Chapter

Adaptation to Mediterranea

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Abstract

The Mediterranean region encompasses countries that surround Mediterranean Sea. Due to its position at the intersection of Eurasia and Africa it has often been a route of human migrations during history, which contributed to its high biodiversity. People living in this area had been exposed to the episodes of natural selection that led to the establishment of specific genetic variations, for which is thought to carry a certain adaptation. Some recent studies have shown that genetic adaptations are probably related to the immune defense against infectious pathogens. One of the most recognizable disease of the region is familial Mediterranean fever (FMF), a prototype of a monogenic autoinflammatory disease. FMF is predisposed by the mutations in the Mediterranean fever (*MEFV*) gene that encodes inflammasome regulatory protein - pyrin. Specific variations of several other genes have been proposed to confer a protection against *Plasmodium malariae* parasite. Some of these are hemoglobin S (HbS), thalassemia, glucose-6-phosphate dehydrogenase deficiency, ovalocytosis, and mutation in the Duffy antigen (FY). In this chapter we will summarize important genetics and pathogenesis features of diseases commonly encountered in the Mediterranean region with a short discussion of potential adaptations that they may carry.

Keywords: familial Mediterranean fever, thalassemia, malaria, hemoglobin S, Duffy antigen, heterozygote advantage

1. Introduction

The Mediterranean region encompasses the lands surrounding the Mediterranean Sea; on the north there is Southern Europe and Anatolia, on the south North Africa, and on the east the countries of Levant. The Mediterranean region has a specific climate, with mild winters and hot, dry summers, which supports the characteristics of the Mediterranean flora and fauna. The region's location at the intersection of Eurasia and Africa has contributed to the high biodiversity of its inhabitants, including people. Beside climate, this region has historically been the most frequent route of human migrations, as it is today.

Given the specific environment influences, several important genetic variations occurred and persist in people living in this area. Some of the genetic adaptations carry a certain degree of protection against infectious agents, but at the same time, when in an inadequate genotype, they can cause health disorders. This chapter will describe and discuss the most common types of genetic variations in the Mediterranean area related to adaptation and / or susceptibility to disease.

2. Mediterranean fever (*MEFV*) gene mutations

2.1 Protein pyrin

One of the most recognizable diseases of the Mediterranean region is Familial Mediterranean Fever (FMF), a prototype of a monogenic autoinflammatory disease, associated with mutations in the *MEFV* gene that encodes for protein pyrin. Autoinflammatory disorders are characterized by dysregulation of innate immune response, unlike autoimmune diseases that are primarily mediated by adaptive immunity. However, approximately in a third of FMF patients pathogenic *MEFV* mutation is not identified, hence the diagnostic criteria for FMF still rely on clinical manifestations [1, 2]. The *MEFV* gene is composed out of 10 exons and 13 introns, which make 781 amino acids (aa) long, multifunctional, protein pyrin. One of its first described functions is the assembly of an inflammasome. Pyrin acts as a pattern recognition receptor (PRR) that senses intracellular danger signals after which it binds to an adaptor protein and oligomerizes to form a pyrin inflammasome. Subsequently, inflammasome recruits and activates caspase-1, which further cleaves pro-inflammatory molecules, such as interleukin (IL)-1β and IL-18 [1, 3, 4].

As a PRR, pyrin seems to recognize downstream effects of a pathogen-driven modification and/or inactivation of RhoA GTPases - molecules that regulate actin dynamics [3]. By sensing a disturbance in actin signaling, pyrin recognizes common virulence mechanisms and starts an immune response. Several pathogen bacteria employ actin cytoskeleton for their invasion and survival, and by secretion of Rho-inactivating cytotoxins they were shown to activate pyrin inflammasome (e.g. *Clostridium, Vibrio parahaemolyticus, Bordetella pertussis, Yersinia pestis*) [5–7].

Besides, pyrin regulates process of autophagy a highly specific degradation of inflammasomes components. With this process pyrin suppresses IL-1β production, thereby preventing an excessive inflammation. Additionally, autophagy-based secretory pathway enables a group of proteins to exit cytoplasm without entering Golgi apparatus, among which is IL-1β [8, 9]. Hence, *MEFV* mutation-induced alterations affect this pathway and may facilitate interleukins secretion.

The pyrin activation requires at least two independent processes: dephosphorylation and pyrin inflammasome maturation involving microtubule dynamics [2, 7]. In FMF patients with pathogenic *MEFV* variants there is a hyperreactive state of the pyrin inflammasome. It seems that the second control mechanism of pyrin activation is lacking, and that pyrin is maintained inactive only by phosphorylation. Mutations in the exon 10 do not impact pyrin phosphorylation but may affect the control mechanism of microtubule dynamics [4, 7].

2.2 The *MEFV* mutations and their effect

According to the Infevers registry (the registry of hereditary autoinflammatory disorders mutations) there are 377 nucleotide variants identified in the *MEFV* gene so far. Most of them are benign and not involved in pathogenesis of FMF.

The FMF has long been considered an autosomal recessive disease, but with description of cases with heterozygote *MEFV* mutations this definition has changed. The mutations may express their effect in either a recessive or a dominant manner, depending on their location in the gene. Generally, those in the exon 10 are considered recessive, while other manifest their effect in a heterozygous state and are considered dominant (gain-of-function). The most frequently identified FMF-causing *MEFV* variants are in the exon 10 and encompass M694V (c.2080A > G), M680I (c.2040G > C), and V726A (c.2177 T > C) missense mutations, with the carrier frequency of ~10% in the populations of the Mediterranean region [2, 4, 8, 10].

In order to achieve a better classification of pathogenic *MEFV* mutations, and to set a guidelines for genetic diagnostic testing of hereditary recurrent fevers, Shinar et al. [11], adopted the final consensus document that proposed a group of bialelic mutations to be used for definition of FMF. This group comprises 14 mutations, 9 of which are clearly pathogenic (M694V, M694I, M680I, V726A, R761H, A744S, I692del, E167D, and T267I), while 5 mutations are designated as of unknown significance (E148Q, K695R, P369S, F479L, and I591T).

Depending on the type of mutations, permissive environmental factors and genetic background, clinical picture of FMF may vary from typical recurrent inflammatory attacks to mild symptoms or asymptomatic cases. The most frequent symptoms are recurrent episodes of fever with serosal inflammation, arthralgia or arthritis, abdominal pain, and localized erythematous skin rash. Episodes are self-limited and usually resolve within 48-72 h. Heterozygous patients usually have milder symptoms and shorter and less frequent attacks. Some asymptomatic carriers may have elevated inflammatory and oxidative stress biomarkers [4, 10, 12, 13]. The most concerning long-term complication is renal amyloidosis, and renal transplantation is the choice in most end-stage renal disease [14, 15]. Standard treatment is a prolonged use of colchicine, although there are resistant cases. One potential cause of resistance is the vitamin D deficiency in these patients [16–18].

The M694V homozygote mutation is mostly associated with early onset of disease and severe course. It seems that environmental factors have stronger influence on this mutation [19, 20]. It is interesting to note the impact of environmental factors, since patients of the same ethnicity have different phenotype depending on the country they live in, *i.e.* the Eastern or Western Europe. Besides, a set of additional factors influence the phenotype, such as patient's age and sex, micro-RNAs, immune factors (HLA I gene A), and microbiota [11, 20–22].

2.3 Potential heterozygote advantage *of MEFV* mutations

The higher frequency of *MEFV* mutations among multiple populations in the Mediterranean region suggests an existence of a heterozygote advantage. Mostly accepted theories explaining this assumption are those that recognize mutations as an adaptation to yet undetermined endemic infectious agent or, more probably, a group of agents. It seems that *MEFV's* exon 10 had been exposed to the episodic positive selection in primates [2, 4, 23, 24].

Several bacteria secrete invasive factors (toxins) that covalently modify RhoA or its regulators. Other may inhibit pyrin inflammasome assembly by keeping it in the phosphorylated state, such as a protein YopM produced by bacteria *Yersinia enterocolitica*. Pyrin activation occurs when RhoA GTPases are disabled to promote their downstream signaling. In that sense, bacteria *Yersinia pestis* (bubonic plague-bacterium) is proposed as a possible agent that had led to the selection of gain-of-function *MEFV* mutations [25]. Other hypotheses imply that mutated pyrin may confer a protection against tuberculosis or brucellosis, but without direct evidence [2]. Potential association between mutated pyrin and defense against tuberculosis is within the processes of autophagy and inflammasome activation. *Mycobacterium tuberculosis* is capable of arresting phagolysosome biogenesis in macrophages and prevents inflammasome activation by its Zn-metalloprotease, while mutated pyrin mediated stimulation of autophagic pathways may overcome this block [2, 26, 27].

2.4 Diversity of *MEFV* mutations

Higher frequency of pathogenic *MEFV* mutations in the Mediterranean basin is mainly explained by a founder effect and balancing selection. The M694V and

V726A mutations seem to emerge in human genome about 2000 years ago, according to their association with specific microsatellite haplotypes in different populations [21, 28]. Presence of the E148Q (c.442G > C) mutation in different ethnic groups in the region supports the hypothesis of its recurrent nature or founder effect, probably stemming from the Asian countries, such as China and India, where E148Q is also frequent [20, 29]. The M694I (c.2082 G > A) mutation is merely present in North Africa and is estimated that occurred in an indigenous population of Berbers before colonizations in the 7th century BC [4, 20].

The M694V, M694I, M680I, and V726A mutations are most common in the Eastern Mediterranean countries, that is in Turkish, Armenian, Arab, and Jewish populations (**Figure 1**). The carrier rate of the mutations in these populations is estimated to be 1:5 to 1:7. Consequently, FMF mainly affects people of these ethnicities [2, 5, 19, 30–32]. The prevalence of *MEFV* mutations and FMF is much lower in the Western Mediterranean countries (France and Spain). Actually, the ethnic origin of these patients is usually from populations with higher mutation frequencies. Also, higher prevalence of homozygous mutations in the East might reflect the consequence of a local custom of consanguinity marriages [20, 33, 34].

Beside differences in *MEFV* mutations distribution between countries, there are variations within countries as well. For example, in Turkey, 94% of diagnosed FMF patients were from central-western parts of the country. However, more than a half of them had a family origin from the eastern provinces, pointing to the migration routes of mutations and disease [19, 35, 36]. The similarity between *MEFV* mutations present in Turks and Jordanians can also be explained by the local migrations, during the Ottoman Empire [20, 37]. The M694V mutation is the most common in Arab FMF patients. Unlike others, Arabs in North Africa have higher rate of M694I mutation that is probably acquired through the intermarriages with the local autochthonous population [20, 31, 38–43].

There is a dissimilar pattern of *MEFV* mutations among Jewish population, as there is a number of distinct Jewish ethnic groups in Europe. Although the aforementioned mutations are mostly present in Jewish FMF patients, their exact frequencies differ depending on a country and ethnic group [16, 23]. For example, high carrier rate of M694V is identified in Jewish FMF patients living in North Africa (Morocco) (11.1%) and Iraq (2.9%), but is rarely observed among Ashkenazim [20], while the V726A is prevalent among Ashkenazi (7.4%) and Iraqi Jews (12.8%) [44]. In the study of *MEFV* mutation prevalence in the Israeli society, M694V was common mutation among non-Ashkenazi Jews, E148Q was observed in

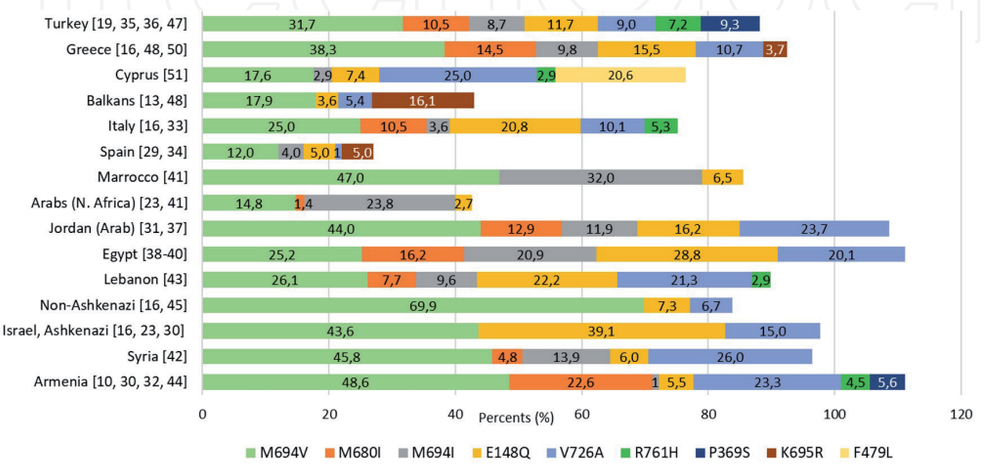

Figure 1.
Allele frequencies of common MEFV mutations in FMF patients (%).

patients of all ethnic groups, while K695R (c.2084A > G) seem to be characteristic mutation present in Jews [45]. In Sephardic Jews, the overall *MEFV* mutation carrier rate is between 1:8 and 1:16 [46], M694V is predominant, while other mutations, such as E148Q, P369S (c.459G > T), K695R and V726A, are rare [16].

The M680I mutation is common in Armenians and is associated with milder phenotype of the disease. Nevertheless, Armenian patients with FMF have common pathogenic mutations as the previous populations, such as M694V (~50%), followed by V726A, M680I, and R761H (c.2282G > A) [10, 30, 32].

The E148Q mutation is the most frequent sequence alteration in the general population, but its clinical significance is still debatable. It is mostly encountered in a heterozygous state in asymptomatic individuals. When in homozygous state it is associated with FMF-like disease, with mild symptoms and later onset of disease. Thus, with regard to the SHARE recommendations heterozygous E148Q does not support the diagnosis of FMF [2, 5, 10, 11].

The P369S and K695R are rare mutations with reduced penetrance, often found in asymptomatic carriers or in complex alleles in FMF patients [23, 30, 47]. They were relatively common in general Ashkenazi Jewish sample in the USA, with the carrier frequency of ~1:5 in FMF patients [16]. The P369S was the most frequent mutation in healthy Armenians and it might ameliorate the phenotypic presentation of the co-existing exon 10 mutations in patients. P369S homozygotes were even observed among the asymptomatic Ashkenazi Jews [16, 30].

Unexpectedly high K695R mutation rate was determined in the countries of Central and South-Eastern Europe. This region is characterized with limited heterogeneity of *MEFV* mutations, with only eight different mutations determined in healthy subjects and FMF patients (K695R, E148Q, V726A, M694V, F756C, I591T, S730F and A744S). The K695R mutation was most common mutation, found in 40% of healthy and 32% of FMF patients, which supports the idea that this is a common mutation for this region [13, 48].

One another *MEFV* variation of unknown significance is the R202Q (c.605G>A), often considered a polymorphism due to its high heterozygous frequency, among healthy individuals. Due to its poor conservation during evolution it is assumed it appeared later compared to other common mutations [49]. The R202Q alteration in homozygous state was associated with FMF-like symptoms in some cases [47, 50]. In our study of *MEFV* distribution in Serbia, 45% of healthy individuals had heterozygous R202Q, while 10% were homozygotes. Although considered healthy, the homozygotes reported self-limited episodes of fever of unknown origin and unspecific abdominal pain [13]. The results indicate a pathogenic role of R202Q homozygosity, perhaps along with other permissive environmental and genetic factors of a patient.

In isolated populations there is a greater chance for arising of specific genotypes. One example of specific *MEFV* mutation distribution is an island of Cyprus, due to its distinct ancestry and relative isolation in the Mediterranean. The *MEFV* mutations carrier rate in the Greek-Cypriot patients suspected for FMF is 1:25, with V726A, M694V, E167D (c.501G > C) and F479L (c.1437C > G) being the most common mutations. F479L is very rare elsewhere but in Greek-Cypriots its frequency is 20.6%. Interestingly, F479L was always co-inherited in *cis* with E167D mutation. It is hypothesized that F479L originated in Cyprus as a founder mutation, from where it spread further [51].

It would be ideal when every population would perform a genetic testing for *MEFV* mutations and accordingly establish a set of the most frequent, which could be used in a screening for suspected FMF patients, as well as other inflammatory conditions, since *MEFV* mutations are found to be a modifying factor in a number of inflammatory and autoimmune diseases [52].

3. Behcet's disease

Behcet's disease (BD) is an autoinflammatory and polygenic disease, more frequent in Mediterranean countries than in rest of Europe. Most cases are identified in countries of the Middle East and along the ancient Silk Route. The highest prevalence among Mediterranean countries is probably in Turkey, with estimated prevalence of 4.2/1000 in Istanbul [53]. This is a rare, sporadic, multi-systemic disease with undetermined cause. The main clinical features are constitutional symptoms and recurrent fever, oral aphthous, genital ulcers, with gastrointestinal, musculoskeletal, neurological, and vascular involvement [54].

Several host genetic factors are implicated in the pathogenesis of BD. The strongest is the association with the major histocompatibility complex HLA–B51 allele, which increases the risk of disease for about 6-fold. Approximately 50% of BD patients possess this HLA variant. Besides, HLA-B51 contributes to the specific clinical features in BD such as less severe disease course, but a higher frequency of ocular manifestations [55, 56].

Behçet's disease can be a comorbidity of FMF, and vice versa, *MEFV* mutations are common finding in BD patients. Some *MEFV* alterations are detected more often in BD patients than healthy subjects, such as P706 polymorphism. In a cohort of Turkish patients, clinical association was found between heterozygous *MEFV* mutation, principally M694V, and vascular involvement [51, 55, 57].

Interestingly, arthritis in BD is self-limiting and nondestructive in nature, pointing to the existence of an inherited protective factor/s. Such a role has been observed for plasminogen activator inhibitor 1 (PAI-1), which levels were higher in synovial fluid of BD patients than healthy. PAI-1 acted protective against destructive arthritis but had promoting effect towards hyperfibrinolysis in BD vasculopathy. However, PAI-1 common polymorphism 4G/5G was not associated with pathogenesis nor development of thrombosis in these patients [58–60].

Besides, several other alterations are described to influence BD occurrence and course, including MHC class 1 polypeptide-related sequence, T cell mediated cytokine dysregulation (of IL-6, IL-8, IL-10), DNA methylation, etc. [55, 61].

4. Pathophysiology of β-thalassemia syndromes

4.1 Introduction

Genetic disorders referred as the ά- and β-thalassemias are caused by defective hemoglobin (Hgb) chains (ά or β) synthesis and are mostly inherited as a Mendelian recessive [62–64]. The name of the disease" thalassemia" is derived from the Greek words: *thalassa* (sea) and *haima* (blood), implicating the geographical region where the disease was initially described due to its high prevalence. B-thalassemia occurs mostly in people with origins near the Mediterranean Sea, Greece, Italy (Sicily, Calabria and Sardinia), Turkey, Middle East, India, Southern China, Sub Saharan Africa, south America and in the populations of Sephardic Jews and Arabs, with Cyprus (14%) and Sardinia (10,3%) having the highest carrier frequency. However, the other form of the disease, ά-thalassemia, is the most common among the people form the Far East, China, Vietnam, Laos, and Cambodia [63, 64].

Although considered as the rare form of the disease, it is confirmed that around 68,000 children annually are born with the various forms of thalassemia syndromes, whereas 1.5-5% of the worldwide population are considered as the carriers of these genetic abnormalities [65–68]. The high frequency of these mutations is considered as an evolutional answer to the malaria infections, providing protection

against *Plasmodium falciparum* for the genetic mutation carriers. The aberrant Hgb synthesis reduces the half-life of erythrocytes which disables completion of parasite maturation cycle [63]. Moreover, the same type of genetic aberrance has been confirmed in consanguineous marriages in some countries [64]. However, high rate of the migrations of populations caused that individuals with thalassemia-syndromes may be found in the US, Australia, Canada, South America and North Europe, making it a global health care burden [65–68]. Moreover, the general epidemiological estimation is that the prevalence of thalassemia-syndromes is about to increase, taking into the consideration the fact that infant mortality declines in low-income and middle-income countries [68].

Thalassemias are heterogeneous, inherited, monogenic, Hgb disorders and are initially classified as ά or β, depending whether genes that control ά- or β-globin chains synthesis are defective. This knowledge implicates that β-thalassemias occur when synthesis of the β-globin chains is reduced (β+) or absent (β-) [62–64]. Moreover, clinical, and hematological manifestations depend on how many of the genes that code β-globin synthesis are defective and whether those defects are homozygous or heterozygous. The phenotype diversity and wide range of disease severity lead to introduction of the concept of β-thalassemia-syndromes.

According, three culprit forms that comprise the β-thalassemia-syndromes are defined and classified by increasing severity of the symptoms: 1) β-thalassemia carrier state, also known as β-thalassemia minor, "heterozygous thalassemia" or "thalassemia trait", 2) β-thalassemia intermedia and 3) β-thalassemia mayor, also referred as "Cooley's anemia" and "Mediterranean anemia", very severe phenotype, that requires blood transfusion for survival (transfusion-dependent anemia) and has a questionable outcome. Besides these forms, there are other identified types of β-thalassemias, that are associated with various Hgb and/or clinical abnormalities or may be autosomal dominant [63, 64]. Persons with most severe forms (major) are homozygotes or compound heterozygotes for β0 or β + genes, intermedia type may be homozygotes or compound heterozygotes, while the mildest form is predominantly heterozygotes [62–64].

In the past two decades, individuals affected with β-thalassemia-syndromes are experiencing tremendous improvement in the quality of life and overall survival, due to the timely diagnosis, adequate therapy, and monitoring of the disease. However, up to date, the only cure for the disease represents allogeneic hemopoietic stem-cell transplantation.

4.2 Molecular basis

The synthesis of β-globin chains in Hgb molecule physiologically is under control of two genes. Any genetic abnormality of the controlling genes, therefore, results in the absence or the reduction of the β-chain. The gene for β-chain is located in the short arm of chromosome 11, sharing the region and being arranged in the order of the development expression, with the functional genes for δ-globin, embryonic ε-globin, the fetal A-γ-globin and G-γ-globin, as well as a pseudogene (ψβ1) [63]. The molecular and clinical diversity of the β-thalassemias emerges from the data that more than 200 genetic mutations have been described up to date [63, 64, 68–70]. Accordingly, clinical, and hematological manifestation and patients' prognosis depend on the basis of imbalance of the ά- and β-chains synthesis, therefore from the type and the extent of the genetic disturbance.

The identified and defined genetic aberrations are silent mutations (silent β-globin), mild mutations (relative reduction of β-globin) and severe mutations (complete absence of β-globin, β0) [68]. Nevertheless, these mutations are identified mostly as single-nucleotide substitutions and insertions of single nucleotides

or small oligonucleotides causing the frameshifts in genes that code β-chains. The typical genetic abnormalities that were described are promoter mutations, being responsible for the milder phenotypes, whereas nonsense, initiation codon, splicing and frameshift mutations have been documented in more severe forms of thalassemia-syndromes, characterized with the complete absence of β-chains [62, 63]. Deletions of the gene are randomly identified aberrations, where the deletional removal of one or several genes from the chromosome 11 causes very rare forms of thalassemias, designated as δβ-, γδβ- and εγδβ-thalassemia [62].

An autosomal recessive pattern of thalassemia inheritance implicates that both parents have to be heterozygotes, owing a copy of a β-globin gene mutation. Possible outcomes in the affected family may be that every child has: 1) 25% chance of being affected, 2) 50% of being an symptom free and carrier, and 3) 25% of not being affected nor a carrier [63, 64].

4.3 Genetic modifiers

Pathophysiological perception why individuals with beta-thalassemia syndromes may clinically appear very heterogeneous, is based on the perseverance of three group of factors that may modify the disease. These factors are designated as genetic modifiers and are explained as genetic variants that induce differences in disease phenotype [64]. Genetic variants that may impact the imbalance of globin chains are categorized as primary modifiers. The other pathogenetic factors that may alleviate the severity of β-thalassemia major are: coinheritance of an ἀ-thalassemia gene and fetal Hgb production, within the β-globin cluster and are classified as secondary modifiers [62, 68].

Coexistence of ἀ-thalassemia enables decreased ἀ-globin chain synthesis, therefore significantly reduces imbalance between the ἀ/non-ἀ-chain in erythrocytes [63]. Increased γ-chain synthesis, in adult life, encounters the excess of ἀ-chains, therefore enables the survival of the erythrocytes that contain fetal hemoglobin, marked as HbF cells. It may be that deletion mutation or point mutation within the β-globin gene cluster simultaneously trigger a rise in fetal Hgb production [62]. According to some research, the increase of HbF synthesis indicates a single nucleotide polymorphism in one of the γ-globin gene promoters or somewhere in the globin locus, resulting in the overexpression of the related gene [62]. It was reported that HbF, that is highly predominant in individuals with severe forms of thalassemia, may account for their improved survival [71]. Moreover, the inverse correlation of HbF levels and factors that reflect disease morbidity was observed, so as the finding that milder phenotypes present with the increased numbers of HbF cells [62, 72]. In addition to this knowledge, it was suggested that certain therapeutic treatments (hydroxyurea) may induce the production of HbF, hence produce less of a need for blood transfusion [73].

Tertiary modifiers are recognized to be genetic and environmental factors that modulate disease complication rates. The results of the molecular studies revealed genetic polymorphisms as possible pathogenetic factors involved in cardiac iron overload, hyperbilirubinemia, and Gilbert disease, osteoporosis, and infections susceptibility, that occur in patients with β-thalassemia syndromes [63, 68, 74–76].

4.4 Pathophysiology

Essential pathophysiological determinant in β-thalassemia syndromes is the uncoupling of the synthesis of the ἀ- and the β-chain, where β-chain synthesis is reduced or absent, resulting in the accumulation of ἀ-globin tetramers in the erythroid precursors [62–64]. This phenomenon eventually leads to an ineffective

erythropoiesis, that is a key feature responsible for various pathophysiological consequences during the course of the disease. Erythrocytes and its precursors (mostly polychromatophilic erythroblasts) are filled with precipitated ȁ-globin tetramers, forming inclusive bodies, causing oxidative membrane damage and subsequent apoptosis [62–64, 77]. Physiologically, biochemical detoxification would be efficient to eliminate harmful proteins from the affected cells. Nevertheless, in the severe forms of β-thalassemias these pathways are inefficient [62].

Premature erythroid cell death in the bone marrow (ineffective erythropoiesis) and in the peripheral blood (hemolysis) cause chronic microcytic-hypochromic hemolytic anemia, that is a persistent finding in persons with thalassemia. Interestingly, hemolysis is less notable in individuals with severe phenotypes of the disease [64]. Chronic hypoxia induces intensive and continuous erythropoietin production, resulting in the great expansion of the bone marrow (25–30 times), subsequent skeletal deformities and the loss of the bone mass [63, 64, 68]. Simultaneously, a compensatory extramedullary hematopoiesis occurs, creating organomegaly, predominantly of spleen and liver [68]. Nevertheless, if the stimulus is extremely potent, all the cell in the body that express hematopoietic potential will be affected, resulting in the formation of the pseudotumors [78]. Hemolysis will trigger the formation of the gall stones and cholelithiasis and also contributes to splenomegaly development. Besides, the thalassemia-syndrome is recognized as a hypercoagulable state, since erythroid precursors, during the ineffective erythropoiesis, may become prothrombotic. Moreover, in association with platelets and coagulation disruption, the condition may result in serious vascular manifestations such as venous thrombosis [63, 64, 68, 79].

Besides ineffective erythropoiesis and anemia, iron overload also represents very important mechanism in the pathogenesis of the thalassemia, contributing to development of complications. Iron deposition within the reticuloendothelial system in the transfusion- dependent forms of β-thalassemia (major and intermedia) represents associated and secondary mechanism in the pathogenesis of iron overload. However, it is well defined that the most important pathogenetic factor in the hemochromatosis development represents increased iron absorption [80], due to the hepcidin downregulation and its deficiency [62, 63].

The apoptosis of the erythroid precursors causes subsequent synthesis and secretion of many factors that most likely inhibit hepcidin synthesis in the liver [62]. Coupled with this, it should be underlined that hepcidin functions as a negative iron regulator, delivering the information between the liver and the red blood cells. Its decreased concentrations result in the increased dietary iron absorption and in release of the iron from its storage (macrophages and hepatocytes). The final result is paradoxically and significant dietary iron absorption, regardless of the iron tissue deposition due to the blood transfusions and eventually hemochromatosis [62].

The identified molecules that are released from the apoptotic erythroid precursors are growth differentiation factor 15, twisted gastrulation 1, and erythroferrone, and all function as hepcidin expression inhibitors [68, 80–83]. The results have been conflicting so far, since some research demonstrated their significant increase in individuals with β-thalassemia [81, 82], while the others confirmed only increase of erythroferrone in animal models [84]. However, their exact function in the pathogenesis is yet to be elucidated. Nevertheless, the substitution of the synthetic hepcidins represents justified therapy option in patients with the severe forms, as already proven experimentally. However, this extensive and progressive iron overload in synergy with anemia may deteriorate already insufficient hematopoiesis. Iron overload, regardless of its pathogenesis, leads to hemochromatosis and organ damage [83, 85].

4.5 Clinical findings

The main clinical features of β-thalassemia syndromes are anemia and iron overload, leading to severe and life threating consequences. The onset and the degree of the symptoms severity depend whether the affected individuals present as a homozygous phenotype (thalassemia major) or as a homozygotes or compound heterozygotes (thalassemia intermedia). Correspondingly, individuals with β-thalassemia minor are usually asymptomatic and may be discovered incidentally, having only the discrete changes in the hematological findings.

The onset of symptoms will appear 12 months after the birth, [67–85], at the moment when HbF production switches to adult and physiological synthesis of HbA is yet to be established [86]. The infants will experience feeding problems, recurrent fevers, diarrhea, enlargement of the abdomen and the growth retardation. If the child has not been diagnosed prenatally, this is the point when the diagnosis of thalassemia is determined, and transfusion indicated [63, 64].

Microcytic-hypochromic hemolytic anemia is an obligatory finding in the affected individuals, predisposing them to progressive paleness and jaundice. Bone marrow expansion secondary to erythroid hyperplasia, lead to significant skeletal changes, creating abnormalities of the face and body. People with severe phenotypes most often experience frontal bossing, depression of the bridge of the nose, mandible and maxilla enlargement with the upper teeth exposure, bone pain, osteopenia and osteochondrosis. If spinal impairment occurs during the childhood, linear growth is delayed, resulting in the discordance in the length of upper and lower limbs [63, 64, 87]. The progressive enlargement of the abdomen is due to the hepatosplenomegaly, whereas the masses of extramedullar hematopoietic tissue may also be found in the chest or spinal column [63, 64].

Iron overload predominates in the most severe clinical phenotypes. Brown pigmentation of the skin, particularly in the areas exposed to the sun, reflects systemic hemochromatosis. Predominant sites for iron deposition tend to be spleen, liver, myocardium, pancreas, and endocrine glands. Although significant liver deposition of iron could be found, its function may be preserved for a long time [62]. Ultimately, liver cirrhosis may develop. Cardiac manifestations stand for the most adverse outcome of iron overload, whereas arrhythmias, dilated cardiomyopathy, and atrial or/and ventricular failure during the course of the disease lead to congestive cardiac failure. Endocrine complications primarily develop due to the insufficiency of the growth hormone (growth retardation) and sex hormones (hypogonadism). Additionally, hormone substitution therapy is commonly required for maintaining normal fertility. Other endocrine disturbances may be very diverse, including diabetes mellitus, hypothyroidism, hypoparathyroidism, hypocorticism, and panhypopituitarism. Pulmonary hypertension may contribute to the complexity of the cardiovascular manifestations by deteriorating left heart function [79, 88].

Other clinical features in β-thalassemia syndromes are osteoporosis, subclinical fractures, nutritional deficiencies, venous thrombosis, chronic B and/or C hepatitis, and infections. The risk of hepatocellular carcinoma in patients who develop liver cirrhosis remains unchanged even if the proper therapy is performed, due to the oxidative DNA damage triggered by chronic iron accumulation [79, 88].

4.6 Laboratory findings and Hgb analysis

Laboratory diagnosis of thalassemia is confirmed based on established red blood cells parameters, qualitative and quantitative Hgb analysis and, when necessary, molecular assessment. Erythrocyte count may be relatively high, whereas Hgb is

reduced <7 mg/dL, mean corpuscular volume (MCV) is between 50 and 70 fL and mean corpuscular Hgb (MCH) 12-20 pg. Peripheral blood smear demonstrates microcytosis, hypochromia, anisocytosis, poikilocytosis (dacrocytes and elliptocytes), along with the erythroblasts. The number of reticulocytes may remain normal, without any diagnostic accuracy. In order to differentiate iron deficiency anemia form the thalassemia-syndromes, few formulas are available to calculate a thalassemic index, but should be performed with caution [63, 64, 89]. In biochemical terms, typical β-thalassemia presents with elevated ferritin levels >12 ng/mL, transferrin saturation increased to 75-100% and unconjugated hyperbilirubinemia [62, 88].

The most accurate method for β-thalassemia differentiation is quantitative HbA2 determination. Considering that physiological HbF in adult population is commonly less than 1.5%, the results for HbA2 ranging between 3.6 and 7% are considered as definite thalassemia values. Nonconclusive or borderline cases, with HbA2 ranging between 3.2 and 3.6%, respectively, require further analysis [86, 89]. Additionally, PCR-based procedures or β-globin gene sequence analysis are necessary for diagnosis confirmation. Besides, in couples with increased risk, a prenatal diagnosis of thalassemia may be achieved by chorionic villi sampling (11th gestational week) or DNA analysis from harvested fetal cells (15-18th gestational week) [63, 64, 89].

4.7 Therapy approach

Conventional management of β-thalassemia syndromes includes blood transfusion, iron chelation, splenectomy and hemopoietic stem-cell transplantation. The introduction of blood transfusion in regular management of β-thalassemia has enormously improved quality of life and survival of the affected individuals [62–68]. The mayor indication for its initiation, in previously diagnosed patients, should be low Hgb level (<7 g/dL), that lasts at least two weeks [64], concerning other clinical signs such as growth retardation, skeletal changes and splenomegaly. The therapeutic aim of transfusion is to maintain Hgb level at 9-10 g/dL or 11-12 g/dL in cases of confirmed cardiovascular disease [63, 64, 68, 86]. Although life-saving approach, blood transfusion has several adverse effects, with iron overload and viral infections (hepatitis B, C) being the most common [62–68].

The knowledge that iron cannot be excreted form the human body and that patients requiring constant blood transfusions tend to develop iron overload, lead to the regular assessment of iron body status. Most conventional method is determination of serum ferritin levels, that may be monitored in order to initiate chelation therapy or may be used as a biomarker of iron chelators efficiency. However, more reliable, yet non-invasive method of tissue iron accumulation has been developed. Magnetic resonance imaging has been successfully used for liver and cardiac iron overload, measuring a tissue iron concentration in mg of iron per gr of dry liver/heart weight [63, 90, 91]. Also, iron binders (chelators) enable its elimination through feces and/or urine and should be initiated after approximately 10-20 performed transfusions or with ferritin levels above 1000 mg/gL [64, 68].

Splenectomy is indicated in the following cases: enlarged spleen with the risk of rupture, severe cytopenia and in patients with the significant blood requirements. In patients with splenectomy, infections and subsequent sepsis remain the leading cause of mortality [63].

However, the only curable therapy for the thalassemia represents hematopoietic stem-cell transplantation [63, 64, 68]. Nevertheless, it was documented that a disease-free survival may be achieved in 80% in matched donors and even 65% in unrelated donors and umbilical blood cord stem-cells transplantation. Nevertheless, this therapy option is still associated with risk and complications, even in the high-income countries [92].

Considering the monogenic nature of the disease, the most challenging, yet possible therapy approach, may be an interference in the globin chains imbalance, achieved by gene therapy and genome editing [68]. Alternative pharmaceutical approaches would be use of agents acting as potent stimulators of late stage erythropoiesis and increased hepcidin expression, throughout its substitution or stimulation of its endogenous production. Even though there has been a substantial progress in the development of therapy options for individuals affected with thalassemia, the best approach to the disease management remains prevention of thalassemia births throughout national screening programs [68].

5. Association of HbS, G6PD and FY gene polymorphisms and malaria

5.1 Introduction

Understanding the molecular mechanisms that underlies the adaptation is of crucial importance in evolutionary biology. Among the plethora of genes that causes adaptive variation in fitness-related features in natural populations, very few are identified [93, 94]. The hemoglobins, oxygen-carrying proteins, tightly connect cell metabolic activities with environmental conditions and thus represent convenient system for analyzing adaptive changes [93, 94]. Also, inherited disorders of hemoglobin are the most common human monogenic diseases [95]. Each year, there are between 300,000 and 400,000 newborns with some of the serious hemoglobin disorders and up to 90% of them are born in low- or middle-income countries [96].

Hemoglobin is the oxygen-carrying protein of red blood cells (RBCs), normally formed of two α-globins and two β-globins that constitute adult hemoglobin A (HbA). Without specific medical treatment, the most severe hemoglobinopathies — HbSS homozygosity (sickle-cell disease) and the thalassemias major are not compatible with life after early childhood. People with HbAS, HbAC, HbCC, HbAE, HbEE, and the thalassemias minor have usually normal life expectancy and are rarely directly associated with morbidity [97].

Plasmodium spp. parasites represent vector-borne pathogens which attack the red cells of reptiles, birds, and primates [98]. Out of five Plasmodium species that parasitize and cause malaria in humans, *P. falciparum* and *P. vivax* are the most common in human populations. *P. falciparum* is endemic in tropical areas worldwide, including Mediterranean [99]. Like the other four Plasmodium species, *P. falciparum* is injected into a human skin via female *Anopheles spp*. mosquitoes as a vector. Then, the sporozoites migrate to the liver where they attack hepatocytes and develop within them for 7–10 days. As a consequence, numerous merozoites are formed which, subsequently, enters erythrocytic stage of RBSs life cycle. In that time, typical features of malaria clinical picture develop [97, 98].

As a disease which is a main cause of morbidity and mortality, malaria caused by *P. falciparum* imposed remarkable evolutionary pressure on the human genome.. Also, malaria caused by *P. falciparum* is in relation with numerous genetic polymorphisms that are responsible for protection against this disease [97, 100]. Hemoglobin mutants S, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and Dufy Antigen/Receptor for Chemokines (DARC) gene mutation are mostly distributed in the areas where *P. falciparum* malaria is endemic. These genes expression have high levels of prevalence in malaria endemic areas which is considered to be the consequence of their protective role against *P. falciparum* [101]. In this context, malaria can be defined as an infectious disease that has pronouncedely higher selective pressure on the human genome in comparison with all other infectious diseases [101]. Polymorphysms of the above-mentioned genes are typical examples

of Haldane's idea of balanced polymorphism. According to this author, balanced polymorphism exists when certain genes have fixed high frequency in susceptible populations since enhanced fitness encompanied with heterozygotes multiple times overweights morbidity and mortality associated with homozygotes and compound heterozygotes [102].

5.2 HbS gene polymorphisms and malaria

Sickle hemoglobin (HbS) is best characterized genetic polymorphism tightly interconnected with malaria. HbS represents a structural variant of normal adult hemoglobin (HbAA) and results from a single point mutation (Glu → Val) on the sixth codon of the beta globin gene [103]. Homozygotes for hemoglobin S (HbSS) have sickle cell disease that further causes high morbidity and mortality. Also, heterozygous for HbS have 10-fold lower risk of dying from malaria compared to homozygous [97, 104, 105]. Heterozygotes (HbAS) have generally asymptomatic sickle cell disease which does not endanger their lives [106].

It has been found that, in the conditions of selection for fitness against malaria, nearly 45 generations (or 1000 years) were necessary to pass until sickle gene frequency reached a stable equilibrium [107]. People with HbAS have 50–90% lower parasite density [105] in comparison with individuals with normal hemoglobin (HbAA). Sub-Saharan Africa is an area with closely 80% of people born with sickle cell anemia and where most *P. falciparum* malaria cases and deaths occur [108]. Besides sub-Saharan Africa, sickle cell anemia is present, although rarely with frequency higher than 20–25%, in the Mediterranean region, the Middle East, and the Indian subcontinent [95]. There is a strong connection between high HbS allele frequency and high malarial endemicity in the world although this finding is based on the observations made in Africa: HbS allele frequency gradually increases from epidemic areas to endemic areas in Africa which is in accordance with the hypothesis that malaria protection by HbS includes the enhancement of innate and acquired immunity to *P. falciparum* [109].

Knowledge of the existing relationship between malaria infection and extension and prevalence of hemoglobinopathies in Mediterranean region are not new [102, 110]. Sickle-cell homozygous persons have short life expectancy and commonly die before adulthood. However, the gene responsible for sickle cell disease "hidden" within the genotype of heterozygous carrier can achieve high frequency due to resistance to *P. falciparum* [111].

There are lots of described biological mechanisms that are considered to be responsible for protection against malaria. First, there were only two mechanisms described regarding a manner in which the presence of HbS in heterozygotes protects against malaria: sickling of circulating infected RBCs and impaired parasite growth and oxidant damage [101]. It has been found that formation of sickle RBSc shapes under low oxygen presure occured more frequently in RBCs infected with *P. falciparum* compared to uninfected RBCs [112]. When parasite triggers sickling of erythrocytes once, sickled cells are removed by macrophages [113]. This action of may macrophages'possibly occurs due to their ability to produce and release numerous cytokines that further recruit more phagocytic cells [114]. In addition, it has been discovered that enchanced sickling was limited to RBCs infected with small Plasmodium forms [115]. On the other hand, impaired parasyte growth and oxygen damage was discovered thanks to *in vitro* studies [112]. In the conditions of normal oxygen pressure, there were no differences in the invasion, growth, and multiplication of *P. falciparum* in HbAS cells compared to HbAA RBCs. In the opposite, hypoxic consitions caused reduced fraction of *P. falciparum* in HbAS cells and a block in the maturation of ring forms to trophozoites and schizonts.

In addition, sickling and destruction of parasites in HbAS and HbSS RBCs at lower oxygen tensions (1–5%) more closely mimiced the micro-aerophilic environment of post-capillary venules *in vivo* [112].

The guiding hypothesis regarding the protective effect against malaria in people with HbAS suggests that decreased *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) [116] expression on infected HbAS RBCs results in lower binding of infected cells to the endothelium [117]. As a consequence, only approximately one-half the cytoadherence was seen in infected HbAS RBCs. Archer and associates have proposed that oxygen-dependent HbS polymerization is a key factor for HbAS malaria resistance [118]. They found that intraerythrocytic *P. falciparum* parasites in HbAS RBCs at low oxygen concentrations arrest in cell cycle before DNA replication and that HbS polymerization is responsible for this growth arrest.

Among the genetic factors responsible for the protection from malaria is one of the complement regulatory proteins – complement receptor 1(CR1). The frequency of CR1 polymorphisms is high in a numerous of malaria endemic areas [100]. A major receptor for RBCs infected with *P. falciparum* is human protein CD36 [119]. CD36 can be involved in malaria by sequestering infected RBCs thus disabling the immune response to this parasite [120]. Some African populations have extremely high frequency of CD36 mutation and this CD36 deficiency causes susceptibility to severe form of malaria [121]. Important genetic factors involved in resistance to malaria are erythrocyte-binding antigens. Special attention was given to erythrocyte binding antigen-175 (EBA-175), a protein that binds to glycophorin A, thus enabling merozoite entry into erythrocytes [122].

An interesting study regarding host genetic factors responsible for malaria resistance was conducted in Senegal, in the population of children and young adults that were 2 to 18 years old. Thanks to the results of this study, three candidate regions in the genome of these children were detected and one of them contains a gene related with the malaria infection in the 5q31q33 region [123].

One of the newest studies revealed that unfavorable microRNA (miRNA) composition in heterozygous HbAS or homozygous HbSS erythrocytes, leads to resistance versus *P. falciparum*. When erythrocytes are infected with *P. falciparum*, a part of erythrocyte miRNAs can translocate into the parasite. LaMonte et al. found that HbAS and HbSS erythrocytes had high number of miR-451 and let-7i integrated into essential parasite messenger RNAs, as well as that these miRNAs, together with miR-223 are negative regulators of parasite growth [124]. miR-451 fuse with transcripts of the regulatory subunit of the parasite's cAMP-dependent protein kinase (PKA-R) and reduce its translation. Therefore, it up-regulates the activity of its substrate PKA and disrupts multiple parasite developmental pathways [124].

Piel and associates created extensive geodatabase of HbS allele frequency and investigated geographical distribution of malaria [125]. Their HbS allele frequency map has shown that throughout majority of the African continent and in localized areas in Mediterranean, this allele is present with the frequency of >0.5%. According to this geodatabase research, in the Chalkidiki region of Greece, southeastern Turkey and in Central Sudan, frequency of this allele was even above 6% [125].

One of the models of how hemoglobiopathies protect from malaria is proposed by Killian associates [126] and reveals association between reduced cytoadherence phenotype and parasitized hemoglobinopathic erythrocytes. This team used conditional protein export system and tightly synchronized cultures of *P. falciparum*. They have showed that exportation of proteins encoded by parasites across the parasitophorous vacuolar membrane is more advanced, faster and increased in amount in parasitized wild type erythrocytes in comparison with hemoglobinopathic erythrocytes.

Severe malaria is in relation with intraerythrocytic life cycle of *P. falciparum* and the pathological cytoadhesive behavior of parasitized erythrocytes [127, 128]. When parasite adheres to the endothelial cells of venular capillaries, it avoids clearance mechanisms of spleen. As a consequence, pathological sequelae form within the affected blood vessel [127, 128].

Pathological consequences of *P. falciparum* malaria can possibly be mediated by adhesion of infected cells to vascular endothelium either to other uninfected red cells (rosetting) or to platelets (clumping). It has also been found that variant of erythrocytes infected with *P. falciparum* do not have noticeable differences regarding their adhesive phenotypes in comparison with erythrocytes of normal individuals infected with this parasite [129].

There are two main phenotypes of parasite-infected RBCs (iRBCs) and both express PfEMP1 [130, 131]. First type of iRBCs mediate iRBCs binding to the endothelial receptors ("cytoadherence") [132] and the second mediate iRBCs binding to uninfected RBCs ("rosetting") [133, 134]. Different iRBCs phenotypes differ in various PfEMP1 that are responsible for binding of iRBCs to microvascular endothelial cells, placental syncytiotrophoblasts or uninfected RBCs [135–137].

Usually, hemoglobin S does not increase IgG responses to various *P. falciparum* proteins [138], but it can potentially enhance IgG responses to PfEMP1, which is the main cytoadherence ligand and virulence factor [139]. In an *in vitro* study, HbAS affected the trafficking system that directs PfEMP1 to the surface of infected erythrocytes. Using cryo-electron tomography, it has been shown that within the cytoplasm of normal RBCs, the parasite proteins are transported to the surface via a parasite-generated host-derived actin cytoskeleton. In addition, hemoglobin oxidation products disrupted this process in HbAS red cells [140].

Exact pathogenic mechanisms of malaria caused by *P. falciparum* are still unknown due to numerous parasite virulence factors, host susceptibility traits, and innate and adaptive immune responses that modify the occurrence of various malaria syndromes [141, 142].

The most important reason for the high frequency of hemoglobin disorders in tropical countries is natural selection through protection of heterozygotes against severe malaria. Protection observe in HbAS is reflected in protection against severe form of malaria and probably, to some extent, against mild malaria [129]. Natural selection is not the only mechanism responsible for high HbS gene frequency [102, 106, 107]. The others are high frequency of consanguineous marriages and epidemiological transition [98]. In addition, different distribution of some hemoglobin disorders in different populations is an example of founder effects by their original inhabitants [143].

Thanks to the studies conducted *in vitro*, various researches united on general hypothesis that protection from malaria is the result of impairment in the invasion and growth of *P. falciparum* parasites into HbAS red cells under conditions of low oxygen tension that were physiologically representative of *in vivo* conditions [112, 144]. Afterwards, a lot of alternative hypothesis have been developed including the one that refers to the enhanced removal of parasite infected HbAS RBCs. This mechanism could be related with sickling of these cells under low oxygen tension [112, 115, 145] causes their premature destruction in the spleen [112]. Specifically, Shear and associates have observed that protective effect of HbS can be lost on the model of transgenic mice that were subjected to splenectomy [146].

There are few researches that suggest that protection against malaria can be achieved not only through innate immunity, but also via acquired immunity. For example, in populations naturally exposed to *P. falciparum*, protective effect of HbAS increases with age [147, 148]. This is in accordance with recent studies on a mouse model which proposes an immuno-modulatory mechanism mediated

throughout hemoxygenase-1 [149]. The problem with these findings when translating into human populations is metabolic difference between sickling disorders of mice and human sickle-cell traits [95].

5.3 Glucose-6-phosphate dehydrogenase (G6PD) gene polymorphisms and malaria

The Glucose-6-phosphate dehydrogenase (G6PD) is a "housekeeping" gene located on long (q) arm of the X chromosome at position 28 – Xq28 [150]. This gene encodes an enzyme named glucose-6-phosphate dehydrogenase that acts in almost all types of cells thus providing normal carbohydrates processing [151]. The most important role of G6PD is in RBCs, where this enzyme is involved in protection of RBCs from damage and early destruction [152].

Glucose-6-phosphate dehydrogenase deficiency is a genetic disorder that mainly affects RBCs, thus causing premature destruction of these cells called hemolysis [153]. Besides causing hemolytic anemia, G6PD has an evolutive advantage regarding the protection against malaria. A consequence of the reduced amount of functional G6PD makes difficult pathway for parasites to invade RBSc [154]. G6PD gene insufficiency is the most frequent in malaria endemic areas. When it comes to Mediterranean, the highest noted frequency of this gene is in Mediterranean parts of Africa, southern Europe and in the Middle East [153].

Interestingly, G6PD deficient patients in Africa, where this type of deficiency is endemic, have milder consequences as well as relatively higher enzyme activity in comparison with patients from Mediterranean and Asia [155].

G6PD deficiency gives especially high protection from *falciparum* malaria infection [156, 157]. Among more than 400 variants of G6PD that differs in biochemical characteristics, enzyme kinetics, physicochemical characteristics, and other parameters [158] is G6PD B+ which is the most common variant of this enzyme. G6PD B+ is used as standard for normal enzyme activity and electrophoretic mobility and, therefore, for identification of other variants. In the area of Mediterranean, special place belongs to G6PD Mediterranean variant [159] which has less than 10% of the enzyme activity of G6PD B+ while its electrophoretic mobility is similar to G6PD B+ [160]. Two-point mutations in gene for this enzyme were identified. One mutation is cytosine to thymine mutation at nucleotide number 563, which causes substitution of serine with phenylalanine [161]. At nucleotide number 1311, change of cytosine with thymine represents a silent mutation [162].

A research conducted by Barišić et al. in the Dalmatinian region of Croatia resulted in discovering a new variant of G6PD named G6PD Split [163]. Change of cytosine to guanine at nucleotide 1442 caused substitution of proline with arginine which led to moderate enzyme deficiency. Besides this novel variant of G6PD discovered in one patient, other 23 unrelated patients with low G6PD activity had five other well-known variants and three patients had uncharacterized forms of G6PD mutations. The most represented form found in nine patients was G6PD Cosenza. G6PD Cosenza was first found in Calabria region of southern Italy and represents the consequence of change of guanine into cytosine at nucleotide 1376. This substitution changes Arginine to Proline [164]. G6PD Cosenza mutation is severe G6PD deficiency frequently jointed with hemolysis.

Around 400 million people from all over the World carry at least one deficient variant of G6PD gene. The frequency of those mutations varies in different populations [165]. In Africans and Afro-Americans G6PD A- is the most common mutation which has a gene frequency of 11%. G6PD B (Mediterranean) is a more severe deficiency usually found in Mediterranean area. Since Mediterranean represents a large

region, the prevalence of this mutation varies from 2 to 20% in Greece, Turkey, and Italy, up to the 70% which is the prevalence characteristic for Kurdish Jews [165, 166].

5.4 FY gene polymorphisms and malaria

In addition to the role it plays in transfusion incompatibility and hemolytic disease of newborns, Duffy Blood Group System is important in medicine due to its association with the invasion of RBCs by the parasite *P. vivax*. Outside Africa, *P. vivax* is the most widespread malaria parasite species, with 40% of cases in the Eastern Mediterranean [167]. Without Duffy antigens on their surface, RBCs are relatively resistant to *P. vivax* [168]. There are six types of Duffy antigens (Fy^a, Fy^b, Fy3, Fy4, Fy5, and Fy6), out of which only Fy3 has a clinical significance. Duffy antigens are also receptors for chemicals secreted by blood cells during inflammation [169].

Duffy-Antigen Chemokine Receptor (DARC) is a glycosylated transmembrane protein receptor which, among other roles, serves as a receptor for *P. vivax*. DARK crosses the membrane seven times and has an extracellular epitope, N-terminal domain responsible for RBC invasion by *P. vivax* merozoites [170, 171]. Two exons (FyA and FyB) of FY gene are encoded by the co dominant FyA and FyB alleles located on human chromosome 1 [172]. The difference between these two alleles is a non-synonymous mutation, specifically substitution of guanine to adenine at nucleotide 125, which was enough to determine the two antithetical antigens [173]. Based on this variation, four phenotypes within Duffy Blood Group System were identified: Fy (a + b-), Fy (a-b+), Fy (a-b-) and Fy (a + b+) [174]. The non-functional allele Fy*O is the consequence of a mutation in the gene promoter at −33 nucleotide that changed thymine to cytosine which abolish its expression in the erythrocyte cell lineage [175, 176].

Individuals with Fy (a-b-) phenotype are resistant to *P. vivax* invasion [177]. This was shown in the study which included 11 volunteers. The individuals affected with malaria were Fy (a+) or Fy (b+). In the countries of West Africa, frequency of the Fy (a-b-) phenotype is a high while the incidence of *P. vivax* malaria is low [178]. Virtual absence of *P. vivax* malaria in populations with widespread DARC negativity is the proof of the substantial importance of the Duffy binding protein (DBP)–DARC interaction [179]. It is important to emphasize that Fy (a − b−) does not protect from *P. falciparum* which therefore can infect RBCs of any Duffy phenotype [169].

While *P. falciparum* can enter human RBCs through series of receptors on their surface, RBCs invasion by *P. vivax* depends on an interaction with the Fy^a or Fy^b antigens [169, 180]. Therefore, in the regions of Africa where Fy (a-b-) phenotype is stable within various ethnic groups, the transmission of *P. vivax* is not usual [181]. On the other hand, individuals with Fy (a-b+) or Fy (a + b-) genotypes that express half the level of Duffy antigens on RBCs compared to Fy (a-b-) homozygotes are less sensitive to blood stage infection by *P. vivax*. Therefore, parasitemia by *P. vivax* might be inhibited by total or partial restriction access of *P. vivax* to Duffy antigen [182, 183].

Phenotypic differences in susceptibility to malaria are the results of Fy gene polymorphism. Individuals that carry Duffy antigen-negative allele hidden within heterozygous genotype have significantly reduced adherence of the DBP ligand domain (DBPII) to erythrocytes [184]. On the other hand, people with Fy^a phenotype have 30–80% lower risk of clinical *vivax* malaria, but not for falciparum malaria [185]. In the countries of Southeast Asia that are the source of *P. vivax*, the Fy^a allele is fixed [186], while Fy^b is represented in the populations in North and Northern-central Europe. This kind of distribution of Fy alleles indicates a selective advantage against *P. vivax* malaria [185].

6. Conclusions

Genetic specificities of Mediterranean region described in this chapter are a good example of how important role genetic diversity plays in adaptability to local environment. Recognition of these specific genetic traits, of the region, is of considerable clinical importance. Carrier identification, genetic counseling and prenatal diagnosis still represent a corner stone in the management of thalassemia-syndromes and various hemoglobiopathies. Due to variable frequencies of different *MEFV* mutations, their genotypes and prevalence should be determined in every population, in order to make reference frameworks for mutation screening when needed.

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Conflict of interest

The authors declare no conflict of interest.

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