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Familial Mediterranean Fever (FMF) or Fabry disease?

Genetic analysis and study of GLA as "modifier gene" of FMF

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1. FAMILIAL MEDITERRANEAN FEVER

Familial Mediterranean fever (FMF) [1] belongs to the group of autoinflammatory syndromes and is considered to be the prototype of hereditary periodic fever syndromes. It is an autosomal recessive disorder caused by mutations of the MEFV gene on chromosome 16p13.3 which encodes pyrin (also known as marenostrin), a protein specifically expressed on the cytoplasm surface of myeloid cells.

This protein plays an important role in the production and secretion of certain inflammatory cytokines [2], such as IL-1β [3], which acts as a negative regulator of apoptosis.

Pyrin is a protein consisting of 781 amino acids, chiefly expressed in mature neutrophils, and consists of four domains: the N-terminal PYRIN domain, a B-box zinc finger domain, a centrally located coiled-coil domain, the C-terminal B30.2 domain [4]. The first of these domains is characterized by six anti-parallel alpha-helices through which the protein binds to extracellular domains to form a macromolecular complex called an inflammasome.

The C-terminal B30.2 is codified by exon 10, and mutations in this exon are usually associated with a severe phenotype of FMF (M694V, M694I, and M680I).

The V726A mutation, which causes a mild phenotype of the disease, is also associated with the B30.2 domain. However, the presence of the V726A mutation along with the M694V or M680I mutation seems to be associated with the development of a severe clinical phenotype.

Pyrin is also localized in the cytoplasm and the nucleus. After its synthesis, a migration of the protein from the cytoplasm to the nucleus via the nuclear pores occurs through the mechanisms of intranuclear signal transduction.

Cazeneuve et al. [5] showed that the five most common mutations in the MEFV gene do not affect the intracellular site of pyrin.

Currently, in fact, the function of pyrin and the impact of its mutations are still under discussion. In 2003, Touitou et al. [6] demonstrated that patients with FMF have reduced transcription of the MEFV gene compared to healthy subjects. Therefore, the pathophysiology of FMF seems to depend on the degree of the defect in mRNA expression.

The discovery of inflammosome [7] has also opened new perspectives on the pathogenesis of auto-inflammatory syndromes. Under normal conditions, protein-protein interaction results in the activation of complex events, which lead to the formation and subsequent release of cytokines, including IL-1β. This mechanism underlies the fundamental pathophysiology of inflammatory syndromes.

In FMF, mutations in pyrin can cause an excessive production of IL-1β in response to minor stimuli.

In regard to this aspect, the role played by the B30.2 domain is currently being debated. Stojanov and Kastner [4] showed that this domain, under normal conditions, inhibits the production of the active form of IL-1β; therefore, mutations in the B30.2 domain would lead to an excessive production of IL-1β.

Tschopp et al. [8] demonstrated that the B30.2 domain interacts with the NALP-3 of caspase-1 (also called cryopyrin, a protein mutated in cryopyrinopathies) and with its substrate, pro-interleukin (IL)-1β.

The authors demonstrated that the overexpression of the B30.2 domain is followed by the activation of caspase-1 and the production of IL-1β, concluding that pyrin is a protein involved in modulating the activity of various proteins involved in inflammasome formation.

Most MEFV gene mutations involve the C-terminal domain (B30.2) of pyrin. Normally, this region binds to and inhibits the catalytic activity of the p20 and p10 subunits of caspase-1; the three most common mutations, which occur in this domain, reduce the effectiveness of this binding and, consequently, the inhibitory action on caspase (Figure 1).

MEFV, the gene responsible for the disease, is composed of 10 exons and has an overall length of 15 kb. To date, more than 70 mutations in the MEFV

gene have been described, and, in many cases, it is not always possible to establish a genotype-phenotype correlation [9].

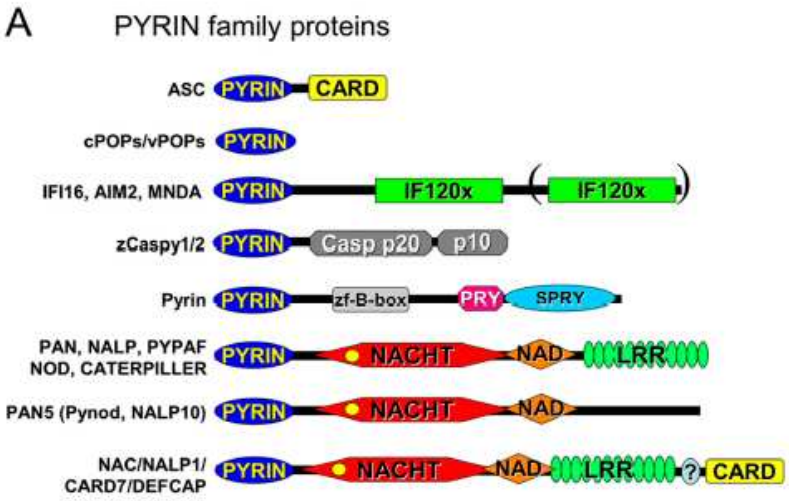

Figure 1. Pyrin structure

The M694V mutation is the most commonly described of the MEFV gene mutations. In 25-60% of cases, febrile episodes debut in the first decade of life, and in 64-95% they manifest by age 20. Padeh et al. conducted a study of large number of Israeli paediatric cases and showed the onset of disease for those under 2 years-old, in contrast to the 2-16 age-group, was characterized by febrile episodes without other associated symptoms, or only abdominal pain [10].

Episodes of fever are usually of short duration (1-3 days) and are associated with abdominal pain (95%); chest pain is described in 33-53% of cases; joint involvement is characterized by oligoarthritis and/or asymmetric

monoarthritis; erysipelas-like erythema in the lower extremities has also been described.

Other clinical manifestations include myalgia, splenomegaly and headache (15%). Laboratory tests reveal a marked neutrophilia and an increase in the erythrocyte sedimentation rate.

The most serious long-term complication is represented by amyloidosis, the most common clinical manifestation of which is proteinuria which can lead to the onset, in certain circumstances, of clinical pictures with kidney damage. In less than 1% of patients, amyloidosis may represent the onset of FMF.

Four ethnic populations have the highest prevalence of FMF (between 1:200 and 1:1000): non-Ashkenazi Jews, Turks, Armenians and Arabs. The south-eastern Mediterranean is considered to be the location of the “original” distribution of the disease. However, the migration of people generally affected by it has favoured the spread of FMF from the eastern Mediterranean to wider geographical areas [11].

Recent studies have expanded the number of groups known to be affected by FMF, extending into the European Union, including the Italians and Greeks. In Italy, the number of patients is estimated to be about 278, including 85 patients from Sicily and 67 patients from Calabria [12]. Rare, isolated cases have also been reported in other ethnic groups, such as that of a Japanese patient.

1.1 CLINICAL MANIFESTATIONS

The fever, which is the key symptom of the disease, varies between 38°C and 40°C, has an average duration of 12-72 hours, is not responsive to antibiotic therapy and undergoes spontaneous resolution. The acute febrile episode associated with abdominal pain may mimic a clinical picture like that of “acute abdomen”, but even in this case the resolution of the symptoms is usually spontaneous [13].

Abdominal pain can be localized and then spread to other areas. When inflammation involves the posterior peritoneum, it can mimic renal colic or pelvic inflammatory disease. For this reason, it has been estimated that 30-40% of patients, those who are not diagnosed promptly, may undergo unnecessary surgeries, such as appendectomies and / or cholecystectomies, without the resolution of their symptoms. During the attack, changes in the patient’s bowel habits may include complaints of constipation and / or diarrhoea.

However, it is important to distinguish between painful episodes associated with acute attacks of FMF and those due to other causes. In FMF, there is typically a resolution of the clinical picture within 24-72 hours.

Joint involvement has been described in about 1/3 of patients. Arthralgias are most commonly arthritis cases and usually involve medium and large joints, such as the ankles and knees. Several cases of sacroiliitis have been reported,

with negative HLA-B27 antigen [14]. Acute episodes of joint pain can be triggered by minor trauma and / or physical effort. Patients with recurrent arthritis have a three-fold higher risk of developing amyloidosis than patients without joint involvement [13].

In addition, protracted febrile myalgia is a rare manifestation of severe forms of FMF, is characterized by the presence of muscle pain, fever, alterations of laboratory tests, and has a variable duration (1-6 weeks).

Chest pain is generally an expression of the inflammation of serous membranes and, therefore, there may be cases of pleurisy and / or pericarditis.

The differential diagnosis is mainly with recurrent idiopathic pericarditis [15], for which colchicine has proved very effective as a standard treatment.

Skin involvement is estimated at around 40% of patients and is manifested in the form of erysipelas, or erythematous events, which are warm to the touch and raised on the skin, range from 10-15 cm in diameter, and are usually located on the backs of the knees, the ankles and the feet.

Although renal involvement is more frequently associated with amyloidosis, non-amyloid renal lesions have also been described, such as mesangial proliferative glomerulonephritis, fibrillary glomerulonephritis, and clinical pictures of vasculitis with renal involvement, such as “polyarteritis nodosa” and “Henoch-Schonlein purpura”.

In intercritical periods, the patient is usually asymptomatic. The most important long-term complication is amyloidosis. Among Sephardic Jews and Turks, FMF is the leading cause of amyloidosis. The localization is mainly associated with persistent or worsening renal proteinuria, up to clinical conditions of nephrotic syndrome and chronic renal failure [13]. The deposition of fibrils can occur in other sites, such as the intestines, heart, endocrine glands, spleen and liver. The incidence of renal amyloidosis has decreased dramatically since the introduction of colchicine therapy. Recently, the incidence of extrarenal manifestations of amyloidosis has increased, following a higher survival rate among patients with renal amyloidosis (treated with dialysis or transplantation).

The diagnosis of FMF is entirely based on clinical signs. Since 1997, however, the MEFV gene has been studied for diagnostic confirmation. The clinical diagnostic criteria were first formulated by Tel-Hashomer [16] (Table 1), from which either two major criteria or one major and two minor criteria were necessary for definitive diagnosis.

Major criteria	Minor criteria
Fever with peritonitis, synovitis, or pleurisy	Recurrent febrile attacks
AA amyloidosis (without risk factors or other chronic inflammatory diseases)	Erysipeloid erythema
Response to colchicine	Family history of periodic syndrome

Table 1. Tel-Hashomer criteria

Recently, in 2009, Yalcinkaya et al. [17] (Table 2) established the following paediatric diagnostic criteria:

Criteria	Description
Fever	Axillary temperature > 38°; duration 6–72 h; ≥ 3 attacks
Abdominal pain	Duration 6–72 h; ≥ 3 attacks
Chest pain	Duration 6–72 h; ≥ 3 attacks
Arthritis	Duration 6–72 h; ≥ 3 attacks; oligoarthritis
Family history of FMF	

Table 2. Yalcinkaya criteria

1.2 GENOTYPE–PHENOTYPE CORRELATION

The genotype-phenotype correlation in patients with FMF is highly variable and not always easy to discern. From the data reported in the literature [18] (Figure 2), the presence of the M694V and M680I mutations in the homozygous and/or compound heterozygous condition is associated with a severe clinical phenotype including the early onset of clinical symptoms such as amyloidosis and arthritis. The E148Q mutation, one of the five most frequent mutations of the MEFV gene, has been associated with a "mild" clinical phenotype.

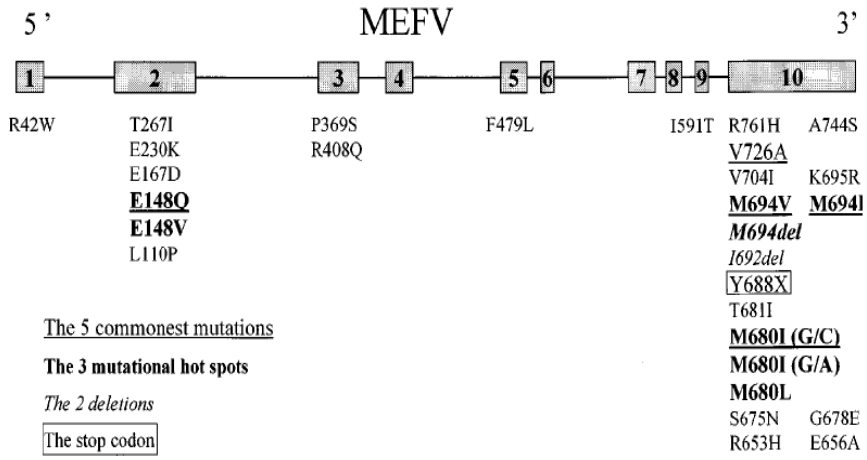

Figure 2. Description of MEFV gene mutations

According to the literature [19], about 1/3 of patients with a single mutation have a phenotypic framework "compatible" with FMF and are responsive to treatment with colchicine.

In support of this hypothesis, we report data from a recent study (Federici et al.) conducted on 386 paediatric patients, of whom 113 were carriers of mutations in the MEFV gene, 46 were homozygotes and / or compound heterozygotes, and 67 were carriers of a single mutation. For these groups, differences that could be associated with different genotypes were analysed, resulting in the identification of five clinical genotypes: 1) patients with two high-penetrance mutations; 2) patients with one high- and one low-penetrance mutation; 3) patients with two low-penetrance mutations; 4) patients with one high-penetrance mutation; 5) patients with one low-penetrance mutation (Figure 3).

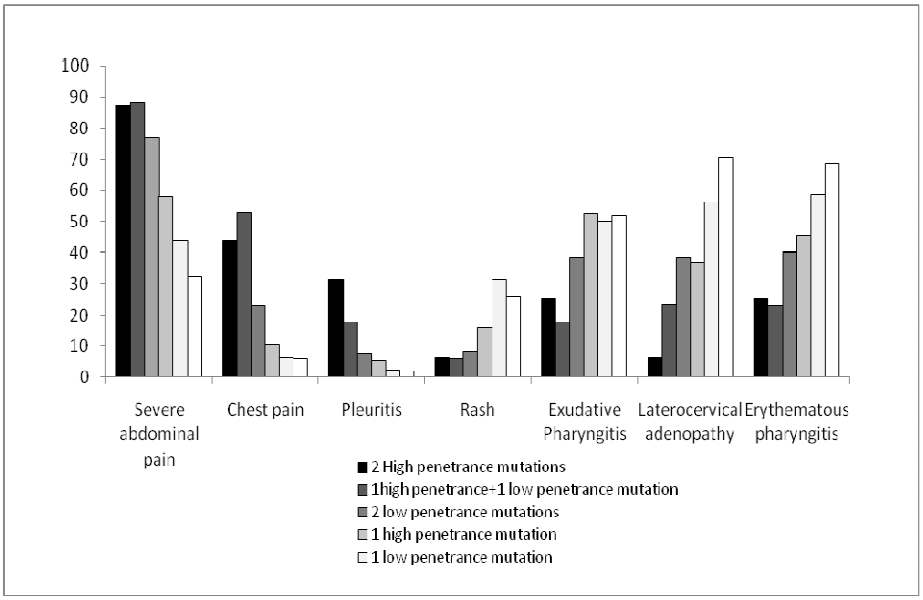

Figure 3. Analysis showing possible differences identifying five clinical genotypes

The clinical heterogeneity of the disease has led to the formulation of the idea of an “FMF-like” clinical framework for subjects with two high-penetrance

mutations and a “PFAPA-like” framework for patients with a single low-penetrance mutation [20].

The hypothesis has been advanced by several authors that subjects with a single mutation in the MEFV gene may have another mutation in a second gene, the two of which interact with each other, leading to a “variable” phenotypic framework.

Another study, conducted on SAA1, coding for SAA1 serum amyloid A, considered the possible role of a modifier gene, finding it to be one of the few examples of modifier genes in humans [21]. The authors therefore concluded that “modifiers genes” have an important role in determining clinical variability in patients with a single mutation in the MEFV gene.

1.3 DIAGNOSIS

Acute episodes of FMF are associated with a non-specific increase in inflammatory markers, such as serum amyloid A, fibrinogen, erythrocyte sedimentation rate (ESR) and C-reactive protein, as well as an increase in white blood cell count, which return to baseline levels during intercritical periods [22]. Typically, chemical-physical examination of the urine detects haematuria and / or proteinuria.

Over the years, a large number of cytokines, chemokines and other pro-inflammatory proteins have been studied in patients with FMF. These include IL-1, 4, 5, 6, 10, 12, 18, TNF-α and γ, cytokines associated with receptors, complement proteins, adhesion molecules, growth factors, immunoglobulins [23]. The overall concept that has emerged from these studies is that FMF is not an autoimmune disease. Therefore, there are no ongoing laboratory diagnostic tests and / or specific instruments designed to detect it. Although more than 70 mutations in the MEFV gene have been described, the majority of cases are caused by four mutations in a single exon: M694V, V726A, M680I, and M694I; the relative prevalence of the different mutations varies depending on the population studied. For example, in the Turkish population, which is the largest ethnic group with FMF, the M694V mutation is the most frequent (51%), followed by M680I (14%) and V726A (9%).

Overall, about 80% of patients with FMF have an identified mutation in the MEFV gene, 57% are compound heterozygotes, 26% have a single mutation, and 16% have no mutations.

1.4 THERAPY

At present, colchicine is the only effective therapy for FMF, improving quality of life with a consequent reduction in the number of attacks or by total resolution of the symptoms.

Akar et al. [24] evaluated the impact of colchicine on subclinical inflammation and symptoms during intercritical periods, documenting an improvement in quality of life and the negativity of laboratory tests after colchicine use. The therapeutic dose of colchicine is established based on body weight: 0.03 mg / kg / day, up to a maximum of 3 mg / day.

The aim of treatment is the prevention of attacks and control of inflammation during subclinical intercritical periods. In cases not responding to treatment with colchicine, it is possible to use several new drugs, including IL-1 inhibitors of three known types: 1) Anakinra [25], an IL-1 receptor antagonist; 2) Rilonacept, a receptor inhibitor; 3) Canakinumab [26], an anti-IL-1 β monoclonal antibody.

2. FABRY DISEASE

Fabry disease (FD) is a lysosomal enzymopathy caused by mutations in the gene encoding the enzyme α-galactosidase A (GLA). This pathological condition, resulting from a progressive congenital metabolic defect, is characterized by the accumulation of lysosomal glycosphingolipids and subsequent cellular and microvascular dysfunction.

The incidence is estimated at 1:40,000 [27] in the general population, although recent newborn screening initiatives have found an unexpectedly high prevalence of disease. In Italy, approximately 1 in 3,100 newborns were found to have genetic alterations associated with FD, and in Taiwan, the incidence was nearly 1 in 1,500. Of the latter group, 86% had the IVS4 +919 G> A intronic mutation which is involved in the alternative splicing of messenger RNA and which has previously been found in patients with a late cardiac variant of FD [28]. The intronic IVS4 mutation +919G>A [29] was also found in a Chinese FD patient with a clinical picture of idiopathic hypertrophic cardiomyopathy.

In FD, the lysosomal enzyme activity of GLA can be absent or deficient, leading to the progressive accumulation of globotriaosylceramide (Gb3 or GL-3), a glycosphingolipid that accumulates within the lysosomes, subcellular organelles which are contained in a variety of cellular structures, including the endothelial cells of capillaries, various kidney cell types (podocytes, tubular,

glomerular endothelial, mesangial and interstitial cells), cardiac cells (cardiomyocytes and fibroblasts) and nerve cells.

Tissue damage begins in childhood or even during foetal development, but unlike many other lysosomal storage disorders, most patients remain clinically asymptomatic during the early years of life [30].

In subjects with FD, it is believed that the lysosomal accumulation of Gb3, and subsequent cellular dysfunction, activates a cascade of events, including apoptosis, the impairment of energy metabolism, the alteration of small vessels, malfunction in the ion channels at the level of endothelial cells, oxidative stress, tissue ischemia and, above all, the development of irreversible cardiac and kidney fibrosis.

2.1 CLINICAL MANIFESTATIONS

The first clinical symptoms may occur during childhood, usually between 3-10 years, generally a few years later in girls than boys.

In turn, the clinical manifestations can vary, depending on the age group. In childhood, symptoms include acroparaesthesia, pain crisis, fever, elevated ESR, hypo/anhidrosis, gastrointestinal symptoms, proteinuria, cornea verticillata, angiokeratoma; in adolescence, manifestations include angiokeratoma, fever, acroparaesthesia, dehydration, renal failure, stroke/heart attack/ transient ischemic attacks (TIA), pulmonary complications [27].

The full form of the disease is rarer than forms characterized by single-organ involvement, which occurs mainly in cardiac, renal and cerebrovascular variants.

In 89% of cases, FD is likely to become chronic, with a tendency to worsen over time. Among the early signs that occur in childhood and which may persist into adulthood, gastrointestinal involvement is described in 30-40% of cases.

Patients usually complain of abdominal pain (postprandial), diarrhoea, nausea and vomiting. These gastrointestinal symptoms may be related to the deposition of Gb3 in the autonomic ganglia of the intestine and mesenteric blood

vessels. In addition, the absence of sweating (anhidrosis) or a decreased ability to sweat (hypohidrosis) are a common clinical symptom among patients.

From the dermatological point of view [27], the most common clinical manifestation are angiokeratomas: these are raised areas of varying size, red-purple in colour, which are typically distributed across the buttocks, groin, abdomen and thighs, although they are sometimes found in mucous membranes, such as the oral cavity.

Histologically, the lesions are small superficial haemangioma that increase in number and size with age and can be either isolated or organized in groups. Other cutaneous manifestations include telangiectasias and subcutaneous oedema.

From the standpoint of ophthalmology [27], FD is characterized by various ocular signs, with involvement of conjunctival and retinal vessels, the cornea and crystal, including: cornea verticillata, subcapsular posterior cataract with a “radial” aspect, tortuosity of conjunctival and retinal vessels.

Tinnitus [27] may be an early symptom as well, as a reduction in the hearing threshold has also been reported in children. The progressive reduction in hearing threshold was seen in 80% of males and 77% of females when assessed by auditory stimulus tests. Despite the absence of severe organ dysfunction, these symptoms, alone or in combination, can cause significant

morbidity and limit the mental and physical development, social integration and development of physiological activities.

The cardiac abnormalities may be present during adolescence in both sexes and can be of various types: conduction abnormalities (reduced PR interval, arrhythmias, altered variability of heart rate valvular insufficiency), orthostatic hypertension and ventricular hypertrophic cardiomyopathy.

The progression of heart damage can occur in young adults with premature coronary artery disease, “non-obstructive” left ventricular hypertrophy, valvulopathy [27] (mitral valve prolapse is the most frequent alteration).

The involvement of the cerebral microcirculation may be responsible for ischemic clinical pictures in young adults. Neurological manifestations, impairments resulting from multifocal cerebral microcirculation, may include TIA, convulsions, parkinsonism, ischemic or hemorrhagic stroke. These first neurological problems are also an expression of the involvement of peripheral somatic nerves and the autonomous nervous system. The onset of symptoms typically occurs in later life, earlier in males than in females.

Pain is one of the first symptoms and can be distinguished between: a) episodic crises (“Fabry crises”), characterized by intense pain that radiates to the extremities of the limbs and other body parts, and b) chronic pain, characterized by burning, tingling and numbness.

Painful crises may be triggered by fever, exercise, stress or sudden changes in temperature. When crises are triggered or accompanied by fever, patients usually present with an increased ESR. For this reason, FD can sometimes be misdiagnosed as other conditions, including rheumatoid arthritis, rheumatic fever, FMF, Raynaud's phenomenon, systemic lupus erythematosus and *"growing pains"*.

The pain may be of reduced intensity in adulthood; for this reason, it is important to research the history of each patient, in particular the presence of acroparaesthesias in childhood [27].

The natural course of Fabry nephropathy in childhood and adolescence is still not largely understood. Warning signs of early kidney damage include the development of microalbuminuria and proteinuria already in second decade of life.

From the histological standpoint [27], irreversible changes in the glomeruli, tubules, interstitial and vascular structures can be observed by renal biopsy, sometimes before the appearance of microalbuminuria. A reduction in the glomerular filtration rate (GFR) is rare in childhood, though more common in adolescence.

As happens in most cases, renal disease worsens with age. Renal lesions are secondary to the storage of Gb3 [31] in glomerular endothelial, mesangial and interstitial cells, and in podocytes. Kidney failure is manifested by

microalbuminuria and proteinuria beginning during either the second or third decade of life.

The clinical signs of respiratory involvement are exertional dyspnea, chronic cough and asthma, and are frequent in both sexes.

Traditionally, females have been considered to be heterozygous *"carriers"* of the defective gene who do not develop symptoms of the disease. Indeed, as reported by several studies in the literature, even women who are heterozygous [32] for mutations in the GLA gene may develop a *"mild"* FD clinical picture. The clinical signs and symptoms are extremely varied in heterozygous females, and this phenotypic heterogeneity is partially due to *"lyonization",* the process by which a copy of the X chromosome is randomly inactivated in all cells of the female, so that heterozygous females constitute a *"mosaic"*.

Furthermore, heterozygous females may be symptomatic, probably as a result of X-chromosome inactivation, which results in a higher percentage developing X-linked genetic diseases [32].

So, generally, heterozygous females may develop damage to organs including the heart, brain *(rarely)* and kidneys *(more slowly than males).* The *"classical"* severe phenotype like the one common for males has only occasionally been observed in females. The possible symptoms include pain, orthostatic hypertension, angiokeratoma, ocular abnormalities, cochlear

involvement and reduction in the hearing threshold, gastrointestinal symptoms and respiratory tract involvement.

The median age of onset of symptoms among women is 13-20 years. There are also **Atypical Variants** of FD, characterizing those patients with mainly renal and cardiac involvement, respectively, as well as **Intermediate Variants**, described in patients who did not show the characteristic signs of FD during the period between 3 and 6 years of life [33].

2.2 DIAGNOSIS

FD has X-linked transmission which is characterized by a deficiency of the lysosomal enzyme GLA. It is encoded by a unique gene, the GLA gene, which is located on the long arm of the X chromosome, in position Xq22 [34].

The GLA gene is composed of seven exons distributed over 12,436 base pairs. There is extensive allelic heterogeneity but no genetic locus heterogeneity. FD can be caused by a variety of point missense or nonsense mutations, splicing mutations, small deletions or insertions, and large deletions. Defects in the gene encoding GLA are very heterogeneous, most of which render the enzyme nonfunctional.

Many families have unique mutations, which could potentially explain the intra-familial variability found for FD. Single nucleotide polymorphisms, such as c.-30G> A, c.-12G> A and c.-10C> T in the 5’ untranslated region (5’UTR), p.Asp313Tyr in exon 6, and sequence variations (VNTR) have been described.

Whether these are true mutations or simply polymorphisms [35] is still undergoing debate. More than 500 mutations in GLA gene have been described in FD patients. About 75% of the genetic alterations causing FD consist of point nonsense or missense mutations; in addition, extensive rearrangements, little insertions or deletions, and defects in the splicing process have also been described.

"Atypical" variants of the hemizygous phenotype, presenting mild symptoms, are mainly associated with point missense mutations and maintain some residual GLA activity.

Attempting to predict the clinical course of the disease which corresponds to a specific mutation is, at present, extremely difficult [36]. Another obstacle to the establishment of genotype-phenotype correlations is the continuous discovery and description of new mutations in the GLA gene.

A recent study of 36 unrelated families has described twenty-two new mutations in the same number of family strains (10 missense, 3 nonsense, 3 splicing defects and 6 small insertions or deletions), while previously-identified mutations were found in the remaining 14 families.

Point mutations, as well as large deletions, have been identified in intronic regions of the GLA gene. These, if they coincide with splicing sites [37], may cause a defect in the processing of pre-RNA and, consequently, lead to the formation of abnormal mRNA.

The location of the individual genetic alterations and the type of specific amino acid substitution do not, at this time, provide guidance criteria regarding the prognosis; in fact, adjacent point missense mutations in the same coding sequence but which correspond to very different clinical pictures have been described.

Recently [27], the key role of the carboxy (C)-terminal region of GLA in the regulation of enzyme activity has been discovered. Mutations that cause a single amino acid substitution [38] in this region are described in *"atypical"* cardiac variants of FD, while the interruption of the amino acid sequence in the C-terminal region is associated with severe clinical expression, even in heterozygotes. Recent studies have also shown that the reduction of enzyme activity [39] can be determined by mutations that do not directly affect the activity itself but which are involved in the maintenance of the structural stability of the enzyme. These proteins are not properly processed and are retained in the endoplasmic reticulum, subject to degradation, which results in a deficiency of protein concentration. It may be important to identify the variety of mutations determining the incorrect *"folding"* [39] of the protein in order to highlight that the incorrect structure can be pathogenetically significant in the clinical pictures of disease (Figure 4).

The demonstration of deficient GLA activity in plasma or leukocytes is the standard laboratory method which, when systematically used to confirm the clinical diagnosis of FD in males, can provide conclusive results. In contrast, affected females may have enzyme activity which falls within the normal range. Therefore, in such cases, GLA gene analysis aimed at identifying mutations responsible for FD is essential.

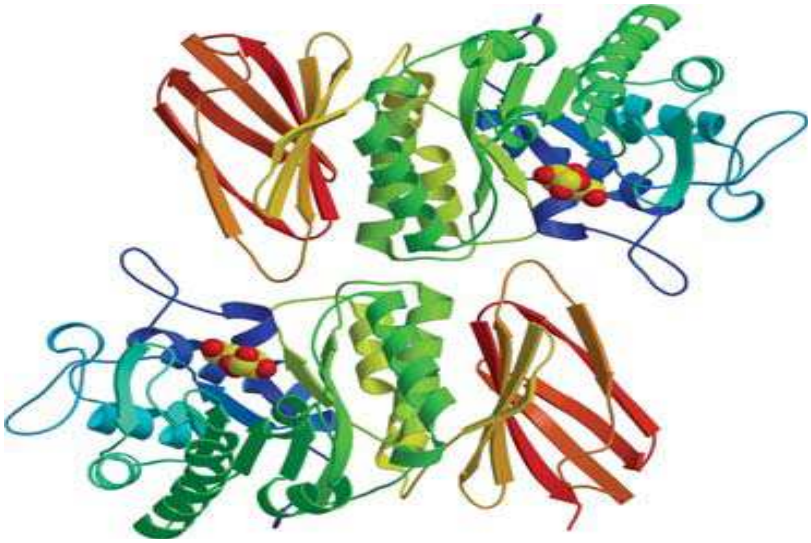

Figure 4. Conformational structure of the enzyme protein

Finally, the dosage of plasma Gb3 [31] has also been proposed and used in the biochemical diagnosis of FD, but it is a method that takes time. Whereas plasma levels of Gb3 are generally lower in females than males, the evaluation of the urinary excretion of Gb3 (Figure 5) is considered a reliable indicator in most patients, both male and females.

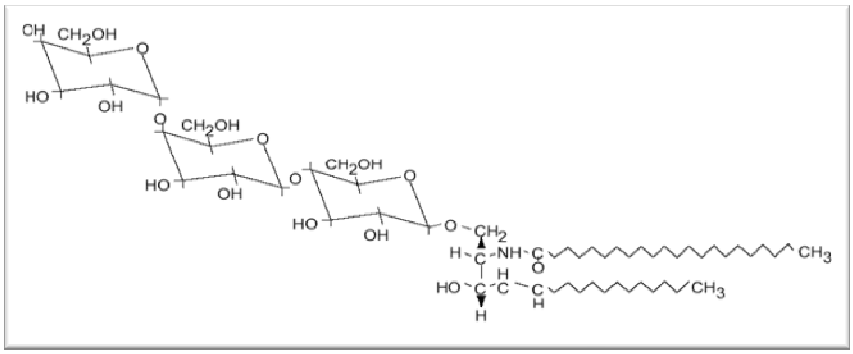

Figure 5: Gb3 structure

However, though the determination of Gb3 levels in plasma and urine analyses is necessary, they do not always correlate with the severity and onset of clinical manifestations. In recent years, lyso-Gb3 (Gb3 deacetylated) has emerged as a possibly more reliable marker than traditional Gb3 because there is a clearer correlation between deacetylated Gb3 levels and the onset of symptoms in FD subjects [40].

2.3 THERAPY

The symptomatic treatment of FD relies on the use of drugs aimed at controlling painful crises. If indicated, nonspecific drug therapy (antiplatelet agents, anticoagulants, antihypertensives, statins) should be continued even while making use of enzyme replacement therapy (ERT) [34, 41] (Figure 6).

Organ/system	Assessment	Guidelines
General	General status, quality of life (SF36® Health survey, EuroQOL or PedsQL® measurement mode), school or work performance, depression, anxiety, drug use, somatic growth	Baseline (at first visit), every 12 months
	Complete physical examination	Baseline, every 12 months
	Genetic counseling	Baseline, on request
	Alpha-galactosidase A activity and genotype	If not previously performed or determined
Kidney	Serum creatinine, ionogram, BUN; morning spot urine for urinary protein/creatinine ratio and albumin/creatinine ratio Urinary Gb_3 (optional)	Baseline. Every 3 months if CKD stage 1 or 2 and >1 g/day of proteinuria or CKD stage 4 Every 6 months if CKD stage 3 Every 12 months if CKD stage 1 or 2 and <1 g/day of proteinuria
Cardiac	Palpitations, angina Blood pressure, rhythm	Baseline, every 6 months Every evaluation visit
	ECG, echocardiography 2-D with Doppler	Baseline, every 12 months
	Holter monitoring	If an arrhythmia is suspected or palpitations are present
	Cardiac MRI	Every other year
	Coronary angiography	If clinical signs of angina
Neurologic	Acroparesthesias, fatigue, fever, heat and cold tolerance, stroke-related symptoms, TIA	Baseline, every 12 months
	Neurologic examination, questionnaires (Brief Pain Inventory)	Baseline, every 12 months
	Brain MRI without contrast	Baseline At time of a TIA or stroke event In females to document CNS involvement Every 3 years
	Magnetic resonance angiography	If cerebral vasculopathy should be excluded
	Comorbid stroke risk factors: Cholesterol (Total, LDL, HDL), triglycerides, Lpa, total plasma homocysteine	Baseline, every 12-24 months
ENT	Tinnitus, hearing loss, vertigo, dizziness	Baseline, every 6 months
	Audiometry, tympanometry, otoacoustic emissions	Baseline, every 12 months thereafter
Ophthalmologic	General ophthalmologic exam (slit-lamp, direct ophthalmoscopy, best corrected visual acuity, visual fields)	Baseline, every 12-24 months
Pulmonology	Cough, exertional dyspnea, wheezing, exercise intolerance	Baseline, every 12 months
	Spirometry	If clinical signs
Gastrointestinal	Postprandial abdominal pain, bloating, diarrhea, nausea, vomiting, early satiety, difficulty gaining weight Endoscopic evaluations	Baseline, every 12 months If symptoms persist or worsen despite treatment
Skeletal	Bone mineral density, 25(OH) vitamin D levels	Baseline

Figure 6. Management of multi-organ Fabry disease

ERT involves the intravenous administration of the deficient enzyme every two weeks. The studies [42] reported in the literature have shown the effectiveness of enzyme therapy in slowing the progression of renal and cardiac damage and in improving symptoms related to the involvement of the peripheral nervous system. Generally well-tolerated, enzyme therapy has been shown to bring about a significant improvement in patients' quality of life after about 1-2 years of therapy with agalsidase A.

3. OBJECTIVE OF THE STUDY

In the absence of a severe clinical picture, some symptoms of FD (recurrent fever, storage disease, renal and abdominal pain) can be confused with those of FMF, causing erroneous diagnoses of FMF and, consequently, the underestimation of FD [43]. For this reason, one of the goals of this research project was to **demonstrate how, in the absence of a severe clinical systemic picture of FD, the clinical overlap between FD and FMF can lead to diagnostic errors.**

This difficulty in correctly differentiating between these diseases supports the idea that an analysis of the GLA and MEFV genes, along with taking a careful family history, should be performed for all patients with a suspected diagnosis of FMF in case their diagnosis turns out not to be genetically justified.

In addition, the genotype-phenotype correlation in patients with FMF is highly variable and not always easy to identify.

This is due, in part, to the fact that 30% of FMF patients with compatible symptoms are carriers of a single demonstrable mutation in the MEFV gene [44, 45]. The hypothesis that subjects with a single mutation in the MEFV gene may have mutations in a second gene has already been taken into account, and the SAA1 gene, which encodes the serum amyloid A, has been identified as one of the few examples of a modifier gene in humans [5]. Hence, modifier genes may

be important in determining the clinical variability among heterozygous patients with MEFV mutations. In addition, the genes of other autoinflammatory syndromes (MVK, TNFRSF1A, PSTPIP1) were considered as potential modifiers of the FMF heterozygote phenotype, but this hypothesis has not produced encouraging results [46].

Among the diseases that begins in childhood, or who have such as frequent symptoms , recurrent episodes of fever and gastrointestinal manifestations, there is also Fabry disease.

So my research project also aimed **to consider the GLA gene as a possible *modifier gene* that may aggravate the clinical phenotype of FMF.**

The idea of studying the GLA gene in subjects with a diagnosis of FMF derived in part from the analysis of two cases of FMF misdiagnosis. These two probands who had been diagnosed with FMF also had symptoms related to FD, such as recurrent fever, acroparaesthesias, cardiac, neurologic and renal dysfunction.

Thus, genetic analysis was performed not only on the MEFV gene, whose mutations are responsible for FMF, but also on the GLA gene responsible for FD. Genetic analysis did not support the diagnosis of FMF [43], as the results showed that, in both probands with suspected FMF, there were no significant mutations in the MEFV gene related to the disease (Table 3).

		GLA								MEFV		
		Enzyme Activity nmol/ml /h	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon2	Exon 3	Exon 10
Family 1	Proband	3,2	-10 C>T	wt	c.370-76 - 80del (homozygotes) + p.S126G	wt	c.640-16A>G (homozygotes)	wt	c.1000-22C>T (homozygotes)	wt	p.P369S	wt
	Father	4,6	wt	wt	c.370-76 - 80del	wt	c.640-16A>G	wt	c.1000-22C>T	p.E148Q	p.P369S	wt
	Mother	2,3	-10 C>T	wt	c.370-76 - 80del + p.S126G	wt	c.640-16A>G	wt	c.1000-22C>T	wt	wt	wt
	Sister	3,2	-10 C>T	wt	c.370-76 - 80del (homozygotes) + p.S126G	wt	c.640-16A>G (homozygotes)	wt	c.1000-22C>T (homozygotes)	p.E148Q	wt	wt

		Enzyme Activity nmol/ml /h	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 2	Exon 3	Exon 10
Family 2	Proband	2,6	-10 C>T p.M51I	wt	c.370-76 - 80del	wt	c.640-16A>G	wt	c.1000-22C>T	wt	wt	p.A744S
	Father	4,3	-10 C>T	wt	c.370-76 - 80del	wt	c.640-16A>G	wt	c.1000-22C>T	wt	wt	p.A744S
	Mother	3,5	p.M51I	wt	wt	wt	wt	wt	wt	wt	wt	wt

Table 3. GLA and MEFV genetic diagram of two families. All mutations are underlined, mutations in the coding regions are in bold red. Normal values of GLA activity assayed in whole blood are ≥ 3.5 nmol/ml/h.

Instead, both subjects were identified as having exonic mutations in GLA (Table 3). These two mutations, p.M51I and p.S126G, have been identified in the literature as responsible for an atypical variant and the classical form of FD, respectively [30, 36, 38]. These amino acid substitutions were also found in other members of both families. In addition, the presence of intronic mutations in the GLA gene (Table 3) aggravated both families' genetic situations [35, 47-51].

These mutations in intronic regions may, according to data in the literature, play a role in both the quality and quantity of GLA transcription and translation [47, 48]. This hypothesis is strengthened, in some cases, by the presence of the accumulation of Gb3 or lyso-Gb3 in the blood and urine of patients with these mutations [51]. In light of these results, MEFV and GLA were analyzed in 186 patients who had been diagnosed with FMF.

4. MATERIALS AND METHODS

We analyzed 186 patients who had been diagnosed with FMF, a group made up of 93 women (age range 7-50 years) and 93 men (age range 5-30 years).

DNA isolation

DNA samples were isolated from whole blood by column extraction (GenElute Blood Genomic DNA Kit, Miniprep, Sigma-Aldrich, USA), and their concentrations were determined using a spectrophotometer.

HRM analysis and DNA sequencing

High resolution melting (HRM) analysis was performed on the DNA samples, using the Light Cycler 480 system (Roche Applied Science, Germany). This system is able to detect any sequence variation in the amplicons of the gene under analysis by using a programme known as “Gene Scanning” software (Roche Applied Science, Germany). HRM analysis is able to detect gene mutations according to the dissociation temperature of two strands (Tm) and subsequent melting curve analysis (Figure 7).

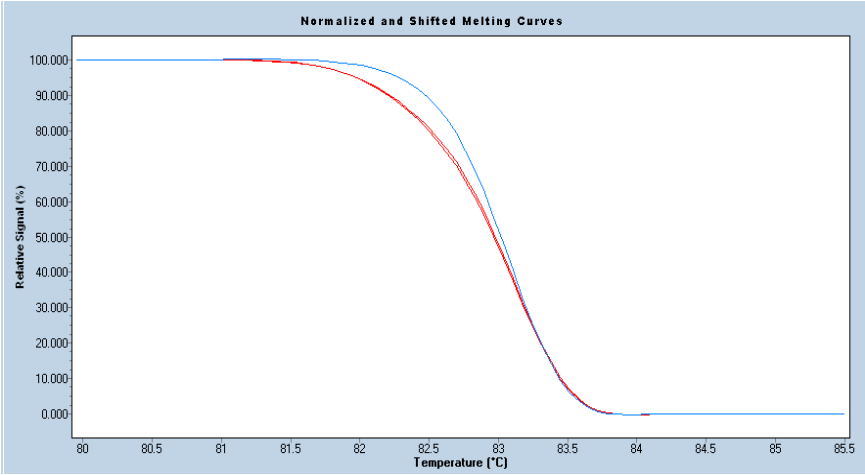

Figure 7. Melting curves of a mutated sample (red curve) and healthy control (blue curve)

The whole process of real-time polymerase chain reaction (PCR) HRM analysis consists of an amplification step for the DNA regions targeted and a subsequent separation of amplicons induced by an increase in temperature. The melting curves generated are subsequently analyzed using the Gene Scanning application.

This method uses the “new generation” SYBR Green dye, the Light Cycler 480 HRM dye, which is able both to bind to all the minor grooves and to the DNA double strand. Therefore, the change in fluorescence is used both to monitor the increase in DNA concentration during real-time PCR and to construct the melting curves during HRM. Three primer pairs were designed for the analysis of exons 2, 3 and 10 of the MEFV gene (Table 4). Seven pairs of primers were designed for the analysis of seven target regions containing the seven exons of the GLA gene and the splicing regulatory sequences flanking them (Table 4).

	Region	Primers
GLA	Exon 1	5′-TCTTACGTGACTGATTATTGGTCT-3′
		5′-CACACCCAAACACATGGAAA-3′
	Exon 2	5′-TGAAATCCCAAGGTGCCTAATA-3′
		5′-GTACAGAAGTGCTTACAGTCC-3′
	Exon 3	5′-ACCTGGTGAAGTAACCTT-3′
		5′-CTCAGCTACCATGGCCT-3′
	Exon 4	5′-GCTGGAAATTCATTTCTTTCCC-3′
		5′-GGATGGTGAGAAGTGGTTG-3′
	Exon 5	5′-AATCTGTAAACTCAAGAGAAGGCTA-3′
		5′-CTTTACCTGTATTTACCTTGAATG-3′
	Exon 6	5′-GATGCTGTGGAAAGTGGTT-3′
		5′-GCCCAAGACAAAGTTGGTAT-3′
	Exon 7	5′-AGAATGAATGCCAAACTAAC-3′
		5′-ATGAGCCACCTAGCCTTG-3′
MEFV	Exon 2	5′-GCCTGAAGACTCCAGACCA-3′
		5′-TCAAGGCTTCTAGGTCGC-3′
	Exon 3	5′-TCCCCGAGGCAGTTTCTGGGCACC-3′
		5′-TGGACCTGCTTCAGGTGGCGCTTA-3′
	Exon 10	5′-TTACTGGGAGGTGGAGG-3′
		5′-AGGGCTGAAGATAGGTTG-3′

Table 4. Primer sequences used to analyze the GLA and MEFV genes

The following real-time PCR conditions were used: 5-30 ng of genomic DNA was amplified in a total volume of 20 µl containing Light Cycler 480 Master Mix (comprising a fluorescent HRM dye; Roche Applied Science, Germany), 3.5 mM MgCl2, 0.2 µM specific PCR primers.

Amplification parameters used in the Light Cycler 480 instrument were: 95°C for 10 minutes, then 40 cycles each at 95°C for 10 seconds, 58°C for 15 seconds and 72°C for 15 seconds (Figure 8).

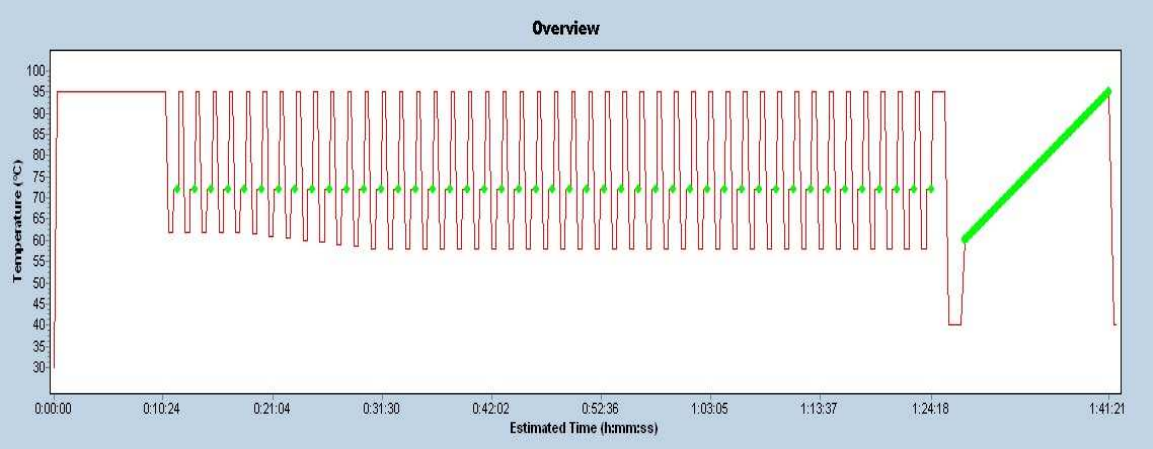

Figure 8. Amplification parameters used in real-time PCR HRM

After amplification, PCR products were melted from 65°C to 95°C (Figure 6), and the melting profiles were analyzed using Gene Scanning software. In order to ensure the detection of hemizygotes in the GLA analysis, male samples were treated by adding a wild type DNA counterpart, in a 1:1 quantity ratio, to produce artificial heterozygotes and induce heteroduplex formation.

PCR products presenting melting curves different in position or shape from those of the wild type control were sequenced to identify the suspected mutations (Figure 7). Purified PCR products were sequenced using the automated DNA sequencer at BMR Genomics.

α-galactosidase activity assay

GLA activity was determined by a modified version of the Dried Blood Filter Paper test described by Chamoles et al. [52]. Briefly, duplicate samples of

whole blood spots (5.5 µl/disk) were incubated at 37°C, against a blank, for 1, 2 and 3 hours, with 70µl of a mixture containing 4-methylumbelliferyl-a-D galactopyranoside 5 mM and N-acetyl-D-galactosamine 0.25 M, in a ratio of 2.5:1 (v:v), both in citrate-phosphate buffer (CPB) 0.15 M pH 4.5.

To control the specificity of the enzymatic reaction, we used the GLA inhibitor deoxygalactonojirimycin-hydrochloride 0.6 mg/ml in CPB. The reactions were stopped at different times by adding 250 µl of ethylendiammine 0.1 M pH 11.4. Released 4-methylumbelliferone (4-MU) was measured using a Wallac Microtiter Fluorometer plate reader (Perkin Elmer, USA) at 355 nm excitation and 460 nm emission wavelength with a calibration curve up to 20 nmol 4-MU sodium salt. Enzymatic activity was measured as U/ml/hr (1 U = 1 nmol) of 4-MU by integrating the three different end point reactions.

5. RESULTS

Molecular analysis of the MEFV and GLA genes was performed on 186 patients with clinical diagnosis of FMF. 21 patients (11.3%) tested for the gene had a MEFV mutation in homozygosis (M680I, R761H, M694), 9 (4.9%) were "compound heterozygotes" (M694V-E148Q, M694V-M694I, M680I-V726A), 57 patients (30.6%) were carriers of a single mutation in the MEFV gene (M680I, M694V, P369S, E148Q, V726A), 99 did not present mutations in MEFV (53.2%) (Table 5).

Patients with suspected diagnosis of FMF	**N. Patients with MEFV gene mutations**
Homozygotes (M680I, R761H, M694)	21
Compound heterozygotes (M694V-E148Q, M694V-M694I, M680I-V726A)	9
Heterozygotes (M680I, M694V, P369S, E148Q, V726A)	57
MEFV negative subjects	99
Total subjects	186

Table 5: Results of MEFV gene analysis conducted on patients diagnosed with FMF

Patients homozygous for MEFV showed a “classical” phenotypic framework, while heterozygous ones manifested phenotypic frameworks of “mild” and / or “PFAPA-like” type (recurrent fever, exudative pharyngitis, aphthous ulcers of the oral cavity, lymphadenopathy).

As stated previously, the determination of genotype-phenotype correlation is not always straightforward for FMF; the clinical manifestations and severity of the phenotype depend on the type of mutation, whether they are of high and / or low penetrance, and the presence of “compound heterozygosity”.

The subjects in which the clinical diagnosis of FMF has not been supported by genetic analysis of MEFV showed some symptoms of FMF, such as recurrent episodes of fever and abdominal pain. The “incidence” of individual clinical manifestations in our case studies is reported in the graph below, according to genotype (Figure 9).

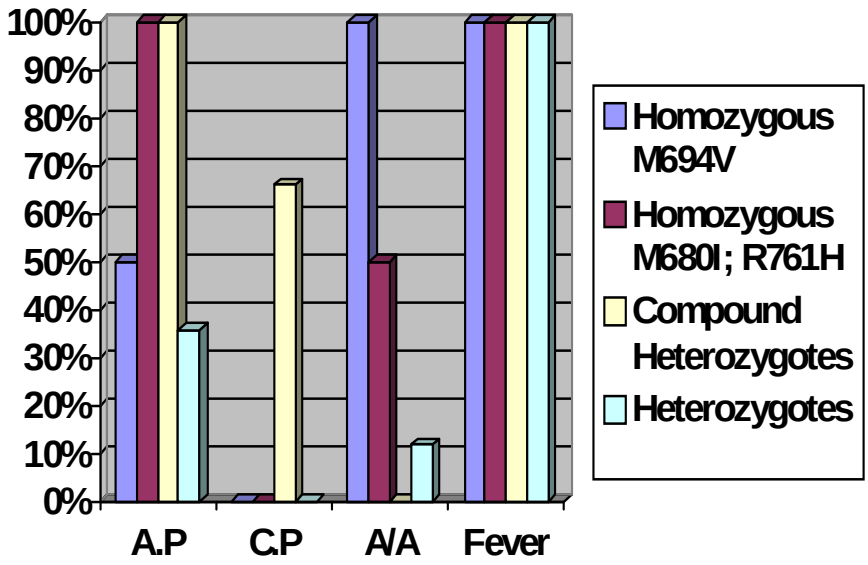

Figure 9. A.P.: abdominal pain; C.P: chest pain; A/A: arthritis/arthralgia, fever

GLA gene analysis of the same group has allowed us to document the presence of GLA gene mutations in 66/186 patients with suspected diagnosis of FMF (Table 6).

	alpha galactosidase A gene								
Haplotype	Exon 1	Intron1/ Exon 2	Intron2/ Exon 3	Intron 3/ Exon 4	Intron 4/ Exon 5	Intron 5/ Exon 6	Intron 6/ Exon 7	Intron 4	Total mutated samples
1	-10 C>T	wt	IVS2-76_80del5	wt	IVS4-16 A>G	wt	IVS6-22C>T	wt	33/186
2	wt	wt	IVS2-76_80del5	wt	IVS4-16 A>G	wt	IVS6-22C>T	wt	6/186
3	-12G>A	wt	wt	wt	IVS4+68 A>G	wt	IVS6-22C>T	wt	3/186
4	-12G>A	wt	wt	wt	IVS4+68 A>G;	wt	IVS6-22C>T	IVS4+866_867delAG	15/186
2+3	-12 G>A	wt	IVS2-76_80del5	wt	IVS4+68 A>G; IVS4-16 A>G	wt	IVS6-22C>T omozigosi	wt	3/186
Exonic mutation	wt	wt	p.S126G	wt	wt	wt	wt	wt	3/186
Intronic mutation	wt	wt	wt	wt	wt	IVS5-33C>G	wt	wt	3/186
Total samples with mutation in GLA gene									66/186

Table 6. Mutations individuated in the GLA gene and the number of patients with these mutations / total number of patients studied

The S126G mutation, responsible for the classical form of FD [36], was found in 3/99 patients diagnosed with FMF but lacking mutations in the MEFV gene, all members of the same family(Table 7).

	Gene alpha galactosidase A									
	Exon 1	Intron1/ Exon 2	Intron2/ Exon 3	Intron 3/ Exon 4	Intron 4/ Exon 5	Intron 5/ Exon 6	Intron 6/ Exon 7	Intron 4	Number of patients without mutations in MEFV gene and with exonic mutations in GLA	Total of patients studied with suspected FMF
Exonic mutation	wt	wt	p.S126G	wt	wt	wt	wt	wt	3/99	186
Total samples with exonic mutation in GLA gene									3/99	

Table 7. Number of patients diagnosed with FMF, negative for MEFV gene mutations, but having an exonic mutation in the GLA gene.

Analysis of enzymatic activity showed that these family members with the S126G mutation in the GLA gene (Table 7) reported values of alpha galactosidase A activity lower than normal range (normal values of α-galactosidase A activity in whole blood are ≥ 3.5 nmol/ml/h).

A careful clinical investigation showed that they manifested certain clinical signs characteristic clinical picture of a systemic disease such as FD, such as heart disease and the coexistence of different symptoms. These subjects, in fact, showed an involvement of systemic signs and symptoms more suggestive of FD compared to patients without mutations in GLA.

Of the 21 patients homozygous for mutations in the MEFV gene, 9 also had intronic mutations in the GLA gene which were transmitted as an haplotype (Table 8).

	alpha galactosidase A gene									
Haplotype	Exon 1	Intronl/ Exon 2	Intron 2/ Exon 3	Intron 3/ Exon 4	Intron 4/ Exon 5	Intron 5/ Exon 6	Intron 6/ Exon 7	Intron 4	Number of homozigous patients in MEFV gene with mutation in GLA	Total of patients studied with suspected FMF
1	-10 C>T	wt	IVS2-76_80del5	wt	IVS4-16 A>G	wt	IVS6-22C>T	wt	9/21	186
Total samples with mutation in GLA gene									9/21	

Table 8. Number of patients homozygous in MEFV also having mutations in the GLA gene

All 21 of the patients homozygous for the MEFV gene showed a classical picture of the FMF phenotype, characterized by recurrent fever, arthralgia, abdominal pain, myalgia and gastrointestinal events. The 9 subjects also having mutations in the GLA gene (Table 8), while responding well to colchicine, maintained high values of SAA> 50 mg /dl, which were normal in the patients without mutations in the GLA gene were found.

Of the 9 patients compound heterozygous for the MEFV gene (Table 9), 3 were also found to have intronic mutations in GLA.

	Gene alpha galactosidase A									
Haplotype	Exon 1	Intronl/ Exon 2	Intron 2/ Exon 3	Intron 3/ Exon 4	Intron4/ Exon 5	Intron5/ Exon 6	Intron6/ Exon 7	Intron 4	Number of compound heterozygous patients in MEFV gene with mutations in GLA	Total of patients studied with suspected FMF
3	-12G>A	wt	wt	wt	IVS4+68 A>G	wt	IVS6-22C>T	wt	3/9	186
Total samples with mutations in GLA gene									3/9	

Table 9. Number of patients compound heterozygous in MEFV also having mutations in the GLA gene

These 3 subjects, compared to the 6 compound heterozygotes in which the investigation of the GLA gene was negative (Table 9) showed, in addition to a PFAPA-like clinical picture, multiple cerebral ischemia, early signs of renal impairment (proteinuria) and reduced hearing threshold.

Of the 57 patients with single mutations in the MEFV gene, 18 also had mutations in the GLA gene. (Table 10).

	alpha galactosidase A gene									
Haplotype	Exon 1	Intron 1/ Exon 2	Intron 2/ Exon 3	Intron 3/ Exon 4	Intron 4/ Exon 5	Intron 5/ Exon 6	Intron 6/ Exon 7	Intron4	Number of heterozygous patients in MEFV gene with mutation in GLA	Total of patients studied with suspected FMF
1	-10 C>T	wt	IVS2-76_80del5	wt	IVS4-16 A>G	wt	IVS6-22C>T	wt	15/57	186
Intronic mutation	wt	wt	wt	wt	wt	IVS5-33C>G	wt	wt	3/57	
Total samples with mutation in GLA gene									18/57	

Table 10. Number of patients heterozygous in MEFV also having mutations in the GLA gene

Clinically, there were no significant differences between subjects with a single mutation in the MEFV gene and those in which mutations were also found in GLA (Table 10). On average, these subjects, apart from some presumptive FMF symptoms (fever and abdominal pain), did not show a characteristic pattern of FMF. In fact, some subjects (with and without mutations in GLA) had other signs which are not common to FMF, such as

angiokeratomas, gastrointestinal events, cardiac arrhythmias, conduction defects, dyspnea and recurrent headaches.

Of the 99 patients who had been diagnosed with FMF but whose MEFV gene analysis did not support such a diagnosis, 33 were found to have mutations in GLA (Table 11).

	Gene alpha galactosidase A									
Haplotype	Exon 1	Intron1/ Exon 2	Intron 2/ Exon 3	Intron 3/ Exon 4	Intron 4/ Exon 5	Intron 5/ Exon 6	Intron 6/ Exon 7	Intron 4	Number of patients without mutations in MEFV gene and with mutations in GLA	Total of patients studied with suspected FMF
1	-10 C>T	wt	IVS2-76_80del5	wt	IVS4-16 A>G	wt	IVS6-22C>T	wt	9/99	186
2	wt	wt	IVS2-76_80del5	wt	IVS4-16 A>G	wt	IVS6-22C>T	wt	6/99	
4	-12G>A	wt	wt	wt	IVS4+68 A>G;	wt	IVS6-22C>T	IVS4+866_867delAG	15/99	
2+3	-12 G>A	wt	IVS2-76_80del5	wt	IVS4+68 A>G; IVS4-16 A>G	wt	IVS6-22C>T omozigosi	wt	3/99	
Total samples with mutations in GLA									33/99	

Table 11. Number of patients without mutations in MEFV but having mutations in the GLA gene

There were no striking clinical differences found between these groups of patients (Table 11). Both the 33 patients with intronic mutations in the GLA gene and the 63 subjects in which the analysis was negative for both genes, except for a few symptoms suggestive of FMF (fever and abdominal pain), showed other systemic signs and symptoms.

All the patients with mutations in intronic region of GLA gene reported values of α-galactosidase A activity within the normal range (normal values of α-galactosidase A activity in whole blood are ≥ 3.5 nmol/ml/h).

6. DISCUSSION

The first objective of this research project was to demonstrate how, in the absence of a severe clinical picture of systemic FD, overlapping clinical features of FD and FMF can lead to errors in diagnosis, as noted in the two cases described in the “objective of the study” section.

The identification of the S126G mutation associated with FD [36] in 3 patients belonging to the same family, in which the presumptive diagnosis was of FMF, leads us to consider the hypothesis of our study to be valid. In fact, the exonic mutation which results in the substitution of a polar amino acid (serine) for part of a non-polar amino acid (glycine) has been associated with FD in the literature.

Thus, some clinical symptoms of FD (recurrent fever, kidney storage disease and abdominal pain) may be confused with those of FMF [43], causing an error in the diagnosis of FMF, and consequently an underestimation of FD. For this reason, a careful genetic analysis of the GLA and MEFV genes, as well as a proper family history, needs to be done for subjects presenting a clinical picture compatible with the two diseases.

The data from our study, obtained from a series of selected patients, can be considered very significant especially when compared to the incidence of FD in the general population. Furthermore we plan to extend the genetic

investigation of the GLA gene to a wider series of patients with diagnosis of FMF, focusing particular attention on those cases that do not respond to colchicine and those in which the diagnosis is not confirmed by the presence of mutations in the MEFV gene.

The second objective of this research project was to consider the GLA gene as a possible modifier gene in FMF.

The choice to study the gene GLA as a possible modifier gene in patients with FMF derives from the fact that 30% of FMF patients with compatible symptoms are carriers of a single demonstrable mutation in the MEFV gene [44, 45] that alone, however, would not be responsible for FMF.

Other studies that were designed to analyze genes other than MEFV in patients with FMF as potential modifier genes have been carried out on SAA1 (serumamyloid A) and genes related to other autoinflammatory syndromes (MVK, TNFRSF1A, PSTPIP1). This hypothesis has produced encouraging results, including the finding that SAA1 is indeed one of the few known examples of a modifier gene in humans.

Regarding our results, the clinical diagnosis of homozygous and compound heterozygous individuals with mutations in the MEFV gene as well as mutations in the GLA gene showed some differences when compared to the subjects with only mutations in the MEFV gene. The former tend to manifest high inflammatory indices and the presence of other non-specific symptoms of

FMF, such as multiple cerebral ischemia, early signs of renal impairment (proteinuria), as well as a reduction in hearing threshold.

So, given the clinical and genetic results obtained, it is possible to hypothesize that, in subjects homozygous and / or compound heterozygous for the MEFV gene, the presence of a mutation in the GLA gene can “aggravate” the clinical phenotype.

In conclusion the following assumptions may be made:

1) in individuals carrying a single mutation in the MEFV gene and manifesting a phenotype compatible with the diagnosis of FMF, but with an incomplete genotype, the presence of a mutation in the GLA gene may support the hypothesis of “gene modifiers”, according to which particular groups of genes change the expression of a simple character which usually depends on a pair of alleles;

2) in individuals carrying a single mutation in the MEFV gene and having an incomplete phenotype, and in those whose genetic analysis of MEFV does not justify diagnosis with FMF, the detection of intronic mutations in the GLA gene could support the diagnosis of another systemic pathology.

These results obtained concerning the interaction between the MEFV and GLA genes are preliminary. We intend, therefore, to extend the study to a larger and more selective patient series.

7. FUNDING

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