newton and Graham, 1997; Dennis Lo *et al.*, 1998].

II.3.2 Amplification Refractory Mutation System

Amplification Refractory Mutation System (ARMS) assay is a simple, rapid, and highly reliable molecular diagnostic method, used for detecting the presence of both MEFV point mutations and factor V Leiden mutation involving single-base changes in our study. A typical ARMS assay comprises two complementary PCRs; each conducted using the same substrate DNA. One reaction includes an ARMS primer specific for the normal DNA sequence that cannot amplify mutant DNA at a given locus. The second reaction includes a mutant-specific primer that cannot amplify normal DNA. A common second primer is used in both reactions as illustrated in Figure II.1. The allele specificity of these primers is conferred by the 3' nucleotide of the primer which complements one allele but not the other. This specificity is maintained by the absence of a 3' to 5'

proofreading activity in Taq DNA polymerase. ARMS assay has also been described in the literature as allele-specific PCR (ASP), PCR amplification of specific alleles (PASA) and allele-specific amplification (ASA) [Newton and Graham, 1997; Dennis Lo *et al.*, 1998].

One important consideration in an ARMS assay is designing primers which are specific to a particular gene of interest. In our case the MEFV gene. Synthesized oligonucleotide primers of both L110P mutation (T→C transition at nucleotide 329) and P706 polymorphism (G→A transition at nucleotide 2118) were designed and supplied by the manufacturer (GibcoBRL Custom Primers-Life Technologies), then used after reconstitution. Primers for other MEFV and factor V Leiden mutations were used as optimized in the Molecular Genetics Laboratory at Makassed Hospital.

Thirty four MEFV mutations, nine MEFV polymorphisms, and factor V Leiden mutation were analyzed in this study. The MEFV mutations L110P, E148Q, E167D, T267I, P369S, R408Q, F479L, M680IGC, M694V, M694I, and V726A, P706 polymorphism, and factor V Leiden mutation were screened by using ARMS assay. Whereas, the remaining MEFV mutations I640M, P646L, L649P, R653H, E656A, D661N, S675N, G678E, M680IGA, M680L, T681I, Y688C, Y688X, I692del2074-2076, I692del2074-2076, M694del, M694L, K695R, V704I, I720M, A744S, P758S, and R761H, and the polymorphisms R652, S683, A701, S703,

F721, V722, D723 and G764 were screened by direct DNA sequencing of exon 10 of the MEFV gene.

Table II.2 summarizes the primers and PCR conditions used in ARMS assay. PCR conditions for both L110P mutation and P706 polymorphism were calibrated to be used in this study, whereas the PCR conditions for the remaining mutations were used as optimized in Molecular Genetics Laboratory in Makassed Hospital.

All PCR amplification reactions were carried out in a final volume of 25 µl and performed in either OmniGene (Hybaid, England) or MiniCycler^{TM} (MJ Research INC., U.S.A.) containing 200 or 400 ng/µl of purified genomic DNA, 0.625U of *Taq* polymerase (Takara, Japan) and its 1X buffer (10mM Tris-HCL, 50 mM KCL, 1.5mM $MgCl_2$), 0.2 mM dNTPs mix and 200 ng/µl of each primer.

PCR amplification conditions included an initial denaturation step at 94ºC for 9 min., followed by 27-30 cycles (mutation dependent) with second denaturation step at 94ºC for 15 sec., annealing (depended on primers used) for 15 sec., and extension at 72ºC for 30 sec., then a final extension step at 72ºC for 10 min..

II.4 AGAROSE GEL ELECTROPHORESIS

The amplified PCR products were resolved by electrophoresis on a 2% agarose gels and stained with ethidium bromide for analysis, as previously described (Sambrook *et al.*, 1989):

- The agarose gel (Techcomp, Ltd.) was prepared in 1X Tris-Acetate EDTA (TAE) buffer (0.04 M Tris-acetate, 0.001 M EDTA). The solution was cooled to 50–60ºC, and then ethidium bromide was added to a final concentration of 0.5µg/ml and mixed thoroughly.
- 10 µl of PCR products were mixed with 3 µl of Glycerol Based-loading buffer which usually prepared as six-fold concentrated solutions (30% Glycerol in distilled water, 0.25% Bromophenol Blue, 0.25% Xylene cyanol FF).
- The obtained 13 µl were loaded in each of the wells formed in gel. DNA molecular weight marker was included in each gel to allow the estimation of the molecular weight of DNA fragments resolved.
- The horizontal gel was run with an overlay of 3–5 mm of 1X TAE running buffer that contained ethidium bromide to a final concentration of 0.5µg/ml.
- A constant voltage of 100 V was applied using a power pack (Owl Scientific Plastic, Inc.). The run was stopped when the bromophenol

blue and xylene cyanol FF have migrated the appropriate distance through the gel.

- Gels were visualized under 300 nm UV Transilluminator (Dinco & Rheunium Industries, Ltd.) and photographed by the Polaroid GelCam camera (Polaroid, U.S.A.).

II.5 DIRECT DNA SEQUENCING

The strategy for molecular detection of MEFV mutations that based on direct exon 10 sequencing after PCR amplification, is a rapid diagnostic aid performed in Molecular Genetics Laboratory at Makassed Hospital, as well as other molecular genetics laboratories protocol.

II.5.1 Exon 10 Amplification Conditions

Exon 10 was amplified with PCR in 100 µl reaction containing 400 ng/µl of genomic DNA, 200 ng/µl of forward 5'CCCGCAAAGATTTGACAGCTGTATC3' and reverse 5'GGACAGATAGTCAGAGGAGCTGTGTTCTT3' primers, 0.2 mM dNTPs mix, 1X buffer (10mM Tris-HCL, 50 mM KCL, 1.5mM $MgCl_2$) and 2.5U of *Taq* polymerase (Takara, Japan). Cycling parameters included an initial denaturation step at 95ºC for 10 min., followed by 35 cycles of 95ºC

for 30 sec., 55ºC for 30 sec., 72ºC for 45 sec., and a final extension step at 72ºC for 10 min..

The quality of the sequencing template is an important factor in the sequencing reaction. Thus the amplified PCR products of exon 10 which equal 435 bp were separated on 2% agarose gel and purified using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech).

II.5.2 Exon 10 Fragments Purification from Gel Bands Protocol

- An empty 1.5 microcentrifuge tube was weighted to the nearest 10 mg.
- Using a clean razor blade or scalpel, the slice of agarose containing the DNA band to be purified was excised, cut as close to the DNA band as possible and transferred to the pre-weighted 1.5 ml tube.
- The tube containing the agarose slice was weight to the nearest 10 mg, and the weight of the empty tube was subtracted to determine the weight of the slice.
- 10 µl of Capture Buffer was added for each 10 mg of gel slice. The contents were vortexed vigorously.
- The tube was incubated at 60ºC for 15 min., until the agarose is completely dissolved.
- During the incubation, one GFX Column was placed in a Collection tube for each purification to be performed.

- After the agarose is completely dissolved, the contents were centrifuged briefly to collect the sample at the bottom of the tube.
- Thereafter, the sample was transferred to the GFX Column and incubated at room temperature for 1 min..
- The GFX Column and Collection tube were centrifuged in a microcentrifuge at full speed for 30 sec. The flow-through was discarded by emptying the Collection Tube into 1.5 ml tube, while the GFX Column was placed back inside the Collection Tube.
- 500 µl of Wash Buffer were added to the Column, and then centrifuged at 15,000 rpm for 30 sec.
- The Collection Tube was discarded, and the GFX Column was transferred to a fresh 1.5 microcentrifuge tube.
- 35 µl of elution buffer (autoclaved double-distilled water) were applied directly to the top of the glass fiber matrix of the GFX Column, incubated at room temperature for 1 min., and then centrifuged at full speed for 1 min. to recover the purified DNA.
- A capillary was dipped in the tube, filled with enough sample of the purified DNA for measurement that prevented from leaking out by using Cristaseal, and quantified by "GENE QUANT II" (Pharmacia Biotech, England).

- The following parameters: OD at 260 nm, 280 nm, 230 nm, 320 nm, $OD_{260}/OD_{280,}$ DNA concentration (μg/μl), and protein concentration (mg/μl) against plain autoclaved double-distilled water as a blank were measured.

II.5.3 Automated Sequencing

Direct DNA sequencing for the purified exon 10 of the MEFV gene was performed by using ABI PRISM 377 automatic sequencer at Hebrew University. Sequencing was performed in one direction, the forward direction. Then sequenced product of each sample was compared to the wild-type MEFV gene obtained from NCBI Gene Bank (web site: www.ncbi.nlm.nih.gov), accession number AF111163.

II.6 STATISTICAL ANALYSIS

Allele frequencies were estimated by direct counting. The distribution of alleles in patients with Behçet's disease was compared with that of normal controls, and significance was tested by the Chi-square (χ^2) test (2 sided), while t test was employed for comparison of two independent means [Bourke *et al.*, 1985]. Significance was assigned to *P* values < 0.05.

CHAPTER III

RESULTS

CHAPTER III

RESULTS

III.1 CLINICAL DATA

Forty four patients who are clinically diagnosed as having BD comprised the study population. Twenty five (25) of them were males and nineteen (19) were females, with a male to female ratio equals 1.3:1.0. The mean ± SD age of the patients at study inclusion was 28.2 ± 12.7 years (range, 6 to 59). The mean age of onset was 21.2 ± 11.2 years (range, 2 to 50). The age at onset of disease is widely variable; most patients began having symptoms when they were under 30 years of age (34/44; 77.3%), whereas, in 22.7% of patients the symptoms appeared in the fourth and fifth decades of life as given in Table III.1.

Twenty eight (64%) patients met the ISG criteria for BD and had definite [D] disease, while twelve (27%) had probable [P], and four patients (9%) were suspected [S] to have BD. Besides, 25% of the patients had a familial BD history. The main clinical characteristics of the forty four BD patients are given in Table III.2 and the frequency of these features is summarized in Table III.3. Recurrent oral aphthous ulcers, with or without other symptoms, is the most frequent feature, seen in all cases (100%). Oral ulcers are the sole compliant in 9% of patients. The second symptom, in

order of frequency, has been genital ulcers (37/44; 84.1%), then arthritis/arthralgia seen in (30/44; 68.2%). Skin disease is found in 61.4% of cases while vascular and neurologic symptoms are observed in 11.4% and 2.3% of cases, respectively. Eye involvement is diagnosed only in 7 patients (15.9%), whereas gastrointestinal involvement and epididymitis in 2 patients (4.5%) and 3 patients (6.8%), respectively. The frequency of the symptoms did not varied according to the age at onset, duration, and phase of the disease. Table III.4 shows the patterns of BD presentation, in which about half of the patients (47.7%) presented with oral ulcers, genital ulcers and skin lesions, followed by the presentation of oral plus genital ulcers in 22.7% of patients. In only 6.8% of patients all the four sites were affected.

III.2 DISTRIBUTION OF MEFV SEQUENCE VARIATIONS

Of the twelve MEFV variations analyzed by ARMS assay, seven MEFV mutations (E148Q, P369S, R408Q, F479L, M694V, M694I, and V726A), and one P706 polymorphism were detected in our samples, while four mutations (L110P, E167D, T267I, M680IGC) could not be identified. The results of a representative ARMS assay for MEFV mutations are shown in Figures III.1 and III.2. As most of MEFV mutations are clustered in exon 10, its sequencing allows the detection of all known and potential new mutations in this region in a single-step reaction. In addition, it confers the

results of ARMS assay. Therefore, patients were further tested for less common mutations by direct DNA sequencing of exon 10 of the MEFV gene. This revealed the detection of A744S and V722M mutations and one polymorphism F721 in our studied patients. Representative results of sequence electropherograms are shown in Figures III.1 – III.3.

Hence, by means of ARMS assay and direct DNA sequencing of exon 10, eleven different MEFV sequence variations were detected in our samples. Eight mutations (M694V, M694I, V726A, E148Q, F479L, P369S, R408Q, and A744S) have been described elsewhere in FMF patients [The French FMF Consortium, 1997; Bernot *et al.*, 1998; Cazeneuve *et al.*, 1999; Aksentijevich *et al.*, 1999]. Four of the eight mutations (V726A, M694V, M694I, and E148Q) have been previously described in BD patients [Touitou *et al.*, 2000; Ben-Chetrit *et al.*, 2002], whereas the other four mutations (F479L, P369S, R408Q, and A744S) were detected in BD patients for the first time in this study. Further, one, V722M (G→A transition at nucleotide 2164), is a novel MEFV mutation that have not been reported before in either FMF or BD patients. The two remaining MEFV sequence variations are MEFV polymorphisms, one of which (F721) is detected for the first time in BD patients, whereas the other polymorphism (P706) was reported before in BD patients [Touitou *et al.*, 2000].

The genetic test pointed out 18 patients (10 males and 8 females) of the 44 (40.9%) with at least one MEFV mutation or polymorphism. Fourteen patients (14/18; 77.8%) were heterozygotes for one mutation or polymorphism, three patients (3/18; 16.7%) were compound heterozygotes and one (1/18; 5.6%) had 3 mutations: E148Q, P369S, and R408Q. The distribution of MEFV genotypes in our BD patients is given in Table III.5. However, no homozygous mutations were detected in our studied BD patients and no mutations were identified in 26 patients (59.1%), but we cannot exclude that some of our patients had still unknown mutations or rare mutations in other exons.

Four patients had two mutated alleles, and fourteen had one mutated allele; altogether 18 patients carried 22 mutations, a frequency of one mutation per 4 chromosomes tested (22/88). Thus, 25% of the total alleles (22/88) tested positive, while 75% of the total alleles (66/88) tested negative. Table III.6 summarizes the frequency of MEFV mutations among Palestinian BD patients. The most frequent MEFV mutation in our patients, who represent one of the populations commonly affected with FMF, was E148Q substitution, identified in 8 alleles (22.2%). Five patients were heterozygotes for E148Q mutation, two of them are brothers. Two patients were compound heterozygotes, once associated with M694V mutation and once with P706 polymorphism. The eight's patient has a complex allele in which E148Q was associated with both P369S and R408Q mutations.

M694V mutation was found in four alleles (11.1%). This mutation was found in heterozygous state in two patients, while in one patient M694V mutation was present with E148Q mutation, and in another with A744S mutation. V726A mutation was detected in three alleles (8.3%). All V726A mutations were detected in heterozygous state. Each of the remaining mutations: M694I, P369S, R408Q, F479L, A744S, and V722M, besides the polymorphisms P706 and F721 was found in one allele (2.8%).

Table III.7 represents the distribution of MEFV genotypes according to BD status, in which a decrease in genetic variation of identified MEFV mutations from definite to suspected disease status can be noted. Out of the 18 BD patients, 11 (61.1%), 5 (27.8%), and 2 (11.1%) were clinically diagnosed as having definite, probable, and suspected BD, respectively, and genetically bearing one, two, or three MEFV mutations or polymorphisms. It is interesting to note that the following mutations: M694V, M694I, E148Q, F479L, P369S, R408Q, and V722M, and the polymorphism P706 were found in the definite BD patients. Whereas, the three mutations V726A, E148Q and M694V and the polymorphism F721 were found in the probable BD patients. Furthermore, M694V, V726A, and A744S mutations were detected in the suspected BD patients. Within the 28 cases of definite BD, compound heterozygosity and complex alleles was detected in 3 cases, heterozygosity was detected in 8 cases; in 17 cases no mutation was

detected at all. Within the 12 cases of probable BD, only heterozygosity was detected in 5 cases; and no mutations were detected in 7 cases. Two genotypes were identified in the four suspected BD patients, one is heterozygous and the other is compound heterozygous. However, our data on probable and suspected BD patients are based on small numbers and therefore they must be interpreted cautiously.

III.3 GENOTYPE – PHENOTYPE CORRELATION

The clinical severity index was calculated for each patient as shown in Table III.8. To determine whether the clinical severity of the BD correlated with MEFV genotype, the patients were pooled according to the presence or absence of MEFV mutations into two groups. Group one consisted of 18 BD patients with MEFV mutations, while the other group consisted of 26 BD patients with none of the targeted MEFV mutations. No significant differences were found among the two groups with regard to the prevalence of oral ulcers, genital ulcers, skin involvement, articular, or gastrointestinal as shown in Table III.9. In addition, both groups were almost similar for age of onset of BD and no correlation between severity of the disease and MEFV mutations were found in this study. *P* value was not significant in all cases. We then analyzed whether the presence of the MEFV mutations predisposed to a particular manifestation of the BD by comparing the

clinical features among subgroups of patients with one or two MEFV mutated alleles versus patients who did not carry MEFV mutation, and again no relation was observed. Here, it is noteworthy to mention that within the cases we examined, no concomitant FMF was noted, nor any symptoms pointing to it, such as short episodes of fever, polyserositis, or erysipelas-like skin lesion.

III.4 DISTRIBUTION OF FACTOR V LEIDEN MUTATION

Factor V Leiden mutation was detected in five (11.4%) of the forty four studied patients with Behçet's disease. These patients (2 males and 3 females) were diagnosed to have definite Behçet's disease; among them four had a history of oral and genital ulcerations, and skin manifestations, and one manifested with oral ulcers, genital ulcers, and eye lesions. All the patients who tested positive were heterozygous for factor V Leiden mutation as illustrated in Figure III.4. Factor V Leiden mutation was detected in one (20%) of five patients with a history of thrombosis and in four (10.3%) of thirty nine patients without a history of thrombosis (*P* value is not significant).

CHAPTER IV

DISCUSSION

CHAPTER IV

DISCUSSION

The present study focuses on the genetic background of BD, that includes the screening for MEFV and factor V Leiden mutations among Palestinian patients. The diversity and the frequency of the mutated alleles in these patients reflect the importance of our study, by providing further genetic argument that the MEFV gene mutations may participate in the expression of inflammatory diseases other than FMF.

Four patients carried two mutated MEFV alleles, and fourteen had one MEFV mutation. Altogether, 22 of the 88 chromosomes studied (1:4) bore an MEFV mutation. Most importantly, 9% of our study cohort carried two mutated alleles. The prevalence of MEFV mutations in our patients with BD (40.9%) significantly exceeds the prevalence previously determined among the general Israeli-Arab population (10.4%) [Shinawi *et al.*, 2000], $P < 0.01$.

Delineation of MEFV sequence variations in our patients led to the identification of nine different MEFV mutations and two MEFV polymorphisms. The E148Q mutation, which is not part of any recognizable motif or domain in the pyrin, is found in all ethnic groups suffering from FMF, and has been suggested to be a mild mutation with low penetrance

[Aksentijevich *et al.*, 1999; Cazeneuve *et al.*, 1999; Yilmaz *et al.*, 2001], represents the most prevalent mutation observed in 22.2% of the total alleles. Hence, the over–representation of E148Q mutation in BD might to be restricted to ethnic groups commonly affected by FMF and could actually be BD–associated since it exceeded that in FMF patients, in our data and in others [Ayesh *et al.*, 2004; Touitou *et al.*, 2000].

Five of the nine MEFV mutations and the two polymorphisms which were detected in 33.3% of alleles, are located in exon 10 and would be predicted to affect the secondary structure of B30.2 C-terminal domain of pyrin, which is crucial for the function of this protein and mutations at or near this region cause a disease [Kastner, 1998]. The M694V mutation which were found to be the most frequent and severe mutation in FMF patients [Touitou *et al.*, 2001], was observed in 11.1% of alleles. While V726A mutation, a milder mutation than M694V [Touitou *et al.*, 2001], was detected in 8.3% of alleles. Both M694V and V726A mutations are widely distributed in the Mediterranean basin [Kastner, 1998]. Although M694I mutation was found mainly in Arabs FMF patients [The French FMF Consortium, 1997], only one allele (2.8%) carried this mutation in this study. Out of the five most common mutations only M680I mutation was not detected in any of our patients. A744S mutation that is mainly confined to Arabs FMF patients [Touitou *et al.*, 2001], accounted for 2.8% of alleles.

The novel missense mutation which was identified for the first time in the present study, V722M (GTG→ATG) is present only in one allele. A mutation that occurs in less than 1% of the alleles in the population is by definition rare and may be disease-causing, whereas a mutation that is found at higher frequency may be a neutral polymorphism. Reports of mutations resembling polymorphism should include the analysis of 100 chromosomes or mention of frequency [Cotton and Scriver, 1998]. Although the functional significance of V722M mutation remains to be demonstrated, it occurs in exon 10, which is part of the rfp domain of the pyrin protein where most mutations, especially severe and frequent ones, are located. Besides, it was not found in about 592 patients, tested for MEFV mutations in Molecular Genetics Laboratory during the last five years [Unpublished Data]. The patient carrying this mutation was not carrying any other previously reported mutations in the MEFV gene.

In a previous study, the P706 polymorphism has been found to be the most frequent MEFV sequence variation detected in 10.5% of probable BD alleles, suggesting that it may be especially associated with incomplete or atypical BD [Touitou *et al.*, 2000]. In the present study, this polymorphism appeared in one definite BD patient. Interestingly, another polymorphism corresponded to silent substitution: F721 (TTC→TTT) was detected for the first time in our BD patient. This polymorphism was detected before in FMF patients in our population [Unpublished Data] and in others

[http://fmf.igh.cnrs.fr]. Note that these two polymorphisms P706 and F721 are within a small genetic region (aa: 692–726), being the target for 14 FMF mutations and polymorphisms, is certainly critical for the MEFV function. A possible explanation how these intraexonic polymorphisms can affect the corresponding protein function may be a decrease in the MEFV mRNA stability. Another hypothesis is that the P706 and F721 polymorphisms impair the gene regulation through a change in the tridimensional conformation of the DNA molecule [Touitou *et al.*, 2000].

Other MEFV mutations accounted for further 8.3% of alleles: P369S in 2.8%, R408Q in 2.8%, and F479L in 2.8%. The substitutions P369S, is six amino acids N-terminal to the B-box zinc finger domain [Aksentijevich *et al.*,1999]. Regarding the R408Q mutation, the substitution replaces a charged residue by a neutral amino acid; besides, this nonconservative mutation lies in a region that comprises the C-terminal residues of this domain [Cazeneuve *et al.*, 1999]. Additionally, F479L resides within the α-helical (coiled-coil) domain [Bernot *et al.*, 1998]. P369S, R408Q, and F479L are mild mutations for FMF phenotype that could readily lead to conformational changes in the pyrin protein [Touitou, 2001]. Interestingly, the L110P mutation which represented previously 2.6% of BD alleles (in a French family) [Touiyou *et al.*, 2000] was not identified in the present study. This mutation was not reported in Arab FMF patients before and we could not detect it in about thirty FMF samples [Unpublished Data]. On the

other hand, P369S, R408Q, F479L, A744S and V722M mutations, and F721 polymorphism are reported for the first time in BD patients in this study. By further studies, a specific relation between these MEFV mutations and BD might be observed.

Three preliminary studies have emphasized the significance of MEFV mutations among BD patients [Touitou *et al.*, 2000; Ben-Chetrit *et al.*, 2002; Atagunduz *et al.*, 2003]. These results indicate the involvement of MEFV mutations in BD susceptibility. However, Touitou *et al.* screened 57 patients with BD (38 definite and 19 probable) from the following ancestries: Arabs, Turks, NAJ, Italians, and French [Touitou *et al.*, 2000]. Ben-Chetrit *et al.* studied 53 patients with BD (Arabs and Jews) who fulfilled the ISG criteria, analysis of their genomic DNA for MEFV mutations revealed that 16 patients bore such mutations. Two of whom were homozygous for V726A and E148Q mutations and displayed FMF manifestations, including episodic peritonitis with fever and arthritis. None of the remaining 14 BD patients with a single MEFV mutation had any FMF manifestation [Ben-Chetrit *et al.*, 2002]. Atagunduz *et al.* investigated three MEFV mutations (M694V, M680I, and V726A) in 57 Turkish patients suffering from BD. Fifteen BD patients were found to carry one single MEFV mutation (26%), compared to 9.1% of controls. Among 20 BD patients with vascular involvement, 11 patients (55%) had MEFV mutations compared to 4 patients (11%) in the non-vascular group, leading

to the conclusion that MEFV mutations may act as a genetic susceptibility factor for vascular BD [Atagunduz *et al.*, 2003].

The frequency of MEFV mutations among patients suffering from Behçet's disease in different studies is given in Table IV.1. Unfortunately, we could not get more information about Atagunduz *et al.* report to be included in the comparison with other studies. The present study represents the first genetic study of MEFV mutations among Palestinian BD patients. MEFV mutated alleles were identified in 25.0% by this research compared to 23.5% of alleles by Touitou *et al.*, 17.0% of alleles by Ben-Chetrit *et al.*, and 13.2% of alleles by Atagundaz *et al.*. The five most common MEFV mutations in FMF (M694V, M694I, V726A, M680I, and E148Q) accounted for 10.4% of the alleles in the different ancestries BD patients [Touitou *et al.*, 2000], a result less than the approximately equal data: 18.2% of alleles among Palestinian BD patients in the present study and 17% of alleles among the Israeli Arabs and Jews BD patients [Ben-Chetrit *et al.*, 2002]. The same observation was noted for M694V mutation, which was reported in 2.6% of alleles by Touitou *et al.*, compared to 4.6% and 4.7% in our study and Ben-Chetrit *et al.* study, respectively. It is important to point out that M694V mutation was recently reported in 9.7% of the alleles [Atagundaz *et al.*, 2003], a result that exceeds those in the previous mentioned reports and in the present study. One interesting finding in our study is the frequency of E148Q mutation observed in 9.1% of alleles. It

was higher than 5.2% and 5.7% of alleles reported in the other two studies [Touitou *et al.*, 2000; Ben-Chetrit *et al.*, 2002].

In addition, a preliminary partial MEFV mutation analysis suggested that about 60% of FMF patients, who also suffer from BD (FMF-BD), have only a single mutated MEFV gene [Livneh *et al.*, 2001]. This report also suggested that FMF may be expressed in individuals harboring only one coding mutation in MEFV. These findings may reflect a more generalized rule, that FMF may be precipitated in carriers of a single mutated FMF gene by factors unrelated to other MEFV allele and suggest that the FMF phenotype in this cohort was associated with the simultaneous presence of BD. The FMF-BD relationship has been cautiously interpreted thus far; which deserves further clarification.

MEFV mutations among Palestinian BD patients show a diverse picture. They include common and rare mutations, severe and mild mutations, and not only heterozygous mutations but also compound heterozygous and complex alleles appeared. One striking feature of FMF is the description of complex alleles with three mutations [Touitou, 2001]. In this study, we identified the first BD patient having three different MEFV mutations: E148Q/P369S/R408Q, an observation that further documents the MEFV molecular diversity of the complex alleles found in BD. In theory, such alleles could arise either sequentially, by the occurrence of independent mutational events, or by intragenic recombinations between

simple alleles; in FMF, MEFV haplotype analyses rather favor the latter hypothesis [Bernot *et al.*, 1998; Aksentijevich *et al.*, 1999]. It is, important to point that 38.6% of BD patients are responding well to colchicine therapy, eight of them are heterozygotes or compound heterozygotes for MEFV mutations. Hence, it is considerable to direct physicians to the possible beneficial effect of colchicine therapy in BD patients having MEFV mutations. Two of four patients included in the present study that were considered to have suspected BD form, have V726A/- and M694V/A744S genotypes, were solely suffering from recurrent aphthous stomatitis (RAS). Keeping in mind that these patients are relatively young, aged (9, 10, 10.5, and 23 years old), and that this symptom sometimes precedes other manifestations by a number of years, it is reasonable to expect the appearance of other main related BD clinical features in the future. Unlike BD, RAS is the most common oral mucosal disease, affecting about 15–20% of the population, with the oral mucosa being the prime and only site of lesions [Freysdottir *et al.*, 1999].

Perhaps the most interesting and provocative finding of this report is the demonstration that approximately half of BD patients (40.9%) has one, two, or three MEFV mutations and polymorphisms, which represents the sensitivity of testing these mutations in our BD samples. Our data, which include 44 BD patients along with the above finding, confirm that these mutations can be used to identify the molecular defect in Palestinian BD

patients. Conversely, the sensitivity could be somewhat lower if there are patients with BD for whom we have not yet detected mutations on their chromosomes. Because only exon 10 was sequenced for all our BD patients but not the remaining exons, our study might have missed all other known and potentially new MEFV mutations.

All MEFV mutations detected in our study were either heterozygous or compound heterozygous. What advantage heterozygotes MEFV mutations might have – if any on BD patients? A number of FMF patients with only one MEFV mutation have been reported, despite the search for new mutations, makes the interpretation of this status difficult in populations where the frequency of the carrier of one copy of the gene is considered to be between 1/10 and 1/5. This finding suggests that other mutations remain to be discovered in other regions of the MEFV gene, but it cannot be excluded that heterozygotes may present mild or sometimes atypical signs of FMF [Dodé *et al.*, 2000; Aksentijevich *et al.*, 1999; Stoffman *et al.*, 2000; Drenth *et al.*, 2001]. Furthermore, it was postulated that carrying a single copy of a mutated gene produced a modified (but not abnormal) inflammatory response that may have been protective against some pathogen or class of pathogens endemic in the Mediterranean basin. It has been suggested that the role of pyrin in the control of inflammation may be consistent with heterozygotes exhibiting heightened response to certain organism(s) [The International FMF Consortium, 1997; Brenner-Ullman *et*

al., 1994]. Recent studies suggested that when pyrin is defective, the negative feedback may not be established resulting in more pronounced Th1 activity and IFN-γ [Aypar *et al.*, 2003]. This level of augmented inflammation may be expected to predispose FMF patients and carriers of the MEFV mutations to have a pro-inflammatory state. This is evident by the report that healthy carriers of the MEFV mutations have modest but significantly elevated baseline plasma levels of the classic acute phase reactants C-reactive protein, suggesting that their general response to inflammatory stimuli may be upregulated [Tunca *et al.*, 1999]. Upregulation of the inflammatory response in persons with pyrin mutations most likely favors inflammation in general and as such predisposes MEFV mutant carriers to BD and other vasculitides [Schwartz *et al.*, 2000; Livneh *et al.*, 2001; Touitou *et al.*, 2000; Gershoni-Baruch *et al.*, 2003]. Moreover, genetically ascertained FMF patients (homozygotes or compound heteroztgotes), significantly expressed lower levels of MEFV mRNA than did healthy controls. Message levels in healthy carriers were intermediate, which supports a true dose-response relationship between the number of mutations and the abundance of MEFV transcripts. The difference between healthy controls and healthy carriers was significant, demonstrating that the decrease in MEFV mRNA expression is related to a molecular defect independent of FMF symptoms [Notarnicola *et al.*, 2002]. Livneh *et al.* hypothesized that a reduced level of functional pyrin, presumably

characterizing heterozygosity, on the one hand, and increased demand for pyrin, by activated granulocytes of BD, on the other, may ultimately lead to FMF attacks in FMF–BD patients [Livneh *et al.*, 2001]. In addition, occasional reports of autosomal dominant FMF have often been discounted, on the basis that asymptomatic FMF carriers are common in certain populations, and give rise to pseudo-dominant inheritance [Booth *et al.*, 2000].

To interpret the correlation data between the genotype and phenotype, it is important to emphasize that all investigated patients were of Palestinian ancestry; they were therefore expected to share common genetic background, thereby allowing correlation studies in a relatively homogenous population. However, the possibility that the presence of MEFV mutations may aggravate the clinical expression of BD was not supported by our observation. That is, the group of BD patients with MEFV mutations was almost identical to the BD group without them regarding age of onset, organ involvement, and severity of the disease (*P* value was not significant). MEFV mutations were also apparently not associated with a particular BD phenotype, and this remains to be confirmed in a wider selection of patients.

The demographic findings obtained from the Middle Eastern countries including ours are shown in Table VI.2. There is a slight preponderance for males in Turkey, Iran, Egypt, Lebanon, Jordan, Greece, and Palestine

whereas males predominate in Iraq, Kuwait, Tunisia, and Saudi Arabia [Saylan *et al.*, 1999]. In these countries the disease is most frequently diagnosed in the third decade of life, in our study 29.5% of patients were diagnosed in the second decade of life, whereas, 25.0% and 22.7% of patients were diagnosed in the first and third decades of life, respectively. The most frequent manifestation of the disease, as seen in this table, consists of aphthous ulcerations. The frequency of this finding is 96–100% in the Middle Eastern countries, while it is 96–98.2% in Japanese patients. Our results of the clinical expression of BD are in line with reports from Arab countries such as Saudi Arabia, Lebanon, Iraq, and Tunisia insofar as the percentage of patients with genital ulcers, skin manifestations, Arthritis, and gastrointestinal involvement in our study is similar to that found in most of the series studied in those countries. The frequency of eye manifestations found in our study is low according to experience rheumatologists, it does not concur with that reported in other Mediterranean and Asian countries, and might be attributed, in part, to the type of patients included or selected in this study. In the Arab Palestinian community 28.8% of marriages among first cousins and 49.3% of marriages among members of the same clan (hamula) are consanguineous, according to the Palestinian Central Bureau of Statistics [Unpublished Data]. A positive family history was noted in quarter (25%) of our forty four patients with BD. Most cases of BD are sporadic in families and the

parents of the patients are unaffected, but a familial aggregation has been previously reported [Koné-Paut *et al.*, 1999]. A high sibling recurrence risk ratio for BD indicates that strong genetic factors play a part in the mechanism for the familial incidence in this disease [Fresko *et al.*, 1998].

Ethnic background is one of the major contributors to the risk of BD [Krause *et al.*, 2001], but the possibility of an environmental influence cannot be ignored by the observation that BD is rare among Japanese immigrants in Hawaii and California, and that the prevalence of BD among citizens of Turkish origin in Germany is lower than that in Turkey [Sakane *et al.*, 1999]. Moreover, two major factors modulating BD severity appeared to be the age at disease onset and patient's sex [Yazici *et al.*, 1984]. Since the extent of organ involvement and prognosis in BD varies widely from one patient to another, it is likely that not only one but several genetic regions contribute to disease risk and severity. No single causative gene for BD has emerged; the evidence indicates that the underlying immune events in BD are triggered by a microbial antigen and subsequently driven by genetic influences which control leukocytes behavior and the coagulation pathways. While HLA-B51 remains the strongest disease marker and is regarded as an important contributor to the risk of BD in different ethnic groups; up to 81% of Asian patients have this allele [Mizuki *et al.*, 2000; Sakane *et al.*, 1999], other polymorphic regions in MICA [Mizuki *et al.*, 1997], TNF [Verity *et al.*, 1999b], ICAM-1 [Kim *et al.*,

2003] and IL-1 [Karasneh *et al.*, 2003], may contribute to disease by modulating the inflammatory response, and thereby influencing the progression of systemic and ocular disease. Here, it may be suggested that MEFV mutations are among these risk factors in the ethnic groups in which are present, whereas other ethnic groups require other genetic predisposing factors. Indeed, most cases with BD and amyloidosis, besides the familial form of BD have been reported from the Middle East and Mediterranean countries rather than Japan where MEFV mutations are mostly prevalent [Akpolat *et al.*, 2002]. These results provide additional evidence that the MEFV mutations are one of the risk factors in the development of BD. Since IL-1β is an important mediator of fever and inflammation that have been reported to be elevated in Behçet's disease [Yosipovitch *et al.*, 1995], and pyrin is among the proteins involved in the IL-1 inflammatory pathway by inhibiting caspase-1 and IL-1β activation through interaction with ASC [Chae *et al.*, 2003], it is tempting to speculate that pyrin play a key role in the pathogenesis of BD.

Vasculitis is the main pathological process in BD that can partially explain the thrombotic phenomena. Why such thrombosis is not so common in other vasculitides, and why it occurs in only about 25% of patients with BD, is still unclear. It is speculated that a combination of hyperactive coagulation pathways, hypoactive anticoagulation mechanisms, or hypoactive fibrinolysis generate a tendency for thrombogenesis in this

subgroup of patients with BD [Kiraz *et al.*, 2002]. Thus, mutations of the genes encoding proteins of these pathways might cause a predisposition to venous thrombosis. The most common inherited coagulation defect associated with venous thrombosis is APCR. In the majority of the cases, APCR is the phenotypic manifestation of factor V Leiden mutation, which leads to a more slow degradation of the mutant factor by APC [Spek and Reitsma, 2000]. Most individuals are heterozygous for factor V Leiden, but homozygous individuals are also rather common. Heterozygotes have a 3 to 8-fold increased risk for venous thrombosis, whereas homozygotes have a much higher risk, about 50- to 100-fold [Spek and Reitsma, 2000]. Different groups investigated factor V Leiden mutation in patients with BD, and their results are conflicting as given in Table IV.3. This genetic coagulation defect could be as high as 37.5% in BD patients with a history of DVT [Gül *et al.*, 1996] or be as low as zero percent [Kiraz *et al.*, 2002]. Likewise, while factor V Leiden mutation was significantly higher among BD patients, there was no significant difference between BD patients with or without venous occlusion [Toydemir *et al.*, 2000]. Relied on several studies particularly Gül *et al.* report in 1996, factor V Leiden mutation was suggested to play a role in the hypercoagulable state of BD [Gül *et al.*, 1996].

Only five BD patients manifesting vascular involvement in the present study, and one of them was diagnosed to have factor V Leiden mutation. On

the other hand, thrombosis is absent in four definite BD patients carrying factor V Leiden mutation. However, heterozygous polymorphism for factor V is not uncommon in the general populations. In Lebanon it is found to be 14.2%, in Syria 13.6%, and in Jordan 12.3% [Irani-Hakime *et al.*, 2000], and accordingly, the presence of factor V Leiden mutation (11.4%) in our cases could be coincidental. Keeping in mind that, the small numbers of patients in our study group specially those suffering from thrombosis prevents any conclusions being reached. Moreover, since most of our BD patients with the mutant factor V do not universally demonstrate venous occlusions, there may be other contributing factor(s).

To complete the picture in this study, it is important to determine the followings: First, since the pathergy test is one of the diagnostic criteria for BD and this was not performed in the investigation of our BD patients, a large number of patients who are considered to have probable or suspected BD may be positive to this test, thereby, increasing the number of patients having definite BD form included in this research. Second, HLA-B51 typing may allow us to apply more accurate genotype-phenotype correlation. Third, statistically, the population comprising this study may not be enough to role out the correlation between MEFV mutations and the clinical manifestations or phenotypes in patients suffering from BD on one hand, and factor V Leiden mutation and venous thrombosis in these patients on the other hand. Therefore, studies of a larger cohort of patients would be

decisive in demonstrating the relation between MEFV mutations and factor V Leiden mutation and BD and if a particular mutation or combination of mutations are associated with BD. Forth, this study should include the screening of Palestinian healthy individuals in parallel to BD patients for the presence or absence of MEFV mutations and factor V Leiden mutation. Finally, it is noteworthy to mention that out of the 44 BD patients included in this study, 32 patients (72.7%) are from Hebron. Therefore, well-selected BD patients not restricted to a certain geographical area are needed.

In conclusion, we demonstrated and genetically confirmed that MEFV mutations do exit in Palestinian BD patients, and we have shown the diversity and the frequency of mutations in these patients which could probably reflect MEFV mutations profile in the Arab Palestinian BD patients. Our findings coincide with other reports, and may further establish this gene as an additional susceptibility genetic factor for BD. This provides important tools for adapting a molecular diagnostic test for the BD population and further investigations. As our study group represents a small sample of BD patients with 34 MEFV mutations and 9 polymorphisms had been screened, we came to the conclusion that the risk of BD is not as simple as explained by the presence of MEFV mutations. In order to solve this relationship, more population-based, epidemiological, well-collected data in world-wide collaborative studies are warranted.

For future researches, I recommend to measure C5a inhibitor in BD patients based on the possible relation between C5a inhibitor and MEFV mutations [Matzner *et al.*, 2000]. To apply a genotype-phenotype correlation data in BD patients, I recommend to screen for all known genetic loci proposed to date to be involved in the pathogenesis or susceptibility of BD, this may include the screening of: HLA-B51, MICA-1, IL-1, ICAM-1, TNF, and MEFV genes altogether [Mizuki *et al.*, 2000; Mizuki *et al.*, 1997; Verity *et al.*, 1999b; Kim *et al.*, 2003; Karasneh *et al.*, 2003; Touitou et al., 2000]. The past 6 years have witnessed the identification of genes causing seven autoinflammatory diseases, in addition to FMF, most of them encodes proteins that are involved in the regulation of apoptosis, inflammation, and cytokine processing [Hull *et al.*, 2003; McDermott, 2002]. Studying the similarities between these diseases and BD may enable us to identify new genes involved in BD pathogenesis.

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Table I.1 The International Study Group criteria for Behçet's Disease*

Finding	Definition
Recurrent oral ulceration	
	Minor aphthous, major aphthous, or herpetiform ulcers observed by the physician or patient, which have recurred at least three times over a 12-month period
Recurrent genital ulceration	
	Aphthous ulceration or scarring observed by the physician or patient
Eye lesions	
	Anterior uveitis, posterior uveitis, or cells in the vitreous on slit-lamp examination; or retinal vasculitis detected by an ophthalmologist
Skin lesions	
	Erythema nodosum observed by the physician or patient, pseudofolliculitis, or papulopustular lesions; or acneiform nodules observed by the physician in a postadolescent patient who is not receiving corticosteroids
Positive pathergy test	
	Test interpreted as positive by the physician at 24 to 48 hours

*For the diagnosis to be made, a patient must have recurrent oral ulceration plus at least two of the other findings in the absence of other clinical explanations [International Study Group for Behçet's Disease, 1990].

Table I.2 Treatment for Behçet's Disease*

Treatment	Dose	Used as first-line therapy	Used as alternative therapy
Tropical corticosteroids			
Triamcinolone acetonide ointment	3 times a day topically	Oral ulcers	
Betamethasone ointment	3 times a day topically	Genital ulcers	
Betamethasone drops	1 –2 drops 3 times daily topically	Anterior uveitis,retinal vasculitis	
Dexamethasone	1.0 –1.5 mg injected below Tenon 's capsule for an ocular attack	Retinal vasculitis	
Systemic corticosteroids			
Prednisolone	5 –20 mg/day orally		Erythema nodosum,anterior uveitis,retinal vasculitis, arthritis
	20 –100 mg/day orally	Gastrointestinal lesions,acute meningoencephalitis,chronic progressive central nervous system lesions,arteritis	Retinal vasculitis,venous thrombosis
Methylprednisolone	1000 mg/day for 3 days IV	Acute meningoencephalitis,chronic progressive central nervous system lesions,arteritis	Gastrointestinal lesions,venous thrombosis
Tropicamide drops	1 –2 drops once or twice daily topically	Anterior uveitis	
Tetracycline	250 mg in water solution once a day topically		Oral ulcers
Colchicine	0.5 –1.5 mg/day orally	Oral ulcers,†genital ulcers,†pseudofolliculitis,†erythema nodosum, anterior uveitis,retinal vasculitis	Arthritis
Thalidomide	100 –300 mg/day orally		Oral ulcers,†genital ulcers,†pseudofolliculitis †
Dapsone	100 mg/day orally		Oral ulcers,genital ulcers,pseudofolliculitis,erythema nodosum
Pentoxifylline	300 mg/day orally		Oral ulcers,genital ulcers,pseudofolliculitis,erythema nodosum
Azathioprine	100 mg/day orally		Retinal vasculitis,†arthritis,†chronic progressive central nervous system lesions,arteritis,venous thrombosis
Chlorambucil	5 mg/day orally		Retinal vasculitis,† acute meningoencephalitis,,chronic progressive central nervous system lesions,arteritis,venous thrombosis
Cyclophosphamide	50 –100 mg/day orally		Retinal vasculitis,acute meningoencephalitis,chronic progressive central nervous system lesions,arteritis, venous thrombosis
	700 –1000 mg/mo IV		Retinal vasculitis,acute meningoencephalitis,chronic progressive central nervous system lesions,arteritis, venous thrombosis
Methotrexate	7.5 –15 mg/wk orally		Retinal vasculitis,arthritis,chronic progressive central nervous system lesions
Cyclosporine‡	5 mg/kg of body weight/ day orally	Retinal vasculitis†	
Interferon alfa	5 million U/day IM or SC		Retinal vasculitis,arthritis
Indomethacin	50 –75 mg/day orally	Arthritis	
Sulfasalazine	1 –3 g/day orally	Gastrointestinal lesions	Arthritis
Warfarin §	2 –10 mg/day orally	Venous thrombosis	Arteritis
Heparin §	5000 –20,000 U/day SC	Venous thrombosis	Arteritis
Aspirin ¶	50 –100 mg/day orally	Arteritis,venous thrombosis	Chronic progressive central nervous system lesions
Dipyridamole	300 mg/day orally	Arteritis,venous thrombosis	Chronic progressive central nervous system lesions
Surgery	—		Gastrointestinal lesions,arteritis,venous thrombosis

* IV denotes intravenously, IM intramuscularly, and SC subcutaneously; †The efficacy of this drug for this use has been reported in controlled clinical trials; ‡Cyclosporine is contraindicated in patients with acute meningoencephalitis or chronic progressive central nervous system lesions; §This drug should be used with caution in patients with pulmonary vascular lesions; ¶Low-dose aspirin is used as an antiplatelet agent [Sakan *et al.*, 1999].

Table I.3 Description of the pyrin mutations

Usual Name	Location in the gene	Alteration	Sequence Variant	Protein Variant	References
R42W	Exon 1	substitution	124C>T	Arg42Trp	http://fmf.igh.cnrs.fr
S108R	Exon 2	substitution	322A>C	Ser108Arg	http://fmf.igh.cnrs.fr
L110P	Exon 2	substitution	329T>C	Leu110Pro	Domingo *et al.*, 2000
334-335INSG	Exon 2	insertion	335-336insG	Glu112fsX241	http://fmf.igh.cnrs.fr
C.390-391INSGAGGGGAAC	Exon 2	insertion	390_391insGAGGGGAAC	Asn130-Gly131insGluGlyAsn	http://fmf.igh.cnrs.fr
E148Q	Exon 2	substitution	442G>C	Glu148Gln	Bernot *et al.*, 1998
E148V	Exon 2	substitution	443A>T	Glu148Val	Tunca *et al.*, 2000
E163A	Exon 2	substitution	488A>C	Glu163Ala	http://fmf.igh.cnrs.fr
E167D	Exon 2	substitution	501G>C	Glu167Asp	Bernot *et al.*, 1998
E230K	Exon 2	substitution	688G>A	Glu230Lys	Timmann *et al.*, 2001
E251K	Exon 2	substitution	751G>A	Glu251Lys	http://fmf.igh.cnrs.fr
T267I	Exon 2	substitution	800C>T	Thr267Ile	Bernot *et al.*, 1998
P283R	Exon 2	substitution	848C>G	Pro283Arg	http://fmf.igh.cnrs.fr
G304R	Exon 3	substitution	910G>A	Gly304Arg	http://fmf.igh.cnrs.fr
E319K	Exon 3	substitution	955G>A	Glu319Lys	http://fmf.igh.cnrs.fr
R329H	Exon 3	substitution	986G>A	Arg329His	http://fmf.igh.cnrs.fr
R354W	Exon 3	substitution	1060C>T	Arg354Trp	http://fmf.igh.cnrs.fr
P369S	Exon 3	substitution	1105C>T	Pro369Ser	Aksentijevich *et al.*, 1999
R408Q	Exon 3	substitution	1223G>A	Arg408Gln	Cazeneuve *et al.*, 1999
E474K	Exon 5	substitution	1420G>A	Glu474Lys	http://fmf.igh.cnrs.fr
H478Y	Exon 5	substitution	1432C>T	His478Tyr	http://fmf.igh.cnrs.fr
F479L	Exon 5	substitution	1437C>G	Phe479Leu	Bernot *et al.*, 1998
V487M	Exon 5	substitution	1459G>A	Val487Met	http://fmf.igh.cnrs.fr
R501G	Exon 5	substitution	1501C>G	Arg501Gly	http://fmf.igh.cnrs.fr
L559F	Exon 7	substitution	1675C>T	Leu559Phe	http://fmf.igh.cnrs.fr
I591T	Exon 9	substitution	1772T>C	Ile591Thr	Touitou, 2001
I640M	Exon 10	substitution	1920C>G	Ile640Met	http://fmf.igh.cnrs.fr

Table I.3 (Continued) Description of the pyrin mutations

Usual Name	Location in the gene	Alteration	Sequence Variant	Protein Variant	References
P646L	Exon 10	substitution	1937C>T	Pro646Leu	http://fmf.igh.cnrs.fr
L649P	Exon 10	substitution	1946T>C	Leu649Pro	http://fmf.igh.cnrs.fr
R653H	Exon 10	substitution	1958G>A	Arg653His	Timmann *et al.*, 2001
E656A	Exon 10	substitution	1967A>C	Glu656Ala	Touitou, 2001
D661N	Exon 10	substitution	1981G>A	Asp661Asn	http://fmf.igh.cnrs.fr
S675N	Exon 10	substitution	2024G>A	Ser675Asn	Dodé *et al.*, 2000
G678E	Exon 10	substitution	2033G>T	Gly678Glu	http://fmf.igh.cnrs.fr
M680L	Exon 10	substitution	2038A>C	Met680Leu	Dodé *et al.*, 2000
M680IGC	Exon 10	substitution	2040G>C	Met680Ile	French FMF consortium, 1997; International FMF consortium, 1997
M680IGA	Exon 10	substitution	2040G>A	Met680Ile	Aksentijevich *et al.*, 1999
T681I	Exon 10	substitution	2042C>T	Thr681Ile	Booth *et al.*, 1998
Y688C	Exon 10	substitution	2063A>G	Tyr688Cys	http://fmf.igh.cnrs.fr
Y688X	Exon 10	substitution	2064C>G	Tyr688X	Notarnicola *et al.*, 2000
I692DEL2074-2076	Exon 10	deletion	2074-2076del	Ile692del	http://fmf.igh.cnrs.fr
I692DEL2076-2078	Exon 10	deletion	2076-2078del	Ile692del	Bernot *et al.*, 1998
M694DEL	Exon 10	deletion	2078-2080del	Met694del	Booth *et al.*, 1998
M694VExon 10	substitution	2080A>G	Met694Va	French FMF consortium, 1997; International FMF consortium, 1997	
M694L	Exon 10	substitution	2080A>T	Met694Leu	http://fmf.igh.cnrs.fr
M694I	Exon 10	substitution	2082G>A	Met694Ile	French FMF consortium, 1997
K695R	Exon 10	substitution	2084A>G	Lys695Arg	Bernot *et al.*, 1998
V704I	Exon 10	substitution	2110G>A	Val704Ile	http://fmf.igh.cnrs.fr
I720M	Exon 10	substitution	2160C>G	Ile720Met	Medlej-Hashim *et al.*, 2002
V726A	Exon 10	substitution	2177T>C	Val726Ala	French FMF consortium, 1997; International FMF consortium, 1997
A744S	Exon 10	substitution	2230G>T	Ala744Ser	Bernot *et al.*, 1998
P758S	Exon 10	substitution	2272C>T	Pro758Ser	http://fmf.igh.cnrs.fr
R761H	Exon 10	substitution	2282G>A	Arg761His	Bernot *et al.*, 1998

Table I.4 Description of the pyrin polymorphisms

Usual Name	Location in the gene	Alteration	Sequence Variant	Protein Variant	References
Y65	Exon 1	substitution	195C>T	Tyr65Tyr	Bernot *et al.*, 1998
D102	Exon 2	substitution	306C>T	Asp102Asp	International FMF consortium, 1997
D103	Exon 2	substitution	309T>C	Asp103Asp	Bernot *et al.*, 1998
P124	Exon 2	substitution	372C>T	Pro124Pro	http://fmf.igh.cnrs.fr
G138	Exon 2	substitution	414G>A	Gly138Gly	International FMF consortium, 1997
L142	Exon 2	substitution	426G>T	Leu142Leu	Medlej-Hashim *et al.*, 2002
A165	Exon 2	substitution	495C>A	Ala165Ala	International FMF consortium, 1997
R202Q	Exon 2	substitution	605G>A	Arg202Gln	Bernot *et al.*, 1998
S301	Exon 3	substitution	901C>T	Ser301Ser	International FMF consortium, 1997
R314	Exon 3	substitution	942C>T	Arg314Arg	Bernot *et al.*, 1998
P393	Exon 3	substitution	1179C>T	Pro393Pro	Bernot *et al.*, 1998
---	Intron 4	substitution	1356+43A>G	---	International FMF consortium, 1997
E474	Exon 5	substitution	1422G>A	Glu474Glu	International FMF consortium, 1997
Q476	Exon 5	substitution	1428G>A	Gln476Gln	International FMF consortium, 1997
R501	Exon 5	substitution	1503C>T	Arg501Arg	Cazeneuve *et al.*, 1999
I506	Exon 5	substitution	1518C>T	Ile506Ile	Cazeneuve *et al.*, 1999

Table I.4 (Continued) Description of the pyrin polymorphisms

Usual Name	Location in the gene	Alteration	Sequence Variant	Protein Variant	References
D510	Exon 5	substitution	1530T>C	Asp510Asp	International FMF consortium, 1997
---	Intron 5	substitution	1588–69G>A	---	Cazeneuve *et al.*, 1999
---	Intron 6	substitution	1610+95C>T	---	International FMF consortium, 1997
---	Intron 8	substitution	1756+8C>T	---	Cazeneuve *et al.*, 1999
IVS8+8 C-T	Intron 8	substitution	1759C>T	---	http://fmf.igh.cnrs.fr
---	Intron 8	substitution	1764–30T>A	---	Cazeneuve *et al.*, 199
P588	Exon 9	substitution	1764G>A	Pro588Pro	International FMF consortium, 1997
R652	Exon 10	substitution	1956G>A	Arg652Arg	http://fmf.igh.cnrs.fr
S683	Exon 10	substitution	2049G>A	Ser683Ser	Bernot *et al.*, 1998
A701	Exon 10	substitution	2103G>A	Ala701Ala	http://fmf.igh.cnrs.fr
S703	Exon 10	substitution	2109C>T	Ser703Ser	http://fmf.igh.cnrs.fr
P706	Exon 10	substitution	2118G>A	Pro706Pro	Bernot *et al.*, 1998
F721	Exon 10	substitution	2163C>T	Phe721Phe	http://fmf.igh.cnrs.fr
V722	Exon 10	substitution	2166G>A	Val722Val	http://fmf.igh.cnrs.fr
D723	Exon 10	substitution	2169C>T	Asp723Asp	http://fmf.igh.cnrs.fr
G764	Exon 10	substitution	2292G>T	Gly764Gly	Medlej-Hashim *et al.*, 2002

Table II.1 Behçet's Disease severity scoring system

Clinical Feature	Points
Oral ulcers	1
Genital ulcers	1
Skin lesions	1
Erythema Nodosum	2
Eye	
Anterior uveitis	1
Posterior uveitis	2
Blindness	3
Articular involvement	
Arthralgia	1
Arthritis	2
Vascular involvement	
Superficial Thrombophlebitis	1
Deep Vein Thrombosis	2
Thrombosis of vena cava superior	3
Epididymitis	2
Gastrointestinal involvement	3
Neurological involvement	4

Table II.2 Primers and PCR conditions used in ARMS assay

Mutation/ Polymorphism	Primers: Sequence (5'-3')	Primers: Type	Reference	DNA concentration (ng/µl)	Tm* (ºC)	Number of cycles	Additives: DMSO† (%)	Additives: Formamide (%)	PCR product bp‡
L110P	CATGCGCACCACGCTCAGCTT	Common	Present Study	400	62	30	4	2	338
	TCCTGGGCTTGTTCTCCCCCA	Normal							
	TCCTGGGCTTGTTCTCCCCCG	Mutant							
E148Q	AGCCCCTGCAGCCTCCCCGCGGA	Common	Unpublished	400	62	28	_	5	228
	CAGCCTGCGGTGCAGCCAGCCCG	Normal	Data						
	CAGCCTGCGGTGCAGCCAGCCCC	Mutant							
E167D	TATTCCACACAAGAAAACGGCACAGATGATTCCGCAGCG	Common	Aksentijevich *et al.*,	200	60	30	_	5	259
	GGGCTCCGGGTCCGAGGCTTGCCCTGCGCGTCCAGGCCC	Normal	1999						
	GGGCTCCGGGTCCGAGGCTTGCCCTGCGCGTCCAGGCCG	Mutant							
T267I	CTGCGCAGAAACGCCAGCTCCGC	Common	Unpublished	200	60	28	2.5	5	222
	TTCTGTTGCCGAGTCCAGATTCGCAGCTG	Normal	Data						
	TTCTGTTGCCGAGTCCAGATTCGCAGCTA	Mutant							
P369S	CCTGTAGCTCCCCTCTTTGCTGGACTGGTTTATATTGTG	Common	Unpublished	200	67	28	_	5	353
	CATGAAAGGAAGAGCCCGGGAAGCCTAAGCC	Normal	Data						
	CATGAAAGGAAGAGCCCGGGAAGCCTAAGCT	Mutant							
R408Q	CCTGTAGCTCCCCTCTTTGCTGGACTGGTTTATATTGTG	Common	Unpublished	200	67	28	_	5	234
	AGTCTGAGTCAGGAGCACCAAGGCCACCG	Normal	Data						
	AGTCTGAGTCAGGAGCACCAAGGCCACCA	Mutant							
F479L	TTCCAGCACGGCTGATGGCAGAGCTG	Common	Unpublished	200	60	28	_	_	273
	TGTACTACTTCCTGGAGCAGCAAGAGCATTTC	Normal	Data						
	TGTACTACTTCCTGGAGCAGCAAGAGCATTTG	Mutant							
V726A	TGGAGGTTGGAGACAAGACAGCATGGATCC	Common	Eisenberg *et al.*,	400	63	28	_	_	246
	TGGGATCTGGCTGTCACATTGTAAAAGGAGATGCTTCCTA	Normal	1998						
	TGGGATCTGGCTGTCACATTGTAAAAGGAGATGCTTCCTG	Mutant							
M680IGC	TTAGACTTGGAAACAAGTGGGAGAGGCTGC	Common	Eisenberg *et al.*,	400	63	28	_	5	219
	ATTATCACCACCCAGTAGCCATTCTCTGGCGACAGAGCC	Normal	1998						
	ATTATCACCACCCAGTAGCCATTCTCTGGCGACAGAGCG	Mutant							
M694V	TGACAGCTGTATCATTGTTCTGGGCTCTCCG	Common	Eisenberg *et al.*,	400	63	28	_	_	211
	TCGGGGGAACGCTGGACGCCTGGTACTCATTTTCCTTCCT	Normal	1998						
	TCGGGGGAACGCTGGACGCCTGGTACTCATTTTCCTTCCC	Mutant							
M694I	CTGTATCATTGTTCTGGGCTCTCCG	Common	Unpublished	400	63	28	_	2.5	194
	GGACGCCTGGTACTCATTTTCCTTC	Normal	Data						
	GGACGCCTGGTACTCATTTTCCTTT	Mutant							
P706	AAGTGGGAGAGGCTGCCTGATG	Common	Present Study	200	61	27	_	5	268
	GCTCCTTTATTAGCAGGCGGGTC	Normal							
	GCTCCTTTATTAGCAGGCGGGTT	Mutant							
Factor V Leiden	CGCAGGAACAACACCATGAT	Common	Endler *et al*,	200	57	27	_	_	N:233
	AACAAGGACAAAATACCTGTATTCATC	Normal	2001						M:246
	GTCTGTCTGTCTCTTCAAGGACAAAATACCTGTATTCTTT	Mutant							

*Tm: Melting Temperature, †DMSO: Dimethyl Sulphoxide, ‡bp: base pair.

Table III.1 Age distribution at onset of the disease

	Number of patients	Percentage (%)
First decade	11	25.0
Second decade	13	29.5
Third decade	10	22.7
Fourth decade	8	18.2
Fifth decade	2	4.6
Total	44	100

Table III.2 Clinical features in Palestinian BD patients

Patient Code	Sex*	Age (yr**)	Address	Age of onset(yr)	Disease status§	Oral ulcers	Genital ulcers	Eye lesions	Skin lesions	Other Features	Family History	Mutation/ Polymorphism
BD1	F	23	Ramallah	19	D	Yes	Yes	No	Yes	Arthralgia	----	Factor V Leiden
BD2	M	47	Hebron	10	D	Yes	Yes	No	Yes	Arthritis, diarrhea, Epididymitis, SIJ†	----	----
BD3	M	12	Hebron	8	D	Yes	Yes	No	Yes	Arthritis, perianal scaring	BD brother	E148Q/-
BD4	M	35	Hebron	10	D	Yes	Yes	No	Yes	Arthritis, DVT‡, Thrombophlebitis, SIJ	Sisters with RAS‖	E148Q/P706
BD5	M	31	Hebron	29	D	Yes	Yes	No	Yes	Arthralgia, SIJ	----	----
BD6	M	39	Hebron	10	D	Yes	Yes	No	Yes	Arthritis, Epilepsy, SIJ	----	E148Q/-
BD7	M	25	Hebron	17	D	Yes	Yes	No	Yes	Arthritis	BD brother + father with RAS	E148Q/-
BD8	M	23	Hebron	10	D	Yes	Yes	Yes	Yes	Arthralgia, SIJ	Brother with RAS	F479L/-
BD9	M	6	Hebron	6	D	Yes	Yes	No	Yes	----	BD father + brother	----
BD10	M	59	Hebron	45	D	Yes	Yes	No	Yes	Arthralgia, Epilepsy	BD sun	----
BD11	M	24	Hebron	16	D	Yes	Yes	No	Yes	Arthritis, SIJ	Sister with RAS	----
BD12	M	47	Hebron	21	D	Yes	Yes	No	Yes	Arthritis, SIJ	Sun with RAS	Factor V Leiden
BD13	F	18	Hebron	14	D	Yes	Yes	No	Yes	Arthralgia, SIJ	Sister with RAS	M694I/-, Factor V Leiden
BD14	F	39	Hebron	32	D	Yes	Yes	No	Yes	Arthralgia, CNS⊥ involvement	----	----
BD15	M	13	Beit Jala	11	D	Yes	Yes	No	Yes	Arthritis	----	----
BD16	F	6	Nablus	2	D	Yes	No	Yes	Yes	Hepatosplenomegaly, recurrent chest infections	----	V722M/-, Factor V Leiden
BD17	M	42	Hebron	36	D	Yes	Yes	No	Yes	SIJ	Children with RAS	E148Q/ P369S/ R408Q
BD18	F	30	Ramallah	24	D	Yes	Yes	No	Yes	Arthritis	----	E148Q/-
BD19	M	25	Ramallah	18	D	Yes	Yes	Yes	No	Stroke	----	----
BD20	M	35	Ramallah	29	D	Yes	Yes	Yes	Yes	Arthritis	----	----
BD21	M	33	Al-Jeeb	28	D	Yes	Yes	No	Yes	Arthritis, diarrhea, Epididymitis	----	----
BD22	F	30	Hebron	27	D	Yes	Yes	No	Yes	Arthritis, Gastrointestinal lesions	BD relatives	M694V/E148Q
BD23	M	39	Ramallah	28	D	Yes	Yes	Yes	Yes	Arthritis, DVT	----	----

Table III.2 (Continued) Clinical features in Palestinian BD patients

Patient Code	Sex*	Age (yr**)	Address	Age of onset(yr)	Disease status§	Oral ulcers	Genital ulcers	Eye lesions	Skin lesions	Other Features	Family History	Mutation/ Polymorphism
BD24	F	32	Hebron	18	D	Yes	Yes	Yes	No	Arthralgia	BD father	----
BD25	F	21	Hebron	18	D	Yes	Yes	No	Yes	Arthritis	----	M694V/-
BD26	M	20	Hebron	18	D	Yes	Yes	No	Yes	Arthritis, DVT, Epididymitis	----	Factor V Leiden
BD27	F	29	Hebron	22	D	Yes	Yes	Yes	No	----	----	----
BD28	M	40	Hebron	39	D	Yes	Yes	No	Yes	Arthritis, DVT,SIJ, Thrombophlebitis	----	----
BD29	F	41	Hebron	33	P	Yes	Yes	No	No	Back pain	----	----
BD30	F	25	Hebron	20	P	Yes	Yes	No	No	Arthritis, SIJ	BD sister	----
BD31	M	27	Hebron	24	P	Yes	Yes	No	No	----	BD relatives	----
BD32	M	52	Bethlehem	50	P	Yes	Yes	No	No	Subcutaneous Thrombophlebitis	----	F721/-
BD33	F	35	Abu Deis	35	P	Yes	Yes	No	No	Back pain	----	----
BD34	F	42	Bethlehem	35	P	Yes	No	No	Yes	Back pain	BD sister	V726A/-
BD35	M	14	Hebron	11	P	Yes	Yes	No	No	----	BD mother + 3 Aunts	----
BD36	F	32	Hebron	22	P	Yes	Yes	No	No	Arthralgia	----	----
BD37	F	33	Hebron	30	P	Yes	Yes	No	No	Arthritis	BD relatives	E148Q/-
BD38	F	19	Hebron	18	P	Yes	No	No	Yes	Arthritis, diarrhea, SIJ	----	----
BD39	F	13	Hebron	9	P	Yes	Yes	No	No	Arthritis	----	M694V/-
BD40	M	33	Jerusalem	32	P	Yes	Yes	No	No	Arthritis	----	V726A/-
BD41	F	10	Hebron	9	S	Yes	No	No	No	Knee pain once	Father with RAS	----
BD42	F	23	Hebron	15	S	Yes	No	No	No	Arthritis, Headache	Father with RAS	----
BD43	M	10.5	Hebron	9.5	S	Yes	No	No	No	Gastrointestinal lesions	----	M694V/A744S
BD44	M	9	Hebron	8	S	Yes	No	No	No	----	----	V726A/-

*M: Male, F: Female, **yr: Year, §D: Definite, P: Probable, S: Suspected, †SIJ: Sacroiliac Joint Involvement, ‡DVT: Deep Venous Thrombosis, ⊥CNS: Central Nervous System, ‖RAS: Recurrent Aphthous Stomatitis.

Table III.3 Frequency of BD manifestations in Palestinian patients

Manifestation	Number of patients n = 44	Percentage (%)
Oral ulcers	44	100
Genital ulcers	37	84.1
Skin lesions	27	61.4
Eye lesions	7	15.9
Arthritis/Arhralgia	30	68.2
Vascular	5	11.4
Neuro-BD	1	2.3
Gastrointestinal	2	4.5
Epididymitis	3	6.8

Table III.4 Predominant symptoms at presentation

Type of presentation	Number of patients	Percentage (%)
Oral only	4	9.1
Oral + genital	10	22.7
Oral + skin	2	4.6
Oral + genital + skin	21	47.7
Oral + genital + eye	3	6.8
Oral + eye + skin	1	2.3
Oral + genital + skin + eye	3	6.8
Total	44	100

Table III.5 MEFV genotypes among the Palestinian BD patients

Genotype	Number of patients	Percentage (%)
E148Q/—	5	11.4
V726A/—	3	6.8
M694V/—	2	4.5
M694I/—	1	2.3
F479L/—	1	2.3
V722M/—	1	2.3
F721/—	1	2.3
M694V/E148Q	1	2.3
M694V/A744S	1	2.3
E148Q/P706	1	2.3
E148Q/P369S/R408Q	1	2.3
—/—	26	59.1
Total	44	100

Table III.6 MEFV gene mutations among Palestinian BD patients

Mutation/ Polymorphism	Number of independent alleles	Percentage (%)
E148Q	8	22.2
M694V	4	11.1
V726A	3	8.3
M694I	1	2.8
F479L	1	2.8
P369S	1	2.8
R408Q	1	2.8
A744S	1	2.8
V722M	1	2.8
P706	1	2.8
F721	1	2.8
Unidentified alleles	13	36.1
Total	36	100

Table III.7 Distribution of MEFV genotypes according to BD patients' status

Disease status*	Genotypes	Number of patients
D		
	E148Q/—	4
	M694V/—	1
	M694I/—	1
	F479L/—	1
	V722M/—	1
	E148Q/ P706	1
	M694V/ E148Q	1
	E148Q/ P369S/ R408Q	1
		11/28
P		
	V726A/—	2
	M694V/—	1
	E148Q/—	1
	F721/—	1
		5/12
S		
	V726A/—	1
	M694V/ A744S	1
		2/4

*D: Definite, P: Probable, S: Suspected

Table III.8 Clinical severity score of BD patients

Patient' Code	Clinical Severity Score	MEFV Genotype
BD1	4	---
BD2	7	---
BD3	5	E148Q/—
BD4	8	E148Q/P706
BD5	4	---
BD6	5	E148Q/—
BD7	5	E148Q/—
BD8	7	F479L/—
BD9	3	---
BD10	4	---
BD11	5	---
BD12	5	---
BD13	4	M694I/—
BD14	8	---
BD15	6	---
BD16	3	V722M/—
BD17	3	E148Q/P369S/R408Q
BD18	1	V726A/—
BD19	5	E148Q/—
BD20	5	---
BD21	10	---
BD22	9	---
BD23	8	M694V/ E148Q
BD24	2	---
BD25	2	V726A/—
BD26	2	---
BD27	3	---
BD28	4	E148Q/—
BD29	4	---
BD30	4	M694V/—
BD31	4	V726A/—
BD32	12	---
BD33	1	---
BD34	3	---
BD35	4	M694V/A744S
BD36	3	F721/—
BD37	4	---
BD38	2	---
BD39	5	M694V/—
BD40	11	---
BD41	3	---
BD42	4	---
BD43	8	---
BD44	2	---

Table III.9 Comparison of various clinical manifestations between BD patients with and without MEFV mutations

Clinical Feature	BD With Mutation, n =18 n (%)	BD Without Mutation, n =26 n (%)	P Value
Oral ulcers	18 (100)	26 (100)	NS*
Genital ulcers	14 (77.8)	23 (88.5)	NS
Eye features	2 (11.1)	5 (19.2)	NS
Skin features	12 (66.7)	15 (57.7)	NS
Arthritis/Arthralgia	12 (66.7)	18 (69.2)	NS
Vascular	2 (11.1)	4 (15.4)	NS
Neuro–BD	0 (0.0)	1 (3.9)	NS
Gastrointestinal	2 (11.1)	0 (0.0)	NS
Epididymitis	0 (0.0)	3 (11.5)	NS
Family history	5 (27.8)	6 (23.1)	NS
Age of onset	19.4±13.1	22.2±9.6	NS
Disease severity	4.4±2.0	5.0±3.1	NS

*NS: Not Significant

Table IV.1 Frequency of MEFV mutations among BD patients in different studies

Mutation/ Polymorphism	Percentage of independent alleles		
	Present study n = 44	**Touitou *et al.*, 2000 n = 57**	**Ben-Chetrit *et al.*, 2002 n = 53**
M694V	4.6	2.6	4.7
M694I	1.1	0.0	1.9
V726A	3.4	2.6	2.8
M680I	0.0	0.0	1.9
E148Q	9.1	5.2	5.7
L110P	0.0	2.6	ND*
F479L	1.1	ND	ND
P369S	1.1	ND	ND
R408Q	1.1	ND	ND
A744S	1.1	0.0	ND
V722M	1.1	0.0	ND
P706	1.1	10.5	ND
F721	1.1	0.0	ND
Identified alleles	26.1	23.5	17.0
Unidentified alleles	73.9	76.5	83.0
Total	100	100	100

* ND: Not Done.

Table IV.2 Frequency of clinical findings of BD in various countries

Country	Number of patients	M/F	Mean age at onset (Mean±SD)	Oral aphthae (%)	Genital ulceration (%)	Papulo-pustular (%)	Erythema nodosum (%)	Eye (%)	Arthritis (%)	CNS (%)	Phlebitis (%)	Pulmonary (%)	GIS (%)	Epididymitis (%)
Turkey	2147	1.03/1	38.5	100	88.2	54.2	47.6	28.9	15.9	2.2	10.6	1	2.8	-
Iran	3443	1.4/1	26.2 ± 9.6	95.8	64	65.8	22.9	57.8	39.9	3.3	6.3	0.7	-	6
Tunisia	716	3.5/1	27	98	79	62	-	51	35	31	23	1.4	4	2
Saudi Arabia	145	3.4/1	29.3	100	87	57	-	56	37	44	25	14	4	4
Iraq	60	3/1	29.4	97	83	48	55	33	48	2	17	-	10	22
Egypt	34	1.6/1	22.9 ± 5.3	100	94	29.4	20.5	41.1	61.7	14.7	5.9	0	-	-
Israel	69	1.3/1	-	100	40	74	-	30	8	28	23	0	26	0
Kuwait	29	3.1/1	33.8	100	93	76	45	69	69	14	-	34	21	-
Jordan	20	2.3/1	32	100	65	35	5	65	55	5	20	5	5	5
Lebanon	100	1.3/1	26	99	78	53	25	63	65	14	11	4	10	2
Greece	70	1.9/1	24	100	78.1	93.7**	-	75	48.4	20.3	7.8	1.5	4.6	10.9
Palestine***	44	1.3/1	21.2 ± 11.2	100	84.1	59.1	11.3	15.9	50.0	2.3	11.4†	0	4.5	6.8

CNS: Central nervous system involvement; GIS: gastrointestinal system involvement; -: not defined; **skin lesions; ***present study; †vascular involvement [Saylan *et al.*, 1999].

Table IV.3 Prevalence of factor V Leiden mutation among BD patients in different studies

All BD Patients (%)	BD patients with thrombosis (%)	BD patients without thrombosis (%)	Controls (%)	References
29.3	-	-	5	Kosar *et al.*, 2002
22.7	-	-	7.1	Kosar *et al.*, 2002
27.4	-	-	19.2	Verity *et al.*, 1999c
25.5	-	-	-	Kosar *et al.*, 2002
0.0	-	-	2	Espinosa *et al.*, 2002
0.0	-	-	0.0	Kosar *et al.*, 2002
23	37.5	9.4	10.3	Gül *et al.*, 1996
19.8	30	9.2	10.6	Kosar *et al.*, 2002
14.3	18.3	10	2	Toydemir *et al.*, 2000
11.4	20	10.3	-	Present Study

-: Not Defined

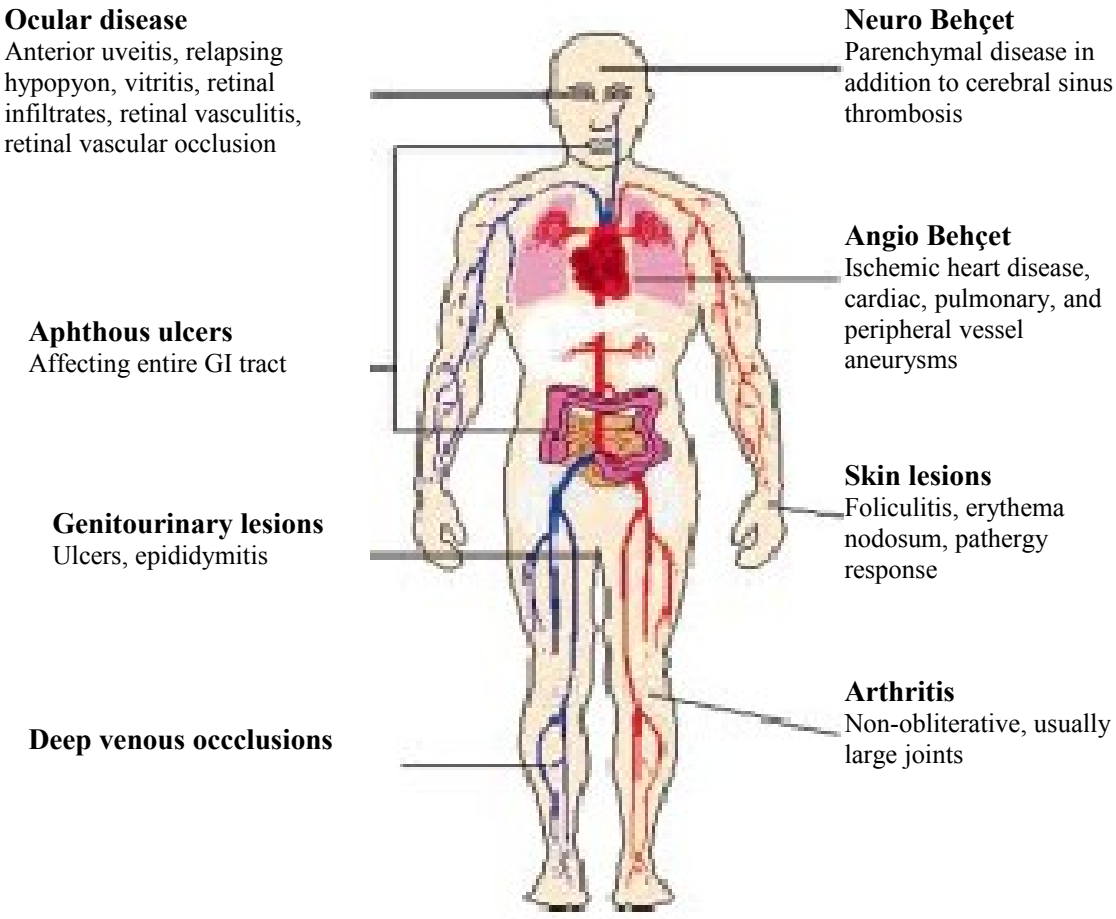

Figure I.1 Spectrum of organ involvement in Behçet's disease [Verity *et al.*, 2003].

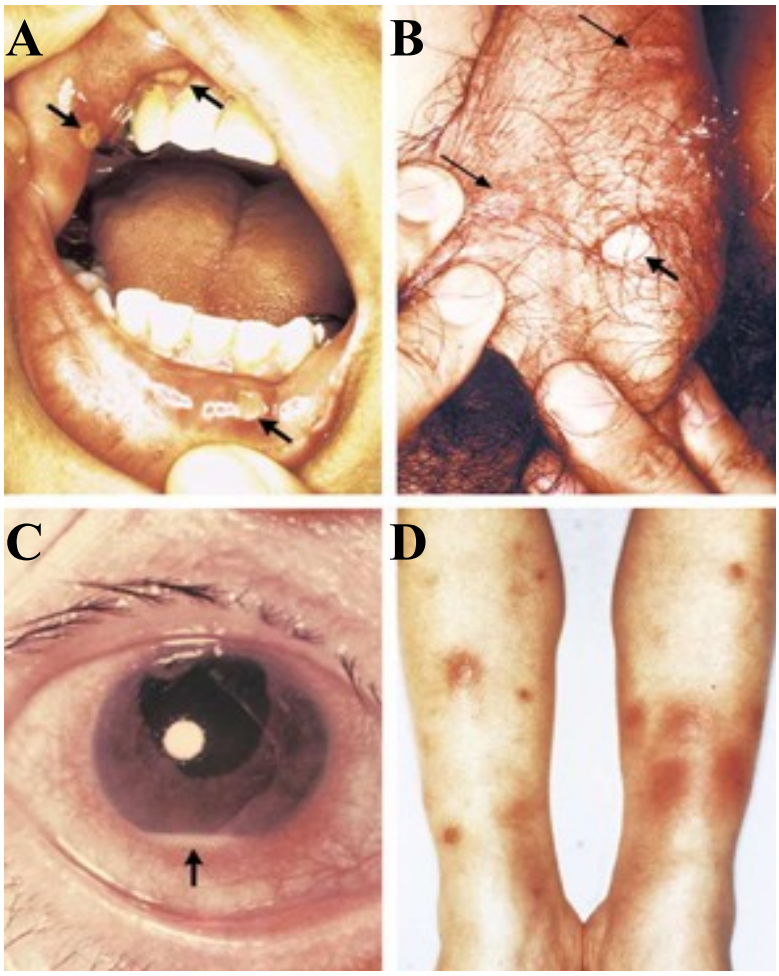

Figure I.2 Characteristic Lesions of Behçet's Disease.
Panel A shows multiple aphthous ulcers on the buccal membrane, gingiva, and labial mucosal membrane (arrows). **Panel B** shows an active genital ulcer (short arrow) and scars (long arrows) on the scrotum. **Panel C** shows hypopyon (arrow) - a horizontal layer of inflammatory cells in the anterior ocular chamber - and deformity of the iris. The bright circle in Panel C is reflected light. **Panel D** shows new and old lesions of erythema nodosum on the front of the legs [Sakane *et al.*, 1999].

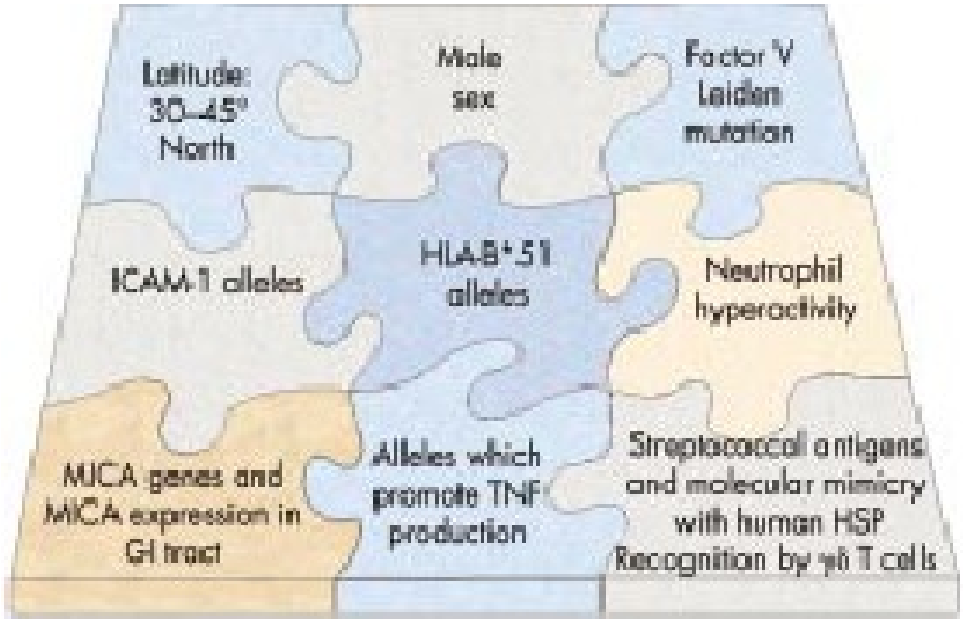

Figure I.3 Behçet's disease is associated with multiple hereditary and environmental risk factors [Verity *et al.*, 2003].

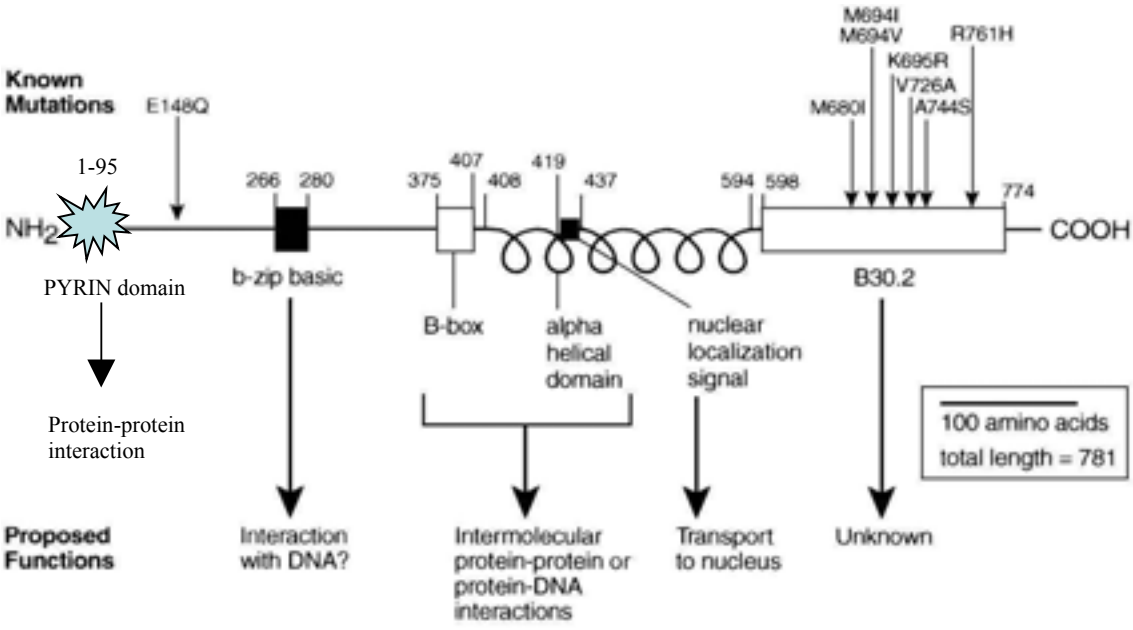

Figure I.4 Pyrin's identified domains and corresponding functions.
The amino and carboxy termini are indicated by NH2 and COOH, respectively. The numbers above the protein segments refer to their amino acid positions on the primary proteins structure [Samuels *et al.*, 1998].

Figure I.5 Proposed Role of Pyrin in Regulating ASC-Mediated Caspase-1 Oligomerization

Left: LPS and proinflammatory cytokines induce ASC, which binds pro caspase-1 through cognate CARD interactions and leads to caspase-1 oligomerization and autocatalysis. Active p10/p20 subunits cleave pro-IL-1β to mature IL-1β, leading to fever and inflammation. Right: LPS and antiinflammatory cytokines induce pyrin, which binds ASC through homotypic PYRIN domain interactions, sequestering ASC and blocking its interaction with caspase-1 [Chae *et al.*, 2003].

Figure I.6 MEFV Gene structure.
The approximate location of MEFV on the subtelomeric region of chromosome 16 followed by a schematic representation of its 10 exons. Mutations are listed under their respective exon, with underlined mutations representing those most commonly identified and one mutation in italics representing the only known truncation mutation [Hull *et al.*, 2003].

Figure II.1 The ARMS assay.
(a) normal homozygous DNA (allele 1); **(b)** heterozygote DNA (allele 1 and 2); and **(c)** affected homozygote DNA (allele 2). In **(a)** only the normal allele is present therefore only the primer specific to allele 1 is incorporated into the PCR product with this substrate DNA. In **(b)** both alleles are present, therefore in their respective PCR reactions, both ARMS primers give rise to PCR product. In **(c)**, with only allele 2 is present, only the allele 2 primer has a complementary substrate and is extended and generates amplified product. AP: ARMS primer; CP: common primer [Newton and Graham, 1997].

L110P

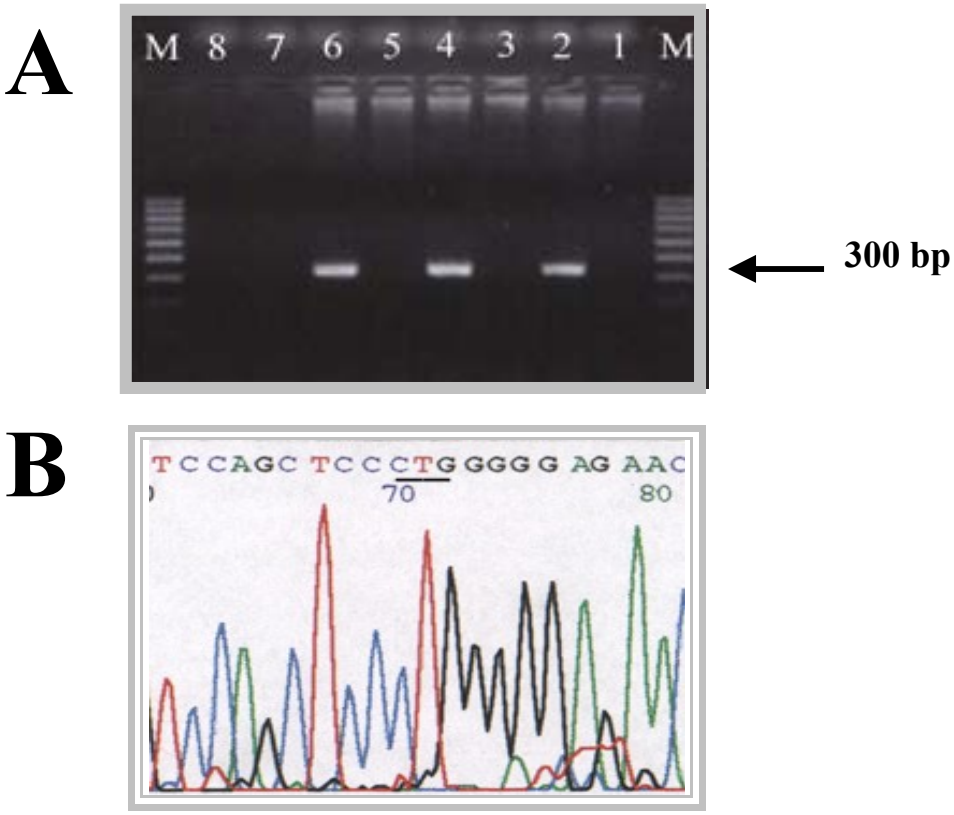

Figure III.1 Gel electrophoresis for ARMS analysis and DNA sequence electropherogram of L110P mutation.
A. Agarose gel electrophoresis of amplified DNA by ARMS assay showing the 338 bp fragment of L110P mutation. Lanes M: DNA molecular weight marker, lanes 1–4: normal patient's samples, lanes 5 and 6: normal control, and lanes 7 and 8: blank sample. Lanes 1, 3, 5 and 7 represent the PCR product of the mutant primer, whereas lanes 2, 4, 6 and 8 represent the PCR product of the normal primer. **B.** DNA sequence analysis of a section of exon 2 in the human MEFV gene utilized by using the forward primer: 5'CCCTAAACGTGGGACAGCTTCATC3' and the reverse primer: 5'CCATTCTTTCTCTGCAGCCGATATAAAG3', showing the normal nucleotide sequence CTG at codon 110 for the normal control individual.

P706

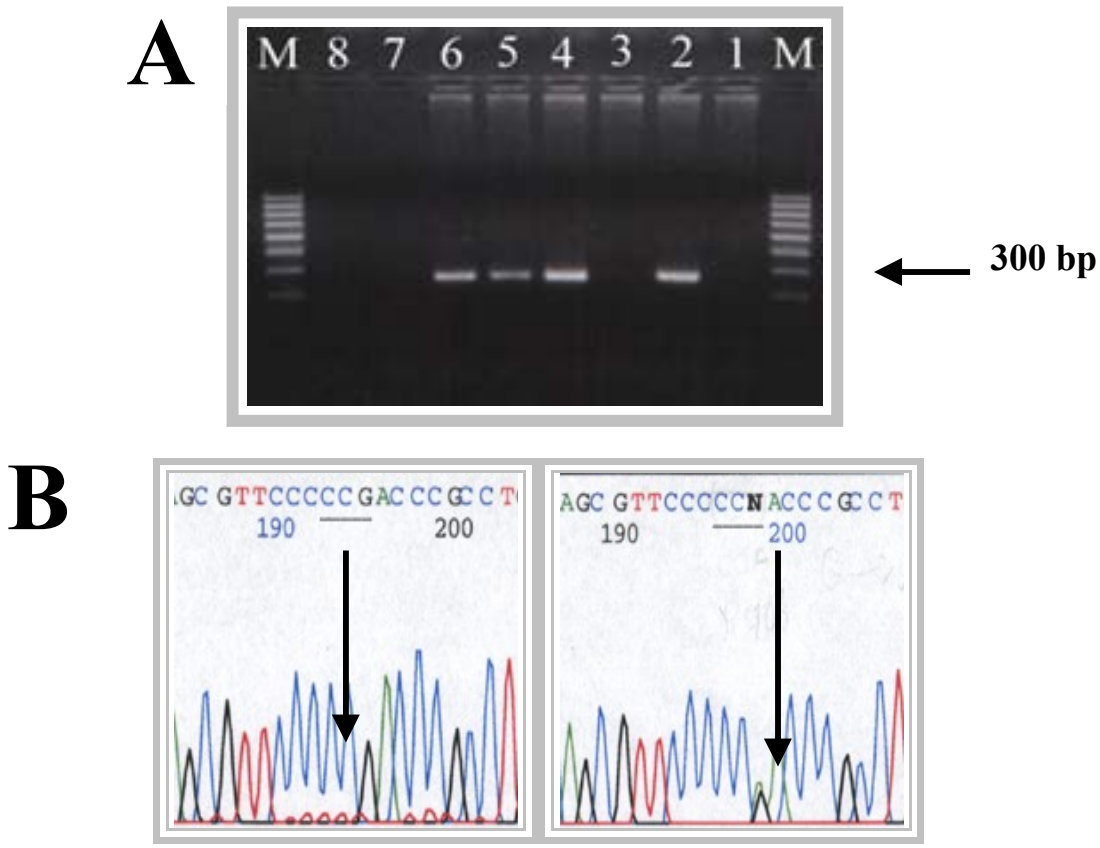

Figure III.2 Gel electrophoresis for ARMS analysis and DNA sequence electropherogram of P706 polymorphism.
A. Agarose gel electrophoresis of amplified DNA by ARMS assay showing the 268 bp fragment of P706 polymorphism. Lanes M: DNA molecular weight marker, lanes 1–4: normal patient's samples, lanes 5 and 6: heterozygote patient sample, and lanes 7 and 8: blank sample. Lanes 1, 3, 5 and 7 represent the PCR product of the mutant primer, whereas lanes 2, 4, 6 and 8 represent the PCR product of the normal primer. **B.** DNA sequence analysis of a section of exon 10 in the human MEFV gene showing the normal nucleotide sequence CCG at codon 706 (left). The G→A substitution changes the sequence of codon 706 from CCG to CCA, but keeping the same amino acid "proline" (right). The downward arrow points to heterozygous alteration.

F721 and V722M

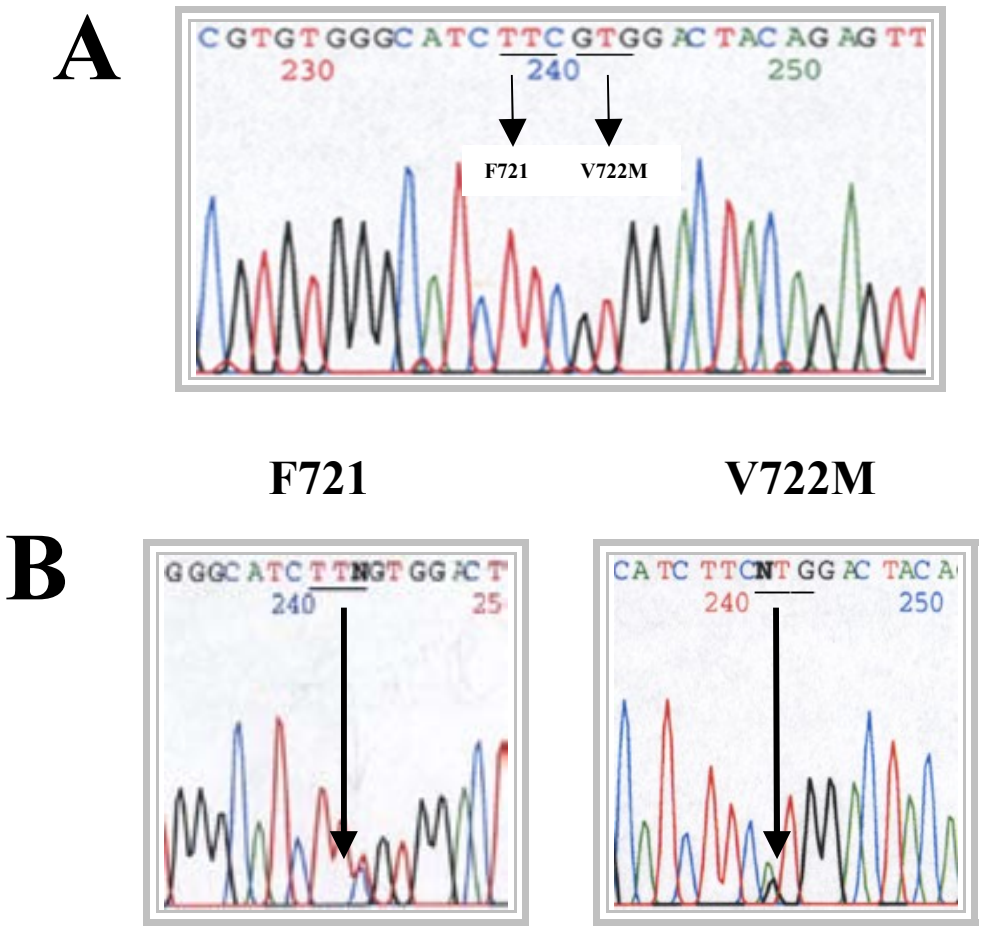

Figure III.3 **DNA sequence electropherograms of F721 polymorphism and V722M mutation.**
A. DNA sequence analysis of a section of exon 10 in the human MEFV gene showing the normal nucleotide sequence TTC at codon 721 and GTG at codon 722. **B.** The C→T substitution changes the sequence of codon 721 from TTC to TTT, but keeping the same amino acid "phenylalanine" (left). The G→A substitution changes the sequence of codon 722 from GTG to ATG and results in a predicted valine-to-methionine substitution (right). The downward arrows point to heterozygous alteration.

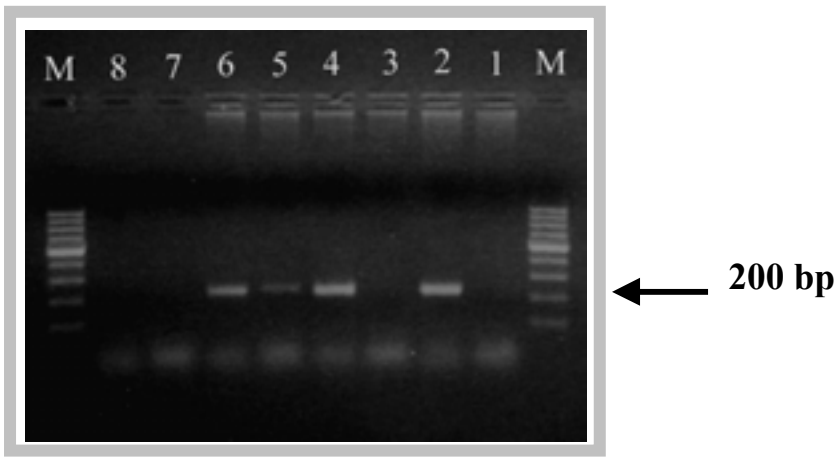

Figure III.4 Agarose gel electrophoresis for ARMS analysis of factor V Leiden mutation.
Gel electrophoresis of amplified DNA by ARMS assay showing both the normal 233 bp and the mutant 246 bp fragments of factor V Leiden mutation. Lanes M: DNA molecular weight marker, lanes 1–4: normal patient's samples, lanes 5 and 6: heterozygote patient sample, and lanes 7 and 8: blank sample. Lanes 1, 3, 5 and 7 represent the PCR product of the mutant primer, whereas lanes 2, 4, 6 and 8 represent the PCR product of the normal primer.