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Molecular Characterization of Glucose-6-Phosphate Dehydrogenase Deficiency among Palestinians in the West Bank

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Thesis Approval

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Jerusalem – Palestine

1437 Hijri / 2016 AD

Dedication

To my father and mother

To my husband

To my brothers and sisters

To my family and friends

To my teachers

To my colleagues

To whoever inspired me positively**.**

Declaration

I certify that this thesis submitted for the degree of Master, is the result of my own research, except where otherwise acknowledged, and that this study (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signed 

Eman Walid Talib Abu Zahriyeh

Date 22 / 8/ 2016

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Abstract

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is considered the most common human enzyme disorder, affecting more than 400 million people worldwide with considerable variation in the enzyme among various racial groups. G6PD deficiency is a public health concern mainly in developing countries. In the West Bank, there are no published studies about the common mutations of *G6PD* gene.

The aim of this study is to investigate the most common genotypes of *G6PD* among Palestinians in the West Bank and to confirm the initial clinical classification of the *G6PD* deficiency by genotyping.

Sixty seven blood samples were collected from patients pre-diagnosed with G6PD deficiency. Samples were tested for G6PD activity by enzymatic assay at Al-Makassed Islamic Charitable Hospital in East Jerusalem. DNA sequencing was used to screen samples for G6PD mutations.

A total of 6 variants were detected in the *G6PD* gene among the study patients, which are: c.202 G>A, c.376 A>G, c.404 A>C, c.406 C>T, c.563 C>T and c.1311 C>T. From the 6 exonic variants detected, 5 were missense mutations resulting in 5 different G6PD variants. The Mediterranean variant (c.563 C>T) was the most frequent variant with a frequency of 62.7% followed by A- (c.202 C>A+ c.367 A>G) and Asahi (c.202 G>A) with a frequencies of 16.4% and 5.9% respectively, while the Valladolid (c.406 C>T) and Cairo (c.404 A>C) have had a low frequency of about 1.5 % each.

Phenotypically, all patients with Mediterranean variant have an enzyme activity less than 10 % which is consistent with its classification as class II. Patients with A- variant have an enzyme activity between 10%-60% which is consistent with its classification as class III. Patients with the Mediterranean variants had lower enzyme activity than patients with A- variants, 0.93 U/gHb compared to 3.45 U/gHb, respectively.

In conclusion, this study characterizes the molecular heterogeneity of G6PD variants among Palestinians in the West Bank. Among these, the Mediterranean variant was the most common in our patients (67%), followed by A- with a percentage of 16.4 %, and then Asahi with a percentage of 5.9 %, and an equal percentage of 1.5 % for Cario, and Valladolid. Phenotypically, G6PD variants detected in this study showed enzyme activity

consistent with their classification as class II or class III. This is the first study of G6PD genotypes in the West Bank.

Table of Contents

List of Tables

List of Figures

Appendices

Definitions

Glucose-6-phosphate dehydrogenase deficiency (G6PD deficiency) is an X-linked recessive inborn error of metabolism that predisposes to hemolysis and resultant jaundice in response to a number of triggers, such as certain foods, and drugs.

Neonatal Jaundice: is a yellow discoloration of the skin and eyes caused by hyperbilirubinemia.

Favism: A condition characterized by hemolytic anemia after eating fava beans (Vicia fava) or being exposed to the pollen of the fava plant.

X-inactivation: (also called lyonization) is a process by which one of the copies of the X chromosome present in female mammals is inactivated.

X-linked recessive: is a mode of inheritance in which a mutation in a gene on the X chromosome causes the phenotype to be expressed in males who are hemizygous for the gene mutation because they have one X and one Y chromosome) and in females who are homozygous for the gene mutation.

Abbreviations

AIVHA: Acute intravascular hemolytic anemia

CNSHA: Chronic non-spherocytic hemolytic anemia.

G6PD: Glucose-6-phosphate dehydrogenase

NADP: Nicotinamide adenine dinucleotide phosphate

PCR: Polymerase chain reaction

PPP: Pentose phosphate pathway

RBC: Red blood cell

ROS: Reactive oxygen species

WHO: World health organization

Chapter one

Introduction

1.1 Background

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is considered the most common human enzyme disorder, affecting more than 400 million people worldwide with considerable variation in the enzyme among various racial groups (Al-Alimi et al., 2010; Riskin et al., 2012). The enzyme is known as one of the most polymorphic enzymes in humans; more than 400 biochemical variants have been reported from various populations. G6PD deficiency is an X-linked hereditary genetic disorder (Persico et al., 1986). All mutations of *G6PD* gene that cause the enzyme deficiency affect the coding sequence. The number of *G6PD* gene mutations associated with enzyme deficiency that have been reported so far according to the Human Gene Mutation Database are about 160 mutations (Ho, Cheng, & Chiu, 2007). The World Health Organizations (WHO) has classified G6PD variants into five groups based on their enzymatic activity and clinical manifestations, among the most common clinical manifestations of this disease are acute hemolysis, chronic hemolysis, neonatal jaundice ("Glucose-6-phosphate dehydrogenase deficiency. WHO Working Group," 1989).

1.2 Structure and function of G6PD enzyme

The monomer of G6PD consists of 515 amino acids, with a molecular weight of about 59 kDa (Cappellini & Fiorelli, 2008). In 1996 a three-dimensional model of the G6PD structure was published (Figure 1), at the same time the crystal structure of human G6PD has been explained and clarified (Au, Gover, Lam, & Adams, 2000). The enzyme is active as a tetramer or dimer and its activity is pH-dependent. Each monomer consists of two domains: the first domain is the N-terminal domain (amino acids 27-200), with a β-α-β dinucleotide binding site (amino acids 38-44), the second larger domain is a β+α domain, consisting of an antiparallel nine-standard sheet (Mason, 1996; Naylor et al., 1996). The dimer interface lies in a barrel arrangement, in this second part of the molecule. Both domains are linked by an α helix, containing the preserved eight-residue peptide that serves as the substrate binding site (amino acids 198-206) (Naylor et al., 1996). By viewing the structure, at (0.3 nm) resolution a molecule of the coenzyme NADP+ is shown in each subunit of the tetramer, distant from the active site and close to the dimer interface. The stability of the active quaternary structure is very important and crucial for normal G6PD activity (Au et al., 2000).

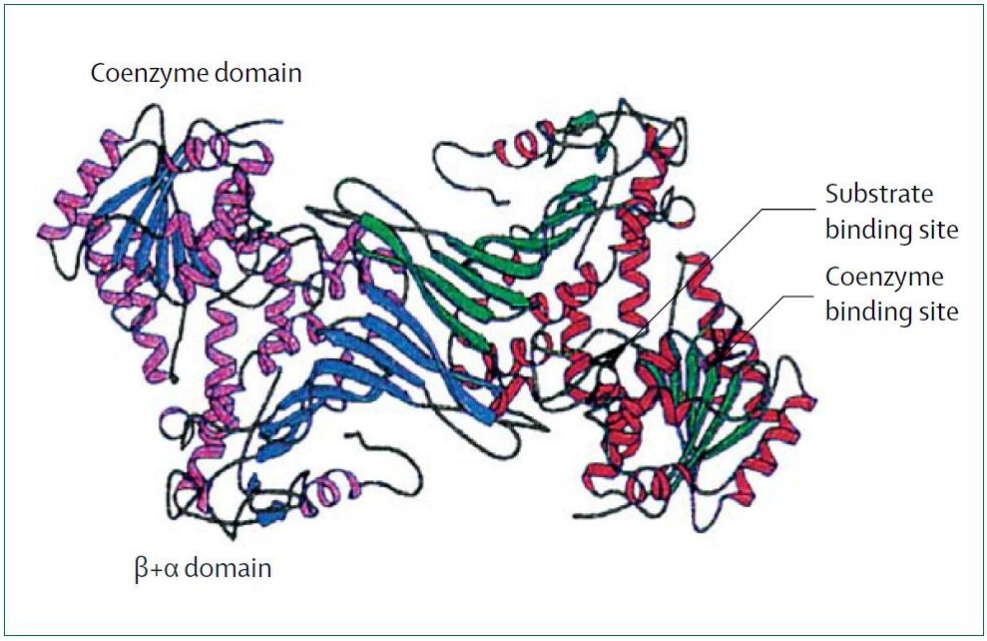

Figure 1.1: Three-dimensional model of active G6PD dimer. The two identical subunits are located across a symmetrical axis (Cappellini & Fiorelli, 2008).

Glucose-6-phosphate dehydrogenase is the rate limiting enzyme in the pentose phosphate pathway (PPP), one of the two enzymatic pathways required for the metabolism of glucose in red blood cells (Al-Alimi et al., 2010). G6PD catalysis the first step in the PPP in which glucose-6-phosphate is oxidized into 6-phosphogluconolactone and at same time converting nicotinamide adenine dinucleotide phosphate (NADP) into its reduced form (NADPH) (Figure 2). NADPH is necessary for maintaining the reduced state of glutathione. Reduced glutathione is used for the reduction of peroxides and preventing the reactive oxygen species (ROS) from accumulating in the red blood cells and affecting the red blood cells membrane's integrity. Any defect in the membrane due to the deficiency of G6PD has a significant effect on the red blood cells' survival leading to hemolysis (Prchal & Gregg, 2005). G6PD is the only enzyme that produces NADPH in red blood cells, since these cells do not have nucleus and mitochondria. Also the G6PD/NADPH pathway is the only source of reduced glutathione which is the only defense against oxidative stress in human red blood cells (Riskin et al., 2012).

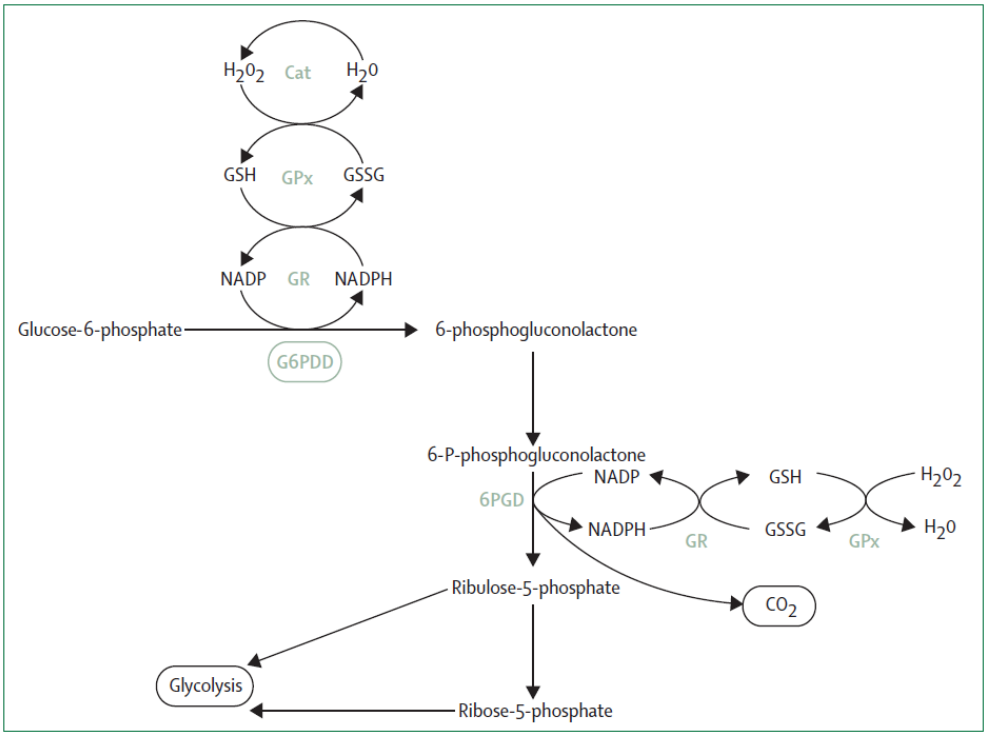

Figure 1.2: Pentose phosphate pathway. NADPH is a result of G6PD and 6-phosphogluconate dehydrogenase. It serves as a proton donor for the regeneration of reduced glutathione, and as a ligand for catalase. Cat: Catalase. GPX: Glutathione peroxidase. GR: Glutathione reductase. G6PDD: Glucose–6–phosphate dehydrogenase. 6PGD: 6-phosphogluconate dehydrogenase. GSH: Reduced glutathione. GSSG: oxidized glutathione (Cappellini & Fiorelli, 2008).

1.3 Genetics and molecular basis of G6PD deficiency

G6PD deficiency is an X-linked recessive genetic disorder; therefore diseases involving this enzyme occur more frequent in males than females (Salvemini et al., 1999). Males are hemizygous for the *G6PD* gene, thus, they can have normal gene expression or be G6PD deficient. Females carrying two copies of the G6PD gene are either homozygous or heterozygous. Heterozygote females are a result of X-chromosome inactivation, such females can be prone to the same pathophysiological phenotype (Beutler, Yeh, & Fairbanks, 1962).

The *G6PD* gene in human is located in the telomeric region of the long arm of the X chromosome (Xq28), next to genes for hemophilia A, congenital dyskeratosis, and color blindness (Figure 3) (Trask, Massa, Kenwrick, & Gitschier, 1991). The gene was cloned in 1986 (Persico et al., 1986), and consists of 13 exons and 12 introns spanning nearly 20 kb, and encoding 515 amino acid protein and a GC- rich (>70%) promoter region. The 5'untranslated portion of the mRNA corresponds to exon 1 and part of exon 2, the initiation codon is in exon 2 (Chen et al., 1991). In the promoter region, there are many binding sites for the transcription factor SPI –GGCGGG and CCGCCC sequences similar to those in other housekeeping gene promoters (Reynolds et al., 1984; Toniolo, Filippi, Dono, Lettieri, & Martini, 1991). The wild-type G6PD is denoted as G6PD B (Cappellini & Fiorelli, 2008).

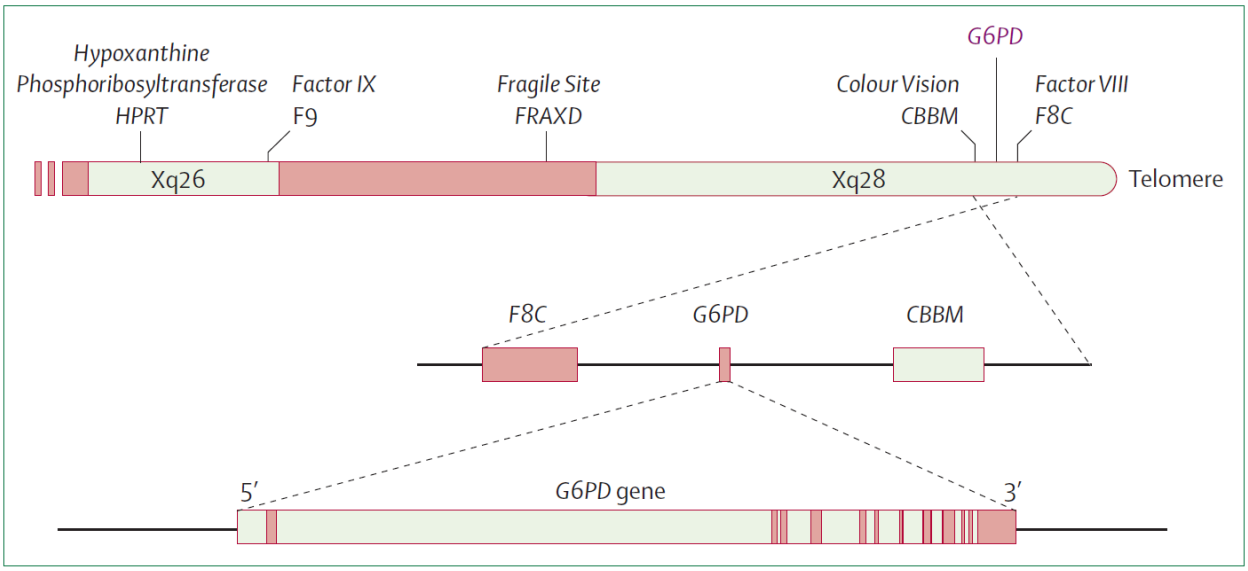

Figure 1.3: Location of G6PD gene on X chromosome (Cappellini & Fiorelli, 2008).

All mutations of *G6PD* gene that cause enzyme deficiency affects the coding sequence (Bulliamy, Luzzatto, Hirono, & Beutler, 1997). The number of mutations associated with enzyme deficiency that have been reported according to the Human Gene Mutation Database are about 160 mutations; most of them are single-base substitutions leading to amino acid replacements. Rarely, a second mutation is present in *cis* (Hirono, Kawate, Honda, Fujii, & Miwa, 2002; Town, Bautista, Mason, & Luzzatto, 1992), and some of these mutations include small deletions, and a stop codon mutation (Franze et al., 1998). No frame shift mutations have been reported which suggests that a complete lack of this enzyme during mammalian development cannot be tolerated and is fatal (Kwok, Martin, Au, & Lam, 2002).

1.4 Epidemiology

G6PD deficiency is considered the most common human enzyme disorder, affecting more than 400 million people worldwide, with considerable variation in the enzyme activity among various racial groups (Nkhoma, Poole, Vannappagari, Hall, & Beutler, 2009). The prevalence of the enzyme deficiency ranges from <3% in Northern America and Europe to 15% - 25% in some regions of Africa and the Middle East (Kaplan & Hammerman, 2000).

It is difficult to define the quantitative contribution of each allele to the overall prevalence of G6PD deficiency in any given population, because the epidemiological studies are based on screening the activity of the enzyme, which is not precise and doesn't extend to global coverage. Lately molecular biology techniques have been used to define the prevalence of G6PD deficiency (Cappellini & Fiorelli, 2008).

In most regions of high prevalence of G6PD deficiency, several polymorphic alleles are found (Bulliamy et al., 1997; Cappellini et al., 1996). G6PD A- (p.Val68Met + p.Asn12Asp) accounts for about 90% of G6PD deficiency in the tropical regions of Africa, and is also frequent in North and South America, in the West Indies, Italy (Cappellini et al., 1994; Martinez di Montemuros, Dotti, Tavazzi, Fiorelli, & Cappellini, 1997), the Canary Islands (Pinto, Gonzalez, Hernandez, Larruga, & Cabrera, 1996), Spain, Portugal and in the Middle East, including Iran, Egypt and Lebanon (Kurdi-Haidar et al., 1990).

The second most common G6PD variant is G6PD Mediterranean (p.Ser188Phe), which is found in all countries surrounding the Mediterranean Sea, and widespread in the Middle East, including Israeli Jews (Karimi et al., 2003), India and Indonesia. In many populations

around the Persian Gulf, G6PD A- and G6PD Mediterranean coexist at different frequencies (Bayoumi et al., 1996).

The Seattle (p.Asp282His) and Union (p.Arg454Cys) variants are other examples of G6PD variants, which were found in southern Italy, Sardinia (De Vita et al., 1989; Fiorelli et al., 1989), Greece, the Canary Islands (Cabrera, Gonzalez, & Salo, 1996), Algeria, Germany, Ireland, and China (Perng, Chiou, Liu, & Chang, 1992) (Figure1.4).

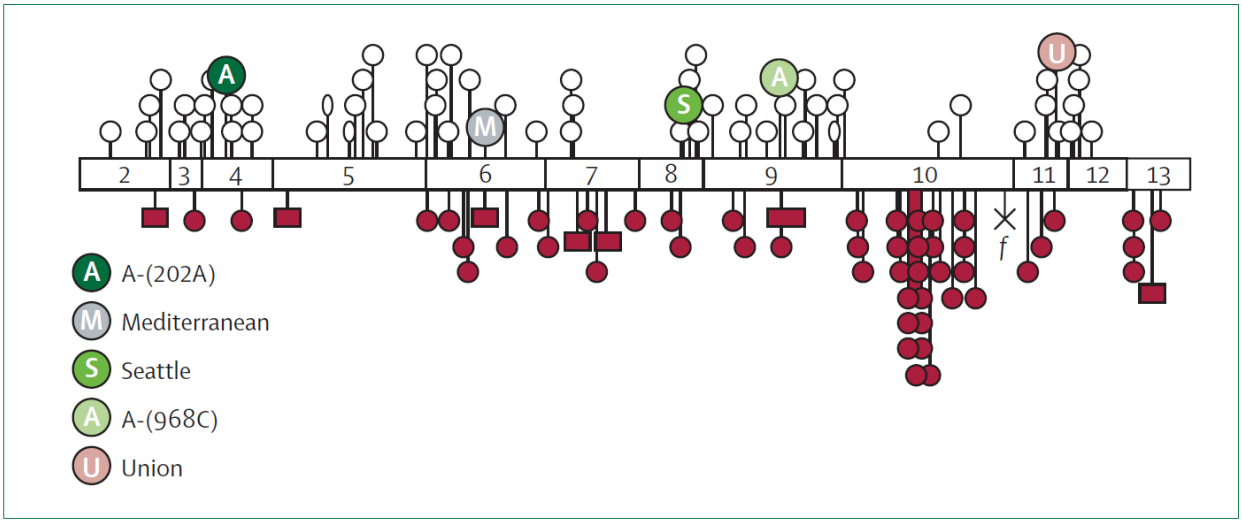

Figure 1.4: Most common mutations along coding sequence of G6PD gene Exons are shown as open numbered boxes. Open circles are mutations resulting in classes II and III variants. Filled circles represent sporadic mutations causing severe variants (class I). Open ellipses are mutations causing class IV variants. Filled squares= small deletion. Cross: a nonsense mutation. F: a splice site mutation. 202A and 968C are the two sites of substitution in G6PD-A (Cappellini & Fiorelli, 2008).

1.5 Diagnosis of G6PD Deficiency

The diagnosis of G6PD deficiency is based on the measurement of enzyme activity by a quantitative spectrophotometric analysis detecting the generation of NADPH from NADP, the amount of NADPH produced will reflect the level of G6PD activity (Kaplan & Hammerman, 2011). In field research, where quick screening of a large number of patients is needed semi quantitative analysis is available. The fluorescent spot test is one of the most common tests used for semi quantitative analysis, it is recommended by the International Committee for Standardization in Hematology (Beutler et al., 1979), it's considered a simple, sensitive, and reliable test. This test indicates G6PD deficiency when the blood fails to fluoresce under ultraviolet light (Minucci, Giardina, Zuppi, &

Capoluongo, 2009). The disadvantage of this test is its low cut off point that will not be helpful in detecting female heterozygotes (Wang, Boo, Ainoon, & Wong, 2009).

Some other qualitative screening tests are also available including the cresyl blue dye decolorization tests. These tests are based on staining the RBCs thus the G6PD deficient RBCs will satin poorly and can be identified microscopically. Among these tests, , the methemoglobin reduction test, in which the NADPH is detected indirectly by measuring the transfer of H+ ions from NADPH to an accepter (Luzzatto, 2006). For better diagnosis quantitative analysis is done specially on female that are missed diagnosed by the qualitative tests. Quantitative measurements are performed by adding a precise amount of hemolyzate to an assay mixture containing G6PD and NADP, the rate of NADPH generation in µmol per min per gm Hb is measured by a spectrophotometer at wavelength of 340 nm at 30°C. The assay is expressed as G6PD U/RBC or U/g Hb, the Hb concentration or the RBC count must be measured from the same blood sample on which the test is being performed (Kaplan & Hammerman, 2011).

A disadvantage of biochemical G6PD testing is that it is not reliable when the test is performed during an episode of acute hemolysis. In this case falsely normal result may be found due to the presence of new and young RBCs (reticulocytes) with higher activity than the old RBCs that were hemolyzed and destructed. The solution for this problem is to perform the test after a couple of weeks after the hemolytic attack, or to determine the *G6PD* gene mutation by Polymerase Chain Reaction (PCR) since the DNA will not be affected by a hemolytic attack (Castro et al., 2005; Lin, Fontaine, Freer, & Naylor, 2005).

1.6 Clinical manifestations

The enzyme is known as one of the most polymorphic enzymes in humans; more than 400 biochemical variants have been reported from various populations (Mason, Bautista, & Gilsanz, 2007). The WHO has classified G6PD variants into five groups based on their enzymatic activity and clinical manifestations ("Glucose-6-phosphate dehydrogenase deficiency. WHO Working Group," 1989) (Table 1.1).

Table1.1: Classification of G6PD variants based on enzyme activity. ("Glucose-6-phosphate dehydrogenase deficiency. WHO Working Group," 1989).

Class	Enzyme activity (%)	Clinical effect
I	Sever deficiency <2%	CNSHA
II	<10 %	Favism, AIVHA, Neonatal Jaundice
III	10-60%	AIVHA, Neonatal Jaundice
IV	60-150%	None
V	>150%	None
AIVHA: Acute intravascular hemolytic anemia (Drug or Infection-Induced). CNSHA: Chronic non-spherocytic hemolytic anemia.		

Among the most common clinical manifestations of this disease are drug or infection induced hemolytic anemia, favism, neonatal jaundice, and chronic non–spherocytic hemolytic anemia, and an asymptomatic form of the disease is observed (Cappellini & Fiorelli, 2008).

1.6.1. Neonatal jaundice:

Neonatal jaundice is one of the most common clinical manifestations of G6PD deficiency (Kaplan & Hammerman, 2009), neonatal jaundice or hyperbilirubinemia is the result of imbalance between the production and conjugation of bilirubin, which will cause severe complications in neonates and can lead to bilirubin encephalopathy and kernicterus. The mechanism whereby G6PD deficiency causes neonatal jaundice is not yet understood. Hemolysis does not seem to contribute as much as impaired bilirubin conjugation and clearance by the liver where screening for G6PD deficiency is not done routinely, evaluating neonates with unexplained hyperbilirubinemia and testing for G6PD deficiency should be done within the first 24 hours of life (Kaplan & Hammerman, 2009).

1.6.2. Chronic non-spherocytic hemolytic anemia:

In some cases, variants of G6PD deficiency cause chronic hemolysis, resulting in congenital non-spherocytic hemolytic anemia. These variants are grouped in class 1 in the WHO classification (Cappellini & Fiorelli, 2008). The G6PD variants causing congenital non-spherocytic hemolytic anemia are sporadic and rise from independent mutations (Fiorelli, Martinez di Montemuros, & Cappellini, 2000). The diagnosis of the complications is based on clinical examinations and findings, the symptoms of this disorder are suspected during infancy and childhood in most cases. Many patients with congenital non-spherocytic hemolytic anemia have a history of sever neonatal jaundice,

chronic anemia exacerbated by oxidative stress that requires blood transfusion, gallstones, splenomegaly, and reticulocytosis. The concentration of lactate dehydrogenase and bilirubin are raised, and the hemolysis is mostly extravascular (Cappellini & Fiorelli, 2008).

1.6.3. Favism:

Favism is the clinical sequelae of the ingestion of fava beans, it was first reported in the early 20th century in the Mediterranean countries in addition to some other counties in the middle east, the far east, and north Africa due to the high consumption of fava beans (Belsey, 1973; Kattamis, Kyriazakou, & Chaidas, 1969). Nowadays favism is believed to be associated with the Mediterranean variant of G6PD deficiency. It should be noticed that not all G6PD - deficient patients undergo favism after the ingestion of fava beans. Many factors affect the development of the disorder, including the health of the patient and the amount of fava beans ingested. Favism can develop after the ingestion of fresh, dried or frozen beans, but mostly after eating fresh beans; the disorder is most frequent in the period when beans are harvested (Meloni, Forteleoni, Dore, & Cutillo, 1983). Isouramil, divicine, and convicine, are thought to be the toxic components of fava beans, increase the activity of the hexose monophosphate shunt promoting hemolysis. Breastfed infants whose mothers have eaten fava beans are also at risk of hemolysis (Arese & De Flora, 1990).

Favism presents as acute hemolytic anemia, mostly after 24 hours of the ingestion of fava beans, clinical symptoms are, hemoglobinuria, elevated levels of bilirubin, acute and severe anemia leading to acute renal failure in some patients, as a result of ischemia or the precipitation of hemoglobin casts. In some cases a patient undergoing severs hemolytic attack can need a blood transfusion (Cappellini & Fiorelli, 2008).

1.6.4. Infection-induced hemolytic anemia:

Infection is the most typical cause of hemolysis in individuals with G6PD deficiency. Hepatitis virus A and B, cytomegalovirus, pneumonia, and typhoid fever are all notable causes. The severity of the hemolysis is affected by many factors including concomitant drug administration, liver function, and age. The total bilirubin levels are elevated by hepatitis as well as hemolysis, which may be a cause of diagnostic error (Cappellini & Fiorelli, 2008).

In sever hemolysis quick blood transfusion can improve the clinical course rapidly. Acute renal failure is a serious complication of viral hepatitis along with G6PD deficiency; pathogenic factors include acute tubular obstruction by hemoglobin casts. In some cases, hemodialysis is needed (Cappellini & Fiorelli, 2008).

1.6.5. Drug-induced hemolytic anemia:

Several drugs have been associated to acute hemolysis in G6PD patients. A specific drug directly causing hemolytic crises in a G6PD patient is difficult to establish for many reasons; first, an agent deems to be safe for some patients and not necessarily safe for others since pharmacokinetics can vary between individuals. Second, drugs with potentially oxidant effects are sometimes given to patients with an underlying illness (such as infection) that could cause hemolytic episode. Third, patients are often taking more than on type of medication. Fourth, hemolysis in G6PD deficiency is a self-limiting process, which means it doesn't always produce clinically significant anemia and reticulocytosis (Cappellini & Fiorelli, 2008).

Usually, safe alternative drugs and medications are available that physicians should be aware of. If no alternative treatment, decisions are based on clinical judgment of risk. Clinical hemolysis and jaundice arise within 24-72 hours of drug ingestion. Hemoglobinuria is a characteristic sign, anemia worsens until days 7-8. After drug termination, hemoglobin concentrations recover after 8-10 days. Heinz bodies are seen in the peripheral blood smear, detected by methyl violet staining, is a typical finding (Cappellini & Fiorelli, 2008; Edwards, 2002).

1.7 Literature review

G6PD deficiency is a public health concern in many countries. It has been estimated that 200 to 400 million people worldwide are affected by G6PD deficiency, and that 7.5% of the global population carry one or two genes for the condition. The disease has been reported in almost all racial groups with prevalence rates ranging from less than 1% in Japan and Northern European populations to as high as 58% in Kurdish Jews (Al-Musawi et al., 2012).

High rates of G6PD deficiency have been reported from tropical regions of Africa, the Middle East, tropical and subtropical regions of Asia, and the Mediterranean margin. Up to

now, more than 160 mutations associated with G6PD enzyme deficiency have been reported. G6PDA - genotype is a specific variant caused by two mutations p.Asn126Asp and p.Val68Met. The p.Val68Met mutation is responsible for 95% of the reported cases of G6PDA- variant in Africa (Shahjahani, Mortazavi, Heli, & Dehghanifard, 2013). From class II variant and with a low prevalence, G6PD-Santamaria is caused by two simultaneous mutations, p.Asn126Asp and p.Asp181Val. This genotype was first identified in Costa Rica (Monteiro et al., 2014) , and also found with low prevalence in northern Italy (Shahjahani et al., 2013). In Latin America, Monteiro et al. (2014) reported that low prevalence rates (<2%) of G6PD deficiency were found in Argentina, Bolivia, Mexico, Peru and Uruguay, while studies performed in Curacao, Ecuador, Jamaica, Saint Lucia, Suriname and Trinidad, as well as some surveys carried out in areas of Brazil, Colombia and Cuba showed high prevalence rates (> 10%) of G6PD deficiency. In addition results showed that G6PD encoded by the G6PD A- (p.Asn126Asp and p.Val68Met) is the most broadly spread genotype across Latin America, identified in 81.1% of deficient individuals surveyed in the continent (Monteiro et al., 2014). The most common dominant variant in Cambodia was G6PD Viangchan (c.871G>A; p.Val291Met) (Louicharoen & Nuchprayoon, 2005), while in Myanmar the Mahidol variant (c.487G>A,p.Gly163Ser) genotype was found to be the most variant distributed among their population (Bancone et al., 2014). In India the Mediterranean variant is more prevalent than the other variants (Shahjahani et al., 2013).

In Tunisia, Benmansour et al. (2015) reported two new class III G6PD variants, G6PD Tunis (c.920A>C: p. Gln307Pro) and G6PD Nefza (c.968T>C: p.Leu323Pro). Four hundred and twenty three patients were screened for G6PD deficiency by a spectrophotometric method to determine the enzymatic activity. All deficient samples were confirmed by molecular analysis to characterize the mutations found in the patient's samples, among the study population 14 different genotypes have been identified including the two novel missense mutations (G6PD Tunis and G6PD Nefza) (Benmansour et al., 2013).

In the Middle East, there are many cases of G6PD enzyme deficiency in Iran, Oman and Saudi Arabia. Many studies were done on cities and provinces of Iran for the prevalence of variants of G6PD enzyme deficiency, and to determine the frequency of G6PD enzyme deficiency and review the most prevalent mutations. The results of these studies and others

indicated a high prevalence of G6PD deficiency in Iran especially of Mediterranean and Chatman variants (Shahjahani et al., 2013). Noori-Daloii et al. (2004) identified the mutations in the *G6PD* gene in patients with favism in Iran and reported that the Mediterranean mutation (c.563C>T; p.Ser188Phe) is predominant in the area (69%) and 26.7% of patients have Chatham mutation at nucleotide (c.1003G>A) among the studied population (Noori-Daloii, Najafi, Mohammad Ganji, Hajebrahimi, & Sanati, 2004) .

G6PD deficiency is the most common hereditary blood disorder among Iraqis, most studies on the molecular basis of the disease was performed on Kurds in Northern Iraq. Al-Musawi et al. (2012) studied the prevalence of G6PD deficiency and the characteristic of deficient variants and their enzyme levels among asymptomatic healthy blood donors in the Arab population of central Iraq. The study identified four polymorphic variants, namely the Mediterranean (c.563C>T), Chatam (c.1003G>A), A- (c.202G>A) and Aureus (c.143T>C) among the sample population, results showed that the Mediterranean variant was detected in 74.3% of the study population. The result of the previous study on Iraqi Arabs complement those reported on Iraqi Kurds, which gave a comprehensive idea about the distribution of G6PD variants in Iraq. An important finding of the study is that a significant number (~ 19%) of G6PD deficient cases remain uncharacterized compared to around ~3% in the earlier studies on Kurds, which may reflect the open admixture with other civilizations throughout the centuries, this finding shows the importance of DNA sequencing to determine whether the uncharacterized mutations were carried by gene flow or they represent novel mutations (Al-Musawi et al., 2012) .

In Kuwait, 2005 a study was performed on individuals from the 5 different provinces of Kuwait, all individuals belonged to known Kuwaiti tribes with minimal recent non-Kuwaiti admixture. The study has characterized the molecular heterogeneity of G6PD variants among ethnic Kuwaitis, the variants found in this study were G6PD Mediterranean, G6PD Chatham, G6PD A- and G6PD Aureus. The findings of this study suggests that gene flow from the Indian sub-continent, sub-Saharan African, and other parts of the Mediterranean may have contributed to the G6PD mutations found on the Kuwaiti population (Alfadhli et al., 2005).

Jaouni et al. (2011) studied the molecular characteristics of G6PD deficiency in Jeddah, Saudi Arabia, the majority of mutations were G6PD Mediterranean, followed by Chatham (Al-Jaouni, Jarullah, Azhar, & Moradkhani, 2011). In a study about neonatal jaundice in

the Makkah region, which aimed to detect the frequency at which the different types of neonatal jaundice occur in Makkah. This study included 239 neonates with neonatal jaundice, 20 anemic neonates and 21 healthy neonates. Al Khotani et al. (2014) reported that the frequency of G6PD deficiency in the studied neonates was around ~6.6 % compared to 2%, 30.3%, 2%, 30.6%, and 14.7% in Yanbu, Al-Hofuf, Riyadh, Al-Qatif, Al Hasa, respectively. Al Khotani and his colleagues concluded that ABO incompatibility and G6PD deficiency are frequent causes of neonatal jaundice in Makkah and recommend the testing and determination of ABO and Rh incompatibility, G6PD deficiency and complete blood counts at birth to avoid serious complications (Alkhotani, Eldin, Zaghloul, & Mujahid, 2014). In another study done on a Saudi population, Alharibi et al. (2014) screened 2100 male subjects for G6PD deficiency to estimate the prevalence of G6PD deficiency among Saudi men, and to investigate the role of the A- variant in the study population. Of the 2100 men participating in the study 100 were shown to be G6PD deficient. The G6PD A- was only found in two subjects and therefore does not appear to have a role in G6PD deficiency in the studied population (Alharbi & Khan, 2014).

In 2014, Osman et al. identified the Mediterranean mutation in Egyptian favism patients. A total of 100 unrelated Egyptian patients were included in the study, and the mutation was found in 87.7% of the patients concluding that G6PD Mediterranean mutation is the most common mutation causing G6PD deficiency among Egyptians with favism (Osman, Zahran, El-Sokkary, El-Said, & Sabry, 2014).

In Lebanon, Inati et al. (2012) determined the prevalence of G6PD deficiency in a Lebanese population 3009 neonates and found that 62 neonates (2.1%) (49 males and 13 females) were G6PD-deficient. Prevalence was 3.1% in males compared with 0.9% in females. There was a higher prevalence of G6PD deficiency among Muslims (2.6%) than among Christians (1.3%), and reported that consanguineous marriages are more common in Muslims in the study population (Inati, Abbas, Boumitri, & Tecle, 2012) .

In Jordan, Karadsheh et al. (2005) studied the molecular heterogeneity of G6PD deficiency in two Jordanian populations, Amman area and the Jordan Valley. The molecular screening for the G6PD mutations resulted in six different mutations and a higher incidence of G6PD deficiency and G6PD A- mutation in Jordan Valley than in the Amman area (Karadsheh, Moses, Ismail, Devaney, & Hoffman, 2005).

Al-Swedan et al. (2012) investigated the most common molecular mutations of G6PD gene among Jordanians in northern Jordan, and examined the correlation between the genotype and phenotype of this enzyme deficiency. Seventy-five blood samples were collected from patients pre-diagnosed with G6PD deficiency on a clinical basis. Al-Swedan reported that the most common variant present in the patients is G6PD Mediterranean, with a frequency of 76.2%, followed by G6PD A- with 19%, and an equal frequency of 1.6% for G6PD Chatham, G6PD Santamaria and G6PD Cairo (Al-Sweedan & Awwad, 2012) .

In Palestine, there are no published studies about the prevalence or mutations of G6PD deficiency except for two studies done in Gaza Strip by Sirdah et al. (2012) on the Gaza Strip population. This study is the first study done on Palestinians in the West Bank.

Sirdah et al. (2012) investigated the molecular heterogeneity of G6PD deficiency in Gaza Strip, Palestine. Eighty unrelated Palestinian children hospitalized for hemolysis were studied. G6PD activity was determined by quantitative spectrophotometry and G6PD mutations were analyzed by sequencing of genomic DNA. The previous study identified three variants of G6PD mutations in the Gaza population which are G6PD Mediterranean, G6PD A- and G6PD Cairo. Other variants present in the Arab and Middle Eastern countries, such as G6PD Chatham and G6PD Aures, were absent from the studied population. The authors also reported a novel mutation that was designated as G6PD Gaza (c.536 G>A; p.Ser179Asn) that was found in a girl as a heterozygous genotype (Sirdah et al., 2012) .

1.8 Problem statement

G6PD deficiency is a public health concern mainly in developing countries. The disease has been reported in almost all racial groups with prevalence rates ranging from less than 1% in Japan and Northern European populations to as high as 58% in Kurdish Jews (Al-Musawi et al., 2012). Determination of the spectrum of mutations in G6PD gene will facilitate the molecular diagnosis of this disorder and aids in better characterization of the disease severity. A previous study by Sirdah et al (2012) has determined the spectrum of G6PD gene mutations among a cohort of G6PD patients in Gaza strip. However, there are no previous reports on the spectrum of G6PD gene mutation among Palestinian patients in West Bank.

1.9 Aims of the study

The aims of this study are to investigate the most common molecular mutations of *G6PD* gene among Palestinian patients, and to confirm the initial clinical classification of G6PD deficiency by genotyping .This study is the first study done in the West Bank.

1.10 Justification

G6PD deficiency is a public health concern mainly in developing countries. In the West Bank, there are no published studies about the common mutations of G6PD gene. Determination of the spectrum of mutations in G6PD gene will facilitate the molecular diagnosis of this disorder and aids in better characterization of the disease severity.

1.11 Hypothesis

Two hypothesis were presented one of them will be proven and the other will be rejected; the null hypothesis (H_0) will be retained, if the frequency of detected mutations in our study are consistent with the Middle Eastern populations findings especially Arabic ones. On the other hand, the alternative hypothesis ($H_{1)}$ will be returned if the H_o was rejected, if the frequencies of detected mutations in our study are inconsistent with the Middle Eastern population's findings especially Arabic ones.

Chapter Two

Methodology

2.1 Materials

Table 2.1: List of materials and instruments used in the study.

Disposables	
Item	**Manufacturer/country**
EDTA tubes	Greiner bio-one. UK
Needles	Medi-Plus. China
Syringes	Medi-Plus. China
Chemicals and kits	
Item	**Manufacturer/country**
DNA extraction kits from blood	MasterPure™ DNA Purification Kit for Blood Version II, Epicentre, USA
lyophilized PCR master mix	AccuPower® HotSart PCR PreMix, BIONEER, South Korea
Gel extraction kit	AccuPower® Gel Purification kit, BIONEER, Korea
G6PD kit	Randox
PCR primers	Metabion, Germany
50 bp leader marker	GeneDirex
TRIS base	MP Biomedical, USA
Chemicals and kits	
Item	**Manufacturer/country**
EDTA-Na bi-basic	AVONCHEM Ltd. UK
Agarose	Hylabs, Israel
Ethidium bromide	Hylabs, Israel
Machines and instruments	

Item	Manufacturer/country
Thermal cycler	Esco Healthcare Ltd. USA
Nano-drop machine	Thermo Scientific. USA
Gel electrophoresis system	Scie-Plas. UK
Gel documentation system	BIO-RAD GEL DOC 2000®, USA
Kone Lab	Thermo scientific, Finland
Cell dyne ruby	Abbott, USA

The sequence of the PCR primers is shown in Table (2.2). All PCR primers were obtained from Metabion (Germany).

Table 2.2: PCR primers used in this study*.

Primer	Sequence 5` → 3`	Amplicon size (bp)
Exons 1+2	F: CAG CGG CAG CGG GTA TG R: GGC CCT GCA ACA ATT AGT TGG	1123
Exons 3+4+5	F: CAC CAA GGG TGG AGG ATG ATG R: AGA GTG GTG GGA GCA CTG	1076
Exons 6+7	F: CTG GGA GGG CGT CTG AAT G R: GCT CTG CCA CCC TGT GC	597
Exons 8+9	F: GCC CTT GAA CCA GGT GAA CA R: TCC AGT GCC CGC ACA CAG	853
Exons 10+11+12+13	F: CAC TGG TCC ACA CCC TGA GA R: GTG CAG GTG AGG TCA AT	956

*All primers used in this study were taken from (Sirdah et al., 2012).

2.2 Methods

2.2.1. Study population:

The study population consisted of patients pre-diagnosed with G6PD deficiency based on clinical bases throughout the West Bank region. Patients had a history of at least one episode of hemolytic anemia or neonatal jaundice with a positive family history of G6PD deficiency. List of patients' names and contact information were obtained from the patients' registration files at their primary clinics and hospitals.

2.2.2. Questionnaire:

An interview-based questionnaire was developed for this study (Appendix A). The questionnaire was designed to collect demographic information and medical history of the patient. Patients were contacted by telephone and the study was briefly explained and then invited for participation in the study. In case of acceptance they were invited to attend the

nearest clinical care center. Patients were asked to provide the information needed to complete the questionnaire. After that, they were asked to donate a blood sample and to provide a written consent, and those who were younger than 18 years were accompanied by their guardian and the guardian was asked to sign a consent form.

2.2.3. Specimen collection, transportation and preservation:

Two samples of venous blood were collected in a K_3EDTA tubes from each patient. Each tube contained one to three milliliters of blood. Specimens were transported from the blood collection site to the main laboratory at Al-Makassed Islamic Charitable Hospital (MICH) in Jerusalem where CBC and G6PD activity were performed. Then samples were transferred to the laboratory at Al- Quds University where genomic DNA was extracted as well as DNA analysis. Samples were processed within one week of collection. Specimens were transported in a temperature-insulating box and kept at 6 $\pm$ 2°C from the time of specimen collection until the preparation of genomic DNA.

2.2.4. G6PD quantitative enzymatic activity:

The enzyme activity was measured using the commercially available G6PD screening test (Randox Laboratories, Ltd, Antrim, UK) at Al-Makassed Hospital in Jerusalem According to the manufacturer's instructions, 0.2 ml of whole blood was washed with 2 ml of 0.9% NaCl solution, the sample was centrifuged at 3000 rmp for 10 minutes after that the supernatant was discarded, and this step was repeated three times. The washed RBCs were suspended in 0.5 ml of Digitonin and the sample was let to stand for 15 minutes at +4°C, the mixture was centrifuged again and the activity was measured on Kone lab machine. G6PD was expressed as U/gHb. The reference values for Randox G6PD screening test methodology is 7 to 20 U/gHb.

2.2.5. Preparation of genomic DNA:

Genomic DNA was prepared from the buffy coat using a commercially available kit (MasterPure™ DNA Purification Kit for Blood Version II, Epicentre, USA). Briefly, whole blood was centrifuged at 3,500 x g for 3 – 5 minutes. Then, 200 µL of the buffy coat were transferred into a 1.5 mL tube, mixed well. This was followed by the addition of 600 µL Red Cell Lysis (RCL) buffer. The tubes were, then, mixed by inversion three times with flicking the bottom of the tubes; in order to re-suspend the WBCs. Then, the mixture

was incubated at room temperature for 10 minutes with shaking every 5 minutes. After that, precipitation of the WBCs was achieved by centrifugation at 10,000 × g for 25 seconds. This, usually, resulted in a small white pellet that settled underneath a clear reddish supernatant, indicating a good RBC lysis step. If the supernatant was not clear or the pellet contained a reasonable amount of intact RBCs, the RBC lysis step was repeated by adding additional RCL buffer. The supernatant was discarded and the pellet was re-suspended in about 25 µL of the left over. Then, the WBCs were lysed by emulsifying the cells in 300µL of the Tissue and Cell Lysis (TCL) solution. The highly viscous solution was pipetted several times in order to insure complete cell lysis. The next step was the protein precipitation step; that was achieved by salting out and involved the addition of 175 µL of the MPC solution (a highly concentrated; 6M, NaCl aqueous solution) followed by vigorous shaking of the solution for 15 – 30 seconds. After that, clear clumps would appear indicating the success of the protein salting out process. Retaining a clear DNA solution and getting rid of the protein debris was achieved by centrifugation at 10,000 ×g for 10 min. Then, the supernatant was transferred into a new 1.5 mL tube and the former tube was discarded. For precipitation of the DNA from the solution, 500 µL of absolute isopropanol were added to each tube, then, tubes were inverted 30 – 40 times. The sample was centrifuged at 10,000 x g for 10 minutes, then, the supernatant was discarded and the pellet was rinsed twice with 500 µL of 70% ethanol and centrifuged each time for 5 min at 13.000×g, and the pellet was left on (40 °C) in order to get rid of the volatile alcohol. Finally, hydration of the DNA was done by the addition of 80 µL of 1/2 TE buffer with pH 7.5, and the DNA solution was preserved at -20°C and later used for DNA amplification.

2.2.6. Assessment of DNA concentration and purity:

Assessment of the DNA concentration and purity were determined spectrophotometrically by the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific®). Purity and DNA concentration were assessed by measuring the optical densities A_{260}, A_{280}, A_{230}. A ratio $A_{260/280}$ greater than (1.8) indicating a good purity and minimum protein interferences of the DNA preparation were used for PCR amplification. All samples gave $A_{260/230}$ ratio greater than (2.0); which infers acceptable salt interferences.

2.2.7. Gene Amplification:

Gene amplification was achieved by Polymerase Chain Reaction (PCR) technique, where lyophilized master mix kit (AccuPower® HotSart PCR PreMix, BIONEER, Korea) was used. The PCR master mix contained a lyophilized mixture of Hot-start Taq DNA Polymerase, Buffer, $MgSO_4$, dNTPs and the loading buffer. Primers and DNA samples as well as the nuclease-free water were added to the mix (Table 2.3) The PCR components were mixed in the provided (0.2 mL) test tubes and the reactions were run using a thermal cycler (Esco Healthcare Ltd. USA) was used. Thermal cycling programs and the annealing temperatures of the primers are shown in (Table 2.3).

Table 2.3: Components of PCR reaction.

Reaction component	Volume (µL)
Lyophilized master mix	0
DNA sample (100 µg/mL)	3
Forward Primer (10µM)	0.5
Reverse Primer (10 µM)	0.5
DEPC- Water	16
Total Volume	20

Table 2.4: Thermal cycling program used for gene amplification.

Step	Duration	Temperature (°C)
Initial denaturation	5 min.	94
35 cycles: Denaturation Annealing Extension	 40 sec. 1min 1min	 94 57 72
Final extension	5 min	72

The PCR products were analyzed by running the PCR product on 1% agarose gel at 100 Volts for 30 min. All bands were then purified from the gel.

The criteria for mutation analysis depended on the prevalence of the given mutation in the Middle East area; we started with the most common mutation (i.e. G6PD Mediterranean) that exists in exon 6, to the less common ones. Homozygote females and positive males were not analyzed further, whereas, negative and heterozygote females and negative males underwent further investigations for the next most common mutations, and the same rhythm was followed towards the least common ones.

2.2.8. Gel purification:

The gel was purified by cutting the DNA fragment from the agarose gel; the slice of DNA was weighed in a 1.5 ml micro-centrifuge tube. Then, three volumes of Gel Binding Buffer were added to one volume of the gel slice. The sample was then incubated at 60°C for 10 minutes, and vortexed every 2-3 minutes during incubation for complete dissolving. After dissolving the gel slice, the color of the mixture must turn yellow indicating that the pH of the mixture is < 7.5 which means that the DNA fragment can effectively bind to the DNA binging filter. For increasing the typical yield, one volume of absolute isopropanol was then added to one volume of the gel slice, and the sample was mixed gently. After that, the mixture was transferred to the DNA binding column and centrifuged for 1 minute at 13,000 rpm. Then, the flow-through was poured off and the DNA binding filter column was re-assembled with a 2.0 ml collection tube. For washing, 500 µl of Buffer 2 was added to the DNA binding column tube and centrifuged for 1 minute at 13,000 rpm. Then, the flow-through was poured off and the washing step was repeated twice. To remove the residual ethanol, the sample was centrifuged at 13,000 for 3 minutes, and then the DNA filter column was transferred to a new 1.5 ml micro-centrifuge tube. Finally, for elution 40 µl of Buffer was added to the center of the binding filter column and centrifuged for 1 minute at 13,000 rpm.

2.2.9. DNA Sequencing:

The PCR products from the 2.2.7 section were subjected to cycle sequencing amplification using either the F or R primers or both. The PCR products were cleaned with sodium acetate and ethanol. The purified amplicon was suspended in formamide-based buffer and loaded onto the DNA sequencer. The DNA sequencing was performed at hy-Labs laboratories in Jerusalem. The DNA sequence results received for the sequencing lab was analyzed visually and then using the BLAST bioinformatics tool.

2.2.10. Ethical considerations:

The Ethical approval for this study was obtained from the Research Ethics committee at Al-Makassed Isalmic Hospital. In addition, all patients or their guardians in case of minors who accepted to participate in this study were briefed about the study objectives and asked to sign an informed consent.

2.2.11. Statistical analysis:

Microsoft Excel 2013 was used to calculate means and standard deviations, and to calculate the percentages and to draw the pie charts.

Chapter Three

Results

3.1 Study population

Sixty seven blood samples were collected from patients pre-diagnosed with G6PD deficiency on clinical bases, including forty seven males (70.1 %) and twenty females (29.9 %), from the West Bank region. The patients' aged ranged from one day to 47 years (Appendex B). All patients enrolled in this study were tested for G6PD enzyme activity and all patients had an activity result below the normal range (7.0-20 U/gHb). Based on the results of the G6PD quantitative enzymatic activity these patients were all included in this study.

Figure (3.1) shows the distribution of the patients participating in this study based on their residency among cities of the West Bank. The highest percentage of G6PD deficiency patients was found in Jerusalem (50.7%, 34 of 67), while the lowest percentage of patients was found in Qalqilya (1.5%, 1 of 67) (Figure 3.1). However, the distribution of patients based on the mother's origin also showed that the highest proportion of patients participating in this study was from Jerusalem (21 of 67), and the lowest proportion was from Haifa and Qalqilya with the same percentage of 1.5 % for each city (Figure 3.2).

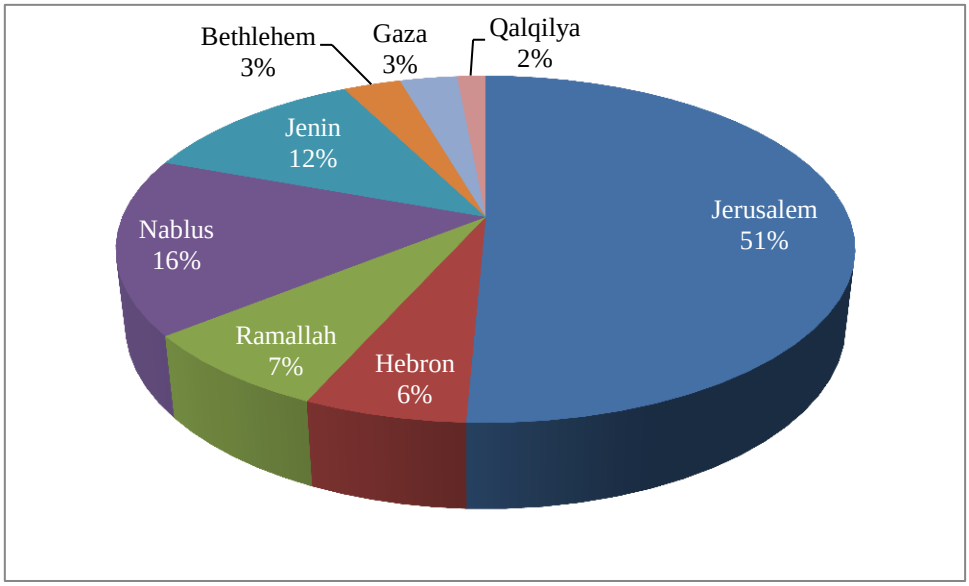

Figure 3.1: Distribution of G6PD deficiency patients based on residency.

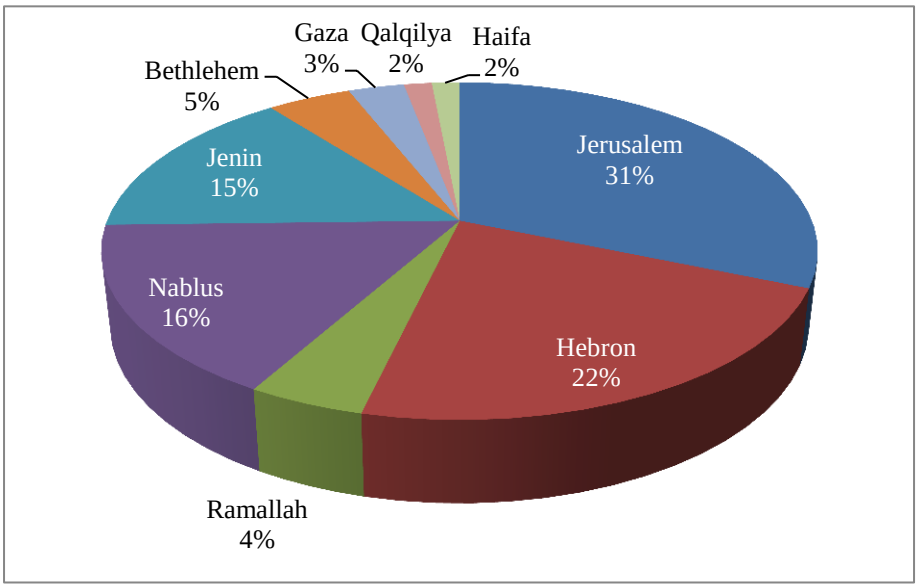

Figure 3.2: Distribution of G6PD deficiency patients based on mother's origin.

3.2 PCR and DNA sequencing

The 5 amplicons spanning the 13 exons of G6PD gene were amplified by PCR. A representative agarose gel of these amplicons is shown in Figure (3.3A). The amplicons were purified from agarose gels and used for DNA sequencing (Figure 3.3).

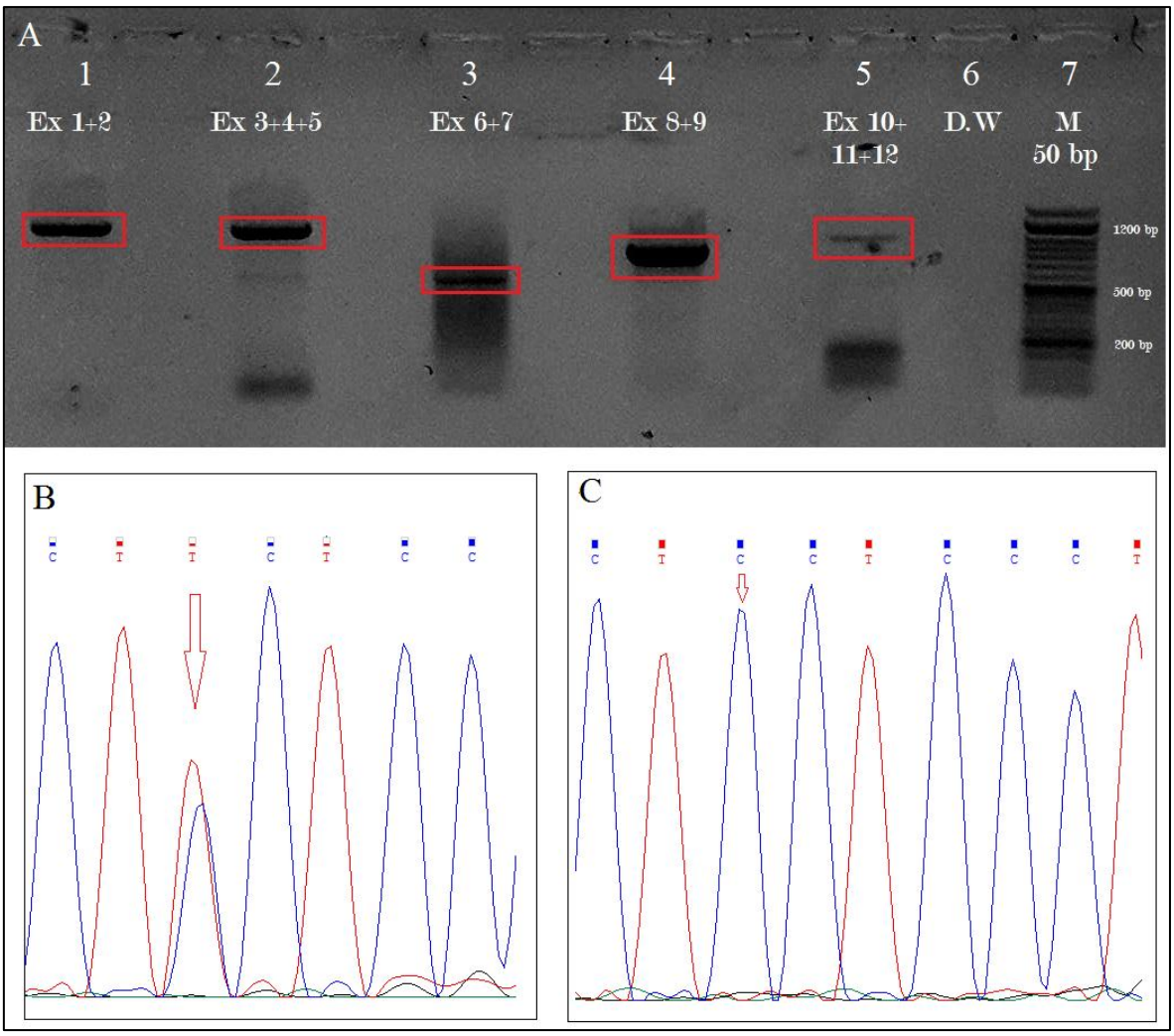

Figure 3.3: Mutation detection in *G6PD* gene. A) Different amplicon sizes of G6PD exons in agarose gel 1%. B) Sequence results for a heterozygous female with the Mediterranean variants (563 C>T). C) A normal sequence result (563 C).

3.3 Detection of G6PD mutations

A total of 6 variants were detected in the *G6PD* gene among the study patients. The exonic variations that were detected in this study were: c.202 G>A, c.376 A>G, c.404 A>C, c.406 C>T, c.563 C>T and c.1311C>T. All of these variations are summarized in Table (3.1).

From the 6 exonic variants detected, 5 were missense mutations resulting in 5 different G6PD variants summarized in Table (3.1). G6PD Mediterranean was the most frequent variant with a frequency of 62.7% followed by G6PD A- and G6PD Asahi with the frequencies of 16.4% and 5.9% respectively, while the G6PD Valladolid and Cairo has had a low frequency of about 1.5 % each .

Table 3.1: Genetic variants detected in G6PD gene among the study patients.

Variant ID[a]	Chr: bp[a]	cDNA change	AA change	Exon number	SNP type
rs1050828	X:154536002	c.202 G>A	p.Val68Met	Exon 4	Non-synonymous
rs1050829	X:154535277	c.376 A>G	p.Asn126Asp	Exon 5	Non-synonymous
rs782322505	X:154535249	c.404 A>C	p.Asn135Thr	Exon 5	Non-synonymous
rs970457	X:154535247	c.406 C>T	p.Arg136Cys	Exon 5	Non-synonymous
rs5030868	X:154534419	c.563 C>T	p.Ser188Phe	Exon 6	Non-synonymous
rs2230037	X:154532439	c.1311 C>T	p.Tyr437=	Exon 11	Synonymous

[a]ENSEMBL Genetic Variation available at: http://asia.ensembl.org/Homo_sapiens/Gene/Variation_Gene/Table?db=core;g=ENSG00000160211;r=X:154531391-154547572. Accessed on August 24th, 2016.

Table 3.2: Frequency of G6PD variants among study patients.

G6PD variant		Frequency, n (%)
G6PD Med	p.Ser188Phe	42 (62.7%)
G6PD A-	p.Asn126Asp + p.Val68Met	11 (16.4%)
G6PD Asahi	p.Val68Met	4 (5.9%)
G6PD Cairo	p.Asn135Thr	1 (1.5%)
G6PD Valladolid	p.Arg136Cys	1 (1.5%)
Undetermined	--	8 (12%)

The first exonic variation found in this study was c.563 C>T (G6PD Mediterranean), which was detected in exon 6, and changes an amino acid from serine (TCC) to phenylalanine (TTC) at position 188 (Figure 3.3), (Table 3.1). The majority of the study patients had this variation; 32 of 47 males, as well as 8 of 20 females with the heterozygous genotype CT and 2 of 20 females with the homozygous genotype TT. The allele frequency for the normal allele (C) was 0.30 in males and 0.70 in females (Table 3.3).

Table 3.3: Allele frequencies of G6PD variants in 67 patients with G6PD deficiency.

Exon	Variation	Allele frequency in males (n=47)	Allele frequency in females (n=20)
Ex 4	202 G>A	G: 0.83 A: 0.17	G: 0.82 A: 0.18
Ex 5	367 A>G	A: 0.85 G: 0.15	A: 0.89 G: 0.11
	404 A>C	A: 0.98 C: 0.02	A: 0.00 C: 0.00
	406 C>T	C: 0.98 T: 0.02	C: 0.00 T: 1.00
Ex 6	563 C>T	C: 0.32 T: 0.68	C: 0.70 T: 0.30

The c.202 G>A variation that is located in the coding region of exon 4, and results in the change between the G and the A allele from a GTG codon to an ATG codon (Figure 3.4), and a change in amino acid from valine to methionine at position 68 (Table 3.1). It was found in 8 out of the 47 males, and 7 out of the 20 females. The allele frequency for normal allele (G) was 0.82 in males and 0.82 in females (Table 3.3).

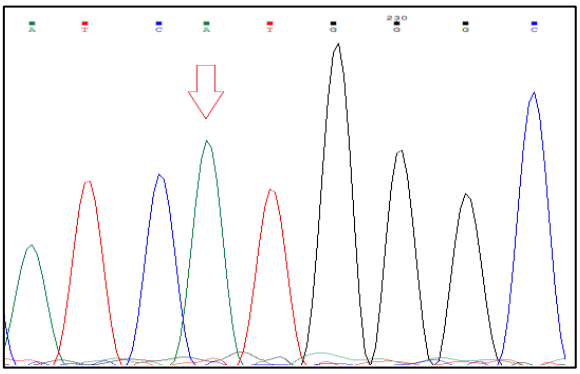

Figure 3.4: Sequence analysis of PCR products of exon 4 (c.202 G>A)

The c.367 A>G (G6PD A) variation was detected and found in exon 5 at position 376 resulting in an amino acid change from asparagine AAT to aspartate GAT at position 126 (Figure 3.5), (Table 3.1). Five out of forty seven males carried this mutation, and 7 of 20 females. The allele frequency for the normal allele A was 0.85 in males and 0.89 in females (Table 3.3).

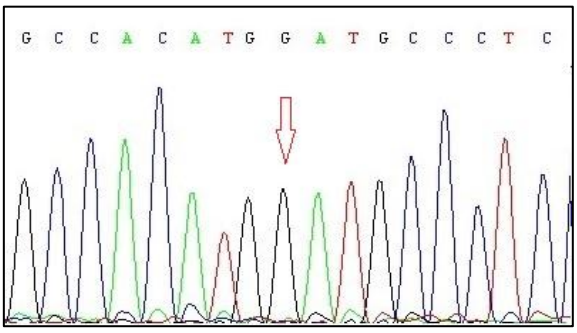

Figure 3.5: Sequence analysis of PCR products of exon 5 (c.367 A>G).

Sequence analysis of PCR products of exon 5 also revealed the c.404 A>C (G6PD Cairo) variation the results in the amino acid change from asparagine (AAC) to threonine (ACC) at position 135 (Figure 3.6), (Table 3.1). This variation was detected in one male. The allele frequency for the normal allele (A) was 0.98 in males (Table 3.3).

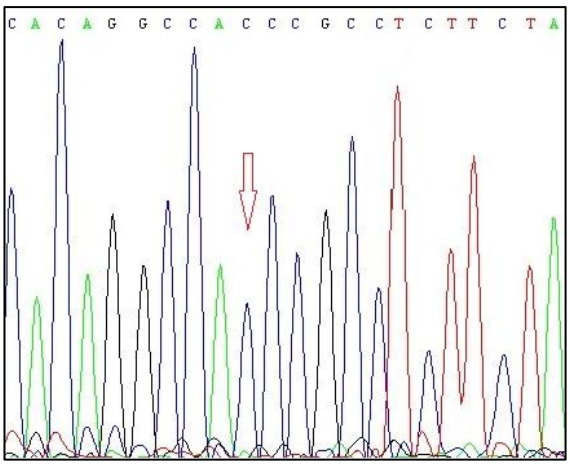

Figure 3.6: Sequence analysis of PCR products of exon 5 (c.404 A>C)

The last exonic variation detected in exon 5 in this study was c.406 C>T (G6PD Valladolid) that changes the amino acid arginine to cysteine C>T (figure 3.7), (Table 3.1). This variation was detected in one male, the allele frequency for the normal allele (C) was 0.98 in males (Table 3.3).

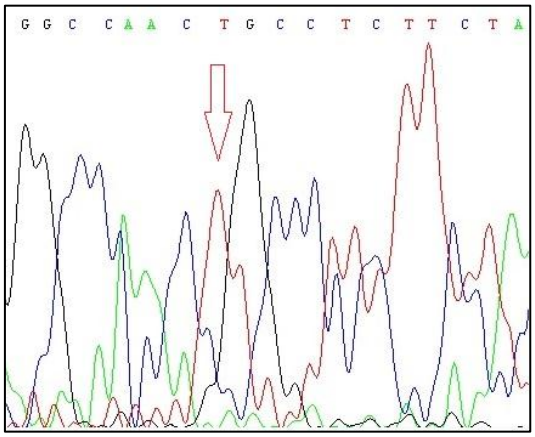

Figure 3.7: Sequence analysis of PCR products of exon 5 (c.406 C>T)

3.4 Genotype-phenotype confirmation

Phenotypically, all patients with G6PD Mediterranean have an enzyme activity less than 10 % (0.93 U/gHb) which is consistent with its classification as class II. G6PD A- and G6PD Asahi patients have an enzyme activity between 10%-60% which is consistent with their classification as class III with an enzyme activity of 3.45 U/gHb, 2.70, respectively (Table 3.4).

Table 3.4: Summary of G6PD enzyme activity.

Variant	**Enzyme Activity, U/g Hb (Reference range: 7-20 U/gHb)**
G6PD Mediterranean	0.39 ± 1.95
G6PD A-	3.45 ± 3.45
G6PD Asahi	2.70 ± 1.6
G6PD Valladolid	2.2
G6PD Cairo	2.3

3.5 Undetected basis for G6PD deficient samples

The genetic cause for G6PD deficiency in seven patients was not determined. No mutations were detected across the 13 exons and exon/intron junctions of G6PD gene and the underlining G6PD mutations remain unknown in these samples. Further analysis of the promoter region and intronic sequences may help understand the genetic basis of G6PD deficiency in these samples.

Chapter Four

Discussion

Sixty seven patients were screened for mutations in the *G6PD* gene, including forty seven males (70.1 %) and twenty females (29.9 %) from the West Bank region, Palestine. The highest percentage of G6PD deficiency patients was found in Jerusalem (50.7%, 34 of 67), while the lowest percentage of patients was found in Qalqilya (1.5%, 1 of 67). While the distribution of patients based on the mother's origin also showed that the highest proportion of patients participating in this study was from Jerusalem (21 of 67), and the lowest proportion was from Haifa and Qalqilya with the same percentage of 1.5 % for each city.

In this study, five missense mutations were detected resulting in 5 different G6PD variants; G6PD Mediterranean was the most frequent variant with a frequency of 62.7% followed by G6PD A- and G6PD Asahi with a the frequencies of 16.4% and 5.9% respectively, while the G6PD Valladolid and Cairo has had a low frequency of about 1.5 %.

The c.563 C>T mutation (G6PD Mediterranean) was found in 42 of the 67 patients (67%) participating in this study (32hemizygous males, 8 heterozygous females and 2 homozygous females). According to the World health Organization (WHO) classification of G6PD variants, G6PD Mediterranean is grouped under class II variants, which have an enzyme activity less than 10 %. In the present, study all patients with this variant were

found to suffer from neonatal jaundice or favism. The enzyme activity for male patients and homozygous females was 0.93 ± 1.95 U/gHb (normal range 7-2 U/gHb).

G6PD Mediterranean is found Arabs, Italians, Greeks, other nations in the Mediterranean area, as well as the Middle Eastern, African and Asian ethnic groups. (Kurdi-Haidar et al., 1990). The highest incident is found among Kurdish Jews (70%) (Beutler, 1994). In addition, Italy has a frequency of G6PD deficiency of 60% (Martinez di Montemuros et al., 1997), Turkey 80% (Oner et al., 2000), and India 60% (Sukumar, Mukherjee, Colah, & Mohanty, 2004). The percentage of G6PD Mediterranean in the patients in this study (67 %) is consistent with findings among other Arab populations in the region; for example, Saudi Arabia (89.1 %) (Al-Jaouni et al., 2011), Kuwait (74.2%) (Alfadhli et al., 2005), Iraq (74.3%) (Al-Musawi et al., 2012), and Jordan (76.2%) (Al-Sweedan & Awwad, 2012), but with a lower percentage in a study conducted in Gaza Strip (35.4%) (Sirdah et al., 2012).

c.202 G>A and c.376 A>G were detected in exon 4 and 5, respectively; these two point mutations give rise to what is called G6PD A- (Town et al., 1992). They were found in 16.4 % of the study patients (5 males and 6 females). Studies from Jordan reported that G6PD A- had a percentage of (19%) (Al-Sweedan & Awwad, 2012), while a study from Gaza had a higher percentage of G6PD A- among their populations study (28.5%) (Sirdah et al., 2012). In addition, studies done on Arabic populations in the region showed a lower frequency of G6PD A- compared to our results, for example; Saudi Arabia had a frequency of 5.8% (Al-Jaouni et al., 2011), and a study done on a Kuwaiti population had a frequency of 12.4% (Alfadhli et al., 2005). Furthermore, prevalence rates for this variant is 2% in Turkey (Oner et al., 2000), 3.7% in Italy (Martinez di Montemuros et al., 1997), and 46% in Algeria (Nafa et al., 1994). According to the WHO classification of G6PD variants, G6PD A- is grouped under class III variants, which are characterized by a moderate to mild deficiency in the enzyme activity (10 % - 60%). Enzyme activity for patients with the G6PD genotype was 3.45 ± 3.2 U/gHb (normal range 7-20 U/gHb). The clinical manifestation among the patients with this variant was neonatal jaundice or favism but with a lower frequency then that seen with the patients with the Mediterranean genotype

The c.202 G>A mutation was found in exon 4 and results in an amino acid change from valine to methionine. This mutation was named G6PD Asahi, it was found in 3 males and one female (5.9 %) of the study population. This mutations was reported in a Jordanian study with a percentage of 3.6 % (Karadsheh et al., 2005). G6PD Asahi is grouped under

class III variants with an enzyme activity from 10% - 150% which is consistent with our result (2.7 $\pm$ 1.6 U/gHb). Patents with this variant suffered from favism or neonatal jaundice.

As for G6PD Cario (c.404 A>C) a variation located in exon 5 was found in one male patient from Nablus (1.5 % of the study patients), with an enzyme activity of about 2.3 U/gHb, and suffered from favism. This mutation was reported once in a Jordanian study with a percentage of 1.3 % (Al-Sweedan & Awwad, 2012), and with a higher percentage in a study done on patients from Gaza (19.6 %) (Sirdah et al., 2012).

The c.406 C>T variation located in exon 5 resulting in an amino acid change from Arginine to Cysteine at position 136 is known as G6PD Valladolid, it was found in one male patient (1.5%) from Jerusalem and suffered from neonatal jaundice with a positive family history of G6PD deficiency. with an enzyme activity of about 2.2 U/gHb. This mutation was reported previously in two cases in a Jordanian study with a percentage of 7.1 % (Karadsheh et al., 2005). Table 4.1 shows a comparison of the G6PD variants found in the presents study and with the Middle Eastern and regional populations.

Table 4.1: Comparison of common G6PD variants in this study with Middle Eastern and regional populations.

Country	G6PD Variant					References
	Mediterranean %	**A- %**	**Asahi %**	**Valladolid %**	**Cairo %**	
West Bank	62.7	16.4	5.9	1.5	1.5	Present study
Gaza Strip	35.4	28.6	-	-	19.6	(Sirdah et al., 2012)
Jordan	76.2	19	3.6	7.1	1.6	(Al-Sweedan & Awwad, 2012)
KSA	89.1	5.8	-	-	-	(Al-Jaouni et al., 2011)
Kuwait	74.2	12.4	-	-	-	(Alfadhli et al., 2005)
Iraq	74.3	6.6	-	-	-	(Al-Musawi et al., 2012)
Algeria	23	46	-	-	-	(Nafa et al., 1994)
Turkey	80-84.2	4-7	-	-	-	(Oner et al., 2000)
Israeli Jews	83.3-95.5	-	-	-	-	(Kurdi-Haidar et al., 1990)

In conclusion, this study characterizes the molecular heterogeneity of G6PD variants among Palestinians the West Bank. Among these, G6PD Mediterranean (c.563 C>T) was

the most common in our patients (67%), followed by G6PD A- (c.202 G>A + c.376) with a percentage of 16.4 %, and then G6PD Asahi (c202 G>A) with a percentage of 5.9 %, and an equal percentage of 1.5 % for G6PD Cario (c.404 A>C), and G6PD Valladolid (c406 C>T). Phenotypically all patients with certain mutations found have had an enzyme activity result consistent with their classification as class II or class III. This is the first study of G6PD genotypes in the West Bank.

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Appendices

Appendix A

استبانة

انا الطالبة – ايمان وليد ابوزاهريه- طالبة ماجستيرفي برنامج العلوم الطبية المخبرية / مسار علم الدم في دائرة العلوم الطبية المخبرية- كليه الدراسات العليا- جامعة القدس .

اقوم ببحث بعنوان تحديد الطفرات الجينية المسببه لمرض نقص انزايم G6PD اوما يعرف بمرض التفول او انيميا الفول في الضفة الغربية في فلسطين

Molecular Characterization of Glucose-6-Phosphate Dehydrogenase Deficiency among Palestinians in the West Bank-Palestine

والذي يهدف الى التعرف على الجينات المسببه لهذا المرض وتحديدها ولدراسه العلاقه بين النمط الجيني والنمط الظاهري لهذا المرض , والكشف عن أي طفرات جديده . حيث سيتم سحب عينات دم من المرضى المصابين بهذا المرض ومن ثم سيتم عمل الفحوصات الوراثيه اللازمه للكشف والتعرف عن الطفرات المسببه لهذا المرض في الضفه الغربيه- فلسطين.

لذلك فان العينات المسحوبه والفحوصات التى ستجرى عليها والنتائج التى سيتم الحصول عليها سوف تعامل بسريه تامه ولغرض البحث العلمي فقط ولن يتم الافصاح عن أي نتيجه الا بموافقه المريض نفسه او ولي امره راجيا من المرضى التعاون لاتمام البحث والحصول على النتائج المرجوه مما قد يساهم في التشخيص الجزيئي والجيني لهذا المرض مما قد يسهل قي وصف ومعرفه مدى حده هذا المرض .

وتقبلوا فائق الاحترام

إيمان ابو زاهريه
دائرة العلوم الطبية المخبرية /مسار علم الدم
كليه الدراسات العليا
جامعة القدس

استبانه

Questionnaire

الاسم: ..... Name:…….....

مكان السكن:..... Residency:

الهاتف:..... Phone number:…….....

تاريخ الميلاد:..... DOB:……

الجنس: ذكر Male انثى Female Gender:

اصل الاب :..... Fathers origin

اصل الام :..... Mothers origin

الديانه: ..... Religion:……

هل توجد صله قرابه بين الاب والام: نعم Yes لا No Parents Consanguinity:

هل يوجد احد مصاب بنقص انزايم G6PD بالعائله نعم Yes لا No Family History

العمر عند التشخيص والبدء بظهور الاعراض..... Age of Onset ……

مستوى الانزايم بالدم..... G6PD Result

نسبه الهيموجلوبين ..... Hb level

التشخيص..... Clinical Manifestation

Declaration

إقرار

التوقيع في اسفل هذه الورقه من قبل المريض – او ولي امره- يقضي بقبول سحب عينه دم من المريض لاجراء الفحوصات اللازمه لغرض البحث العلمي والاطلاع على ملف المريض من قبل الباحث ان لزم الامر لاخذ أي معلومات قد تفيد البحث وله جزيل الشكر.

By signing the bottom of this page by the patient or guardian entails the permission to take a blood sample from the patient to perform the necessary tests for the scientific research. And look into the patients medical files by the researcher if needed to take any information that may help the research, and we are very thankful.

Signature / التوقيع

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Appendix B

Summary of the patients' demographic and clinical information.

Sample code	Residency	Gender	Age of onset	Mothers origin	Parent's Consanguinity	Family History	Clinical Manifestation	G6PD level	Hb level	G6PD variant
1	Jerusalem	M	At birth	Jenin	Y	Y	NJ	0.3	16.9	Med
2	Jerusalem	M	At birth	Hebron	N	N	NJ	1.7	17	Med
3	Jerusalem	M	At birth	Jerusalem	N	Y	NJ	2.2	10.5	Valladolid
4	Jerusalem	M	At birth	Jerusalem	Y	Y	NJ	2	16	Med
5	Jerusalem	M	At birth	Jerusalem	Y	N	NJ	1.9	15.8	Med
6	Jerusalem	F	At birth	Jerusalem	Y	N	NJ	6	15.1	G6PD A-
7	Jerusalem	M	At birth	Jerusalem	N	Y	Nj	0.6	16.8	Med
8	Ramallah	F	At birth	Bethlehem	N	N	NJ	4.1	16.7	Asahi
9	Jerusalem	F	At birth	Jerusalem	N	N	NJ	6	16	ND
10	Jerusalem	M	At birth	Jerusalem	N	Y	NJ	0.4	11.7	Med
11	Jerusalem	F	At birth	Jerusalem	N	Y	NJ	4.7	11.4	Med
12	Hebron	M	4	Hebron	N	Y	Favism	0.6	14.4	Med
13	Ramallah	M	2	Haifa	Y	N	Favism	2	14.2	Asahi
14	Nablus	M	3	Nablus	Y	Y	Favism	0.7	11.7	Med
15	Nablus	F	2	Nablus	Y	Y	Favism	5.1	13.7	G6PDA-
16	Nablus	M	4	Nablus	N	Y	Favism	0.5	12.9	Med
17	Nablus	M	5	Nablus	N	Y	Favism	2.2	13.6	Asahi
18	Nablus	M	2	Nablus	Y	Y	Favism	2.3	15.2	Cairo
19	Nablus	M	1	Nablus	N	Y	Favism	0.7	12.2	Med
20	Nablus	F	6	Nablus	Y	Y	Favism	5.8	12.1	G6PD A-
21	Nablus	M	7	Nablus	N	Y	Favism	2.6	13.3	Asahi
22	Nablus	M	3	Nablus	N	Y	Favism	0.7	12.4	Med
23	Nablus	F	4	Nablus	N	Y	Favism	2.7	12.4	G6PD A-
24	Nablus	M	2	Nablus	Y	Y	Favism	3.1	8.6	G6PD A-
25	Bethlehem	F	7	Bethlehem	N	Y	Favism	1.3	12.2	Med
26	Bethlehem	M	6	Bethlehem	N	Y	Favism	0.1	13.2	Med
27	Hebron	M	3.5	Hebron	N	Y	Favism	0.8	14.9	G6PD A-
28	Jerusalem	F	6	Jerusalem	Y	Y	Favism	3.9	10.8	G6PD A-
29	Jerusalem	M	7	Jerusalem	Y	Y	NJ	4.2	15.1	G6PD A-
30	Jerusalem	F	2.6	Jerusalem	Y	Y	Favism	1.8	12.8	G6PD A-
31	Jerusalem	M	25	Hebron	N	Y	Favism	0.09	14.4	Med
32	Jerusalem	F	4	Hebron	N	Y	Favism	1.2	12.7	Med
33	Jerusalem	M	10	Jerusalem	N	Y	Favism	0.7	15.7	ND
34	Jerusalem	M	1 month	Hebron	Y	N	NJ	1.8	12.9	ND
35	Jerusalem	M	At birth	Jerusalem	N	N	NJ	0.5	17.5	Med
36	Jerusalem	F	20	Hebron	N	Y	Favism	0.4	12	Med

37	Hebron	M	5	Hebron	N	Y	Favism	0.4	11.9	Med
38	Ramallah	M	11	Ramallah	N	N	Favism	1.5	14.9	G6PD A-
39	Ramallah	M	2	Ramallah	Y	Y	Favism	3.1	15.3	G6PD A-
40	Jerusalem	F	2 days	Hebron	N	Y	NJ	0.9	17.3	Med
41	Jenin	M	6	Jenin	Y	Y	Favism	6.1	12.9	ND
42	Jerusalem	M	at birth	Bethlehem	N	Y	NJ	0.12	18.1	Med
43	Jerusalem	M	at birth	Jerusalem	N	Y	NJ	0.05	16.8	Med
44	Jerusalem	M	1 weak	Hebron	N	Y	NJ	3.3	11.6	Med
45	Jerusalem	M	3	Hebron	N	Y	Favism	0.3	13.2	Med
46	Jerusalem	M	4	Hebron	N	Y	Favism	0.6	12.3	Med
47	Ramallah	M	3	Jenin	Y	Y	Favism	0.1	15.9	Med
48	Jerusalem	F	at birth	Hebron	N	N	NJ	5.3	21.4	G6PD A
49	Jerusalem	M	at birth	Jerusalem	N	N	NJ	0.02	11	Med
50	Jenin	M	6	Jenin	Y	Y	Favism	0.5	11.6	Med
51	Jenin	M	8	Jenin	N	N	Favism	0.4	14.3	Med
52	Gaza	M	2.5	Gaza	N	N	favism	4.7	4.06	ND
53	Jerusalem	M	At birth	Ramallah	N	N	NJ	3.8	16.5	Med
54	Jenin	M	12	Jenin	N	N	Favism	0.7	12.7	Med
55	Qalqilya	M	4	Qlqilya	N	N	Favism	0.02	13.8	Med
56	Jenin	F	2	Jenin	N	Y	Favism	1.3	11.9	Med
57	Jenin	M	At birth	Jenin	N	Y	NJ	3	11.6	Med
58	Gaza	M	3	Gaza	Y	Y	Favism	2.2	12.1	Med
59	Jerusalem	F	At birth	Jerusalem	N	N	NJ	6.3	16.3	Med
60	Jerusalem	M	At birth	Jerusalem	N	N	NJ	1.3	15.9	Med
61	Jenin	F	3	Jenin	N	N	Favism	4.4	12.5	Med
62	Jenin	M	2	Jenin	N	Y	Favism	5.1	14.9	ND
63	Jerusalem	M	4	Jerusalem	N	Y	Favism	0.3	12.1	Med
64	Hebron	F	5	Hebron	N	N	Favism	5.3	13.4	Med
65	Jerusalem	M	7	Jerusalem	N	N	Favism	2	12.2	Med
66	Jerusalem	F	at birth	Hebron	N	N	NJ	4.3	17.3	Med
67	Jerusalem	F	8	Jerusalem	N	Y	Favism	3.9	10.3	ND
M: Male, F: Female, Y: Yes, N: No, NJ: Neonatal Jaundice, Med: Mediterranean, ND: Not Detected.										

التوصيف الجزيئي لنقص نازعة الهيدروجين من الجلوكوز 6 فوسفات في الشعب الفلسطيني في الضفة الغربية – فلسطين

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إن نقص إنزيم نازعة الهيدروجين من الجلوكوز 6 فوسفات (G6PD) هي أكثر الاضطرابات الإنزيمية البشرية شيوعا. و حيث أن إنزيم G6PD متعدد الأشكال للغاية؛ فقد تم تعريف أكثر من 400 نوع في مختلف الشعوب. إن نقص إنزيم G6PD هو مصدر قلق على الصحة العامة في البلدان النامية بشكل رئيسي. إلا أنه لم يتم نشر أي دراسة حول الطفرات الشائعة لجين G6PD في الضفة الغربية.

تهدف هذه الدراسة إلى التعرف على الأنماط الوراثية الأكثر شيوعا من الإنزيم G6PD بين الفلسطينيين في الضفة الغربية ودراسة العلاقة بين النمط الجيني والنمط الظاهري لنقص هذا الإنزيم.

تم جمع سبع و ستين عينة دم من المرضى مسبقي التشخيص بنقص إنزيم G6PD. تم قياس نشاط إنزيم ال G6PD للعينات بوساطة فحص الأنزيمية في مستشفى جمعية المقاصد الإسلامية الخيرية في القدس الشرقية. تم استخدام تسلسل قواعد الحمض النووي لتشخيص العينات لطفرات G6PD.

تم الكشف عما مجموعه 6 متغيرات في جين G6PD بين المرضى الذين شملتهم الدراسة. كانت الاختلافات في الإكسونات التي تم الكشف عنها في هذه الدراسة: c.202 G> A، c.376 A> G، c.404 A> C، c.406> C T، c.563> T C و c.1311 C>T. من المتغيرات ال6 الإكسونية المكتشفة، تم الكشف عن 5 طفرات مغلطة مما أدى إلى حدوث 5 متغيرات مختلفة من إنزيم G6PD. كان G6PD البحر الأبيض المتوسط المتغير الأكثر شيوعا بنسبة 62.7٪، تليها G6PD A- وG6PD Asahi بنسبة 16.4٪ و 5.9٪ على التوالي، في حين أن G6PD بلد الوليد والقاهرة كانا بنسبة منخفضة حوالي 1.5٪.

كان النمط الظاهري لجميع المرضى الذين يعانون من G6PD البحر الأبيض المتوسط لديهم نشاط إنزيمي بنسبة أقل من 10٪، وهو ما يتسق مع تصنيفها ضمن الطبقة II. مرضى G6PD A- لديهم نشاط إنزيمي بين 10٪ -60٪ وهو ما يتسق مع تصنيفها ضمن الطبقة III. وكانت نسبة النشاط الإنزيمي للمرضى الذين يعانون من متغيرات البحر الأبيض المتوسط أقل من أولئك الذين يعانون من G6PD A- 0.93 U/gHb و 3.45 U/gHb.

في الختام، هذه الدراسة يعين التجانس الجزيئي لمتغيرات G6PD بين الفلسطينيين في الضفة الغربية. ومن بين هؤلاء، كان G6PD البحر الأبيض المتوسط (c.563 C> T) الأكثر شيوعا بين المرضى (67٪)، يليه G6PD A- (c.202 G> A + c.376) بنسبة 16.4٪، و ثم G6PD أساهي (c.202 G> أ) بنسبة 5.9٪، ونسبة متساوية بـ 1.5٪ لل G6PD (c.404 A> C) القاهرة، وG6PD بلد الوليد (c406 C> T). بالنسبة للنمط الظاهري، كان نتيجة النشاط

الإنزيمي لكل المرضى الذين يعانون من بعض الطفرات بما يتفق مع تصنيفها ضمن فئة II أو III. هذه الدراسة هي الأولى عن النمط الجيني ل G6PDفي الضفة الغربية وفلسطين.