An-Najah National University

Faculty of Graduate Studies

PREVALENCE AND MOLECULAR CHARACTERIZATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY AMONG ASYMPTOMATIC PALESTINIANS IN THE NOTHERN DISTRICT OF THE WEST BANK

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Dedication

I dedicate this thesis to my amazing parent's mom and dad, my sisters (Hadeel & Atheer) for standing by my side in all their efforts through these tough and beautiful years

To my supervisors

To my friends

To everyone loves science and strives to obtain it

Finally, yet importantly, May Allah accept this research as useful science and grant me his mercy, Amen.

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Declaration

I, the undersigned, declare that I submitted the thesis entitled:

PREVALENCE AND MOLECULAR CHARACTERIZATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY AMONG ASYMPTOMATIC PALESTINIANS IN THE NOTHERN DISTRICT OF THE WEST BANK

I declare that the work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

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Date: 9/10/2023

List of Contents

List of Tables

List of Figures

List of Appendices

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Abstract

Red blood cells are highly dependent on the cytoplasmic enzyme glucose-6-phosphate dehydrogenase (G6PD) to protect them from harmful oxidative damage. Mutations in the G6PD gene cause the X-linked recessive condition known as G6PD deficiency. With an estimated 400 million victims worldwide, this deficiency is the most widespread enzyme deficiency in the world.

G6PD deficiency is an important issue for the public's health, particularly in developing countries. In the West Bank of Palestine, no investigations have been conducted regarding the occurrence of G6PD deficiency in asymptomatic individuals, nor have the common mutations of the G6PD gene been studied. Evaluating the prevalence of G6PD deficiency among randomly chosen healthy blood bank donors in the West Bank is the major purpose of this study. This will be achieved by employing the Methemoglobin reduction test to identify cases of G6PD deficiency and subsequently examining enzyme activity. The study will also delve into the molecular basis of G6PD deficiency and characterize the specific deficient variants found among the affected donors.

From September 2020 to January 2021, a cross-sectional study was undertaken, encompassing a span of five months. The study involved 1380 healthy blood donors from three governmental hospitals located in the West Bank (specifically, Tulkarm, Nablus, and Jenin). Out of the 1380 donors, atotal of 32 were identified as having G6PD deficiency. Consequently, the prevalence of G6PD deficiency in the sampled population was determined to be 2.3%.

The molecular analysis of G6PD deficient samples revealed the presence of three variants: c.563 C>T, c.376 A> G, and c.202 G>A. Among these variants, the Mediterranean variant (c.563 C>T) was the most prevalent, accounting for 53.1% of the cases. Following this, the A- variant (c.376 A>G) and the Asahi variant (c.202 G>A) were observed with frequencies of 21.9% and 9.4% respectively. Notably, all samples with the Mediterranean variant exhibited enzyme activity levels below 10%, which aligns with its classification as class II.

On the other hand, samples with the A- and Asahi variants displayed enzyme activity ranging from 10% to 60%, consistent with their classification as class III.

In summary, this study successfully ascertained both the prevalence and molecular diversity of G6PD deficiency among asymptomatic Palestinians residing in the West Bank. The Mediterranean variant was found to be the most common, with a prevalence rate of 2.3%, followed by the A- variant and the Asahi variant.

Keywords: G6PD Deficiency; G6PD Variants; Methemoglobin Reduction Test.

Chapter One

Introduction and Literature Review

1.1 General Background

All organisms, with the exception of Archaea, which are primarily anaerobic, and a few obligatory surface (like Mycoplasma genitalium) and intracellular (like Rickettsia prowazekii) parasites, contain G6PD enzyme (1). Every cell in the human body has the enzyme glucose-6-phosphate dehydrogenase (G6PD), which is commonly found in the cytoplasm. It is a necessary housekeeping enzyme that takes part in numerous cellular processes. Its main function is to protect red blood cell proteins from damage caused by oxidative stress (1). The first reaction of the pentose phosphate pathway, where glucose 6- phosphate is oxidized to 6-phosphogluconolactone, is catalyzed by G6PD. NADPH (Nicotinamide Adenine Dinucleotide Phosphate) and reduced glutathione (GSH) are produced as a result of that process (2).

Being the most common inherited enzyme disorder in humans, G6PD deficiency affects 200–400 million people worldwide, or around 10% of the world's population. Males are affected more frequently than females because it is an X-linked disorder (3).

In certain instances, a deficiency in the G6PD enzyme within red blood cells can lead to the abnormal rupture of the cell membrane, resulting in hemolytic anemia (4). The severity and likelihood of hemolysis are determined by the extent of enzyme deficiency, which correlates with the biochemical characteristics of each specific G6PD variation (5).

1.2 Structure, Genetics and Deficiency of G6PD

The glucose 6-phosphate dehydrogenase (G6PD) gene, is located near the genes for hemophilia A, congenital dyskeratosis, and color blindness at position 28 on the long arm of the X chromosome (6). As seen in Figure 1.1, it is made up of 12 introns, referred to as non-coding regions, and 13 exons, which are coding sequences.

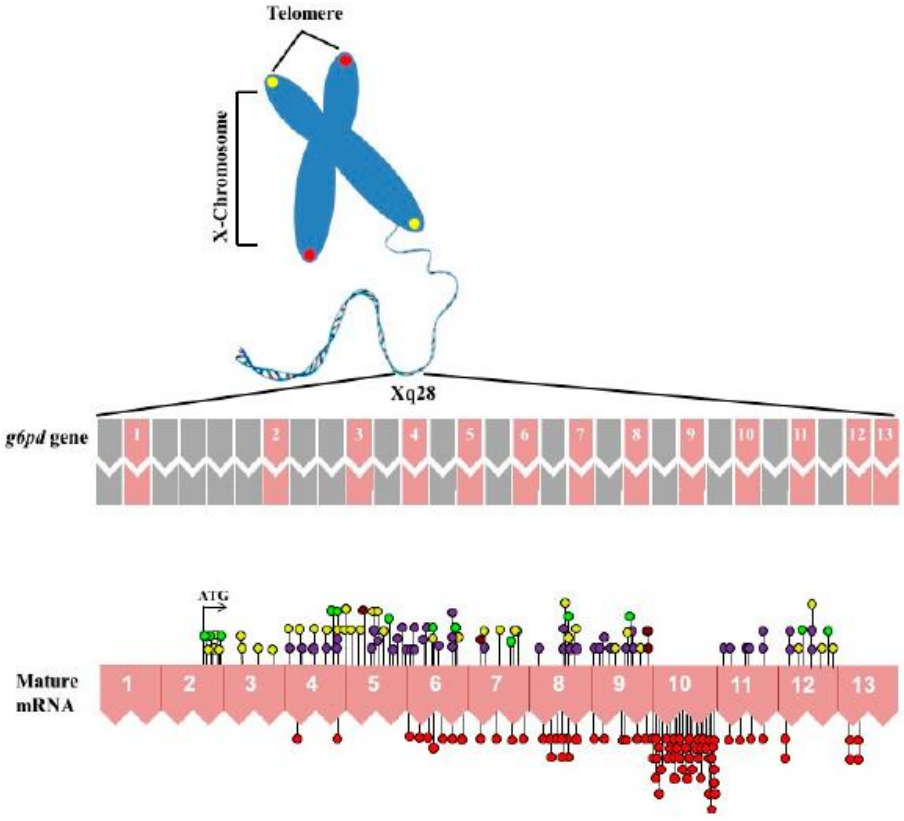
Due to an uncommon situation where the first 600 base pairs of the 5' of the G6PD gene, which are part of exon 1 and belong to exon 2, are not translated, the start codon ATG is located at base 115 of the 127 base pair of exon 2. Two ATG codons out of reading frame have been identified in this untranslated 5' sequence, and two sub-regions

have significant levels of guanine and cytosine (80%), which is a hallmark of genes with constitutive expression (7).

A number of characteristics can be seen in the G6PD gene's promoter region, including a high percentage of GC (over 70%), the absence of the CAAT element, which is commonly observed in positions 70 to 90 of different eukaryotic genes, the substitution of the typical TATA box with the ATTAAAT sequence, which is located 202 bp from the ATG start codon, and the presence of at least nine CCGCCC sites, which appear to be involved in gene regulation. A genetic disorder known as generalized G6PD insufficiency is brought on by mutations that interfere with the entire G6PD gene (8).

Figure 1.1

The G6PD Gene Location on X Chromosome



Note. From Glucose-6-phosphate dehydrogenase: Update and analysis of new mutations around the world byManzo et al 2016. Copyright © 2016 by the authors; licensee MDPI, Basel, Switzerland. (9)

At the Figure above, the introns and exons are visually represented by gray and pink color boxes, respectively. The exons of the human G6PD gene are labeled numerically from 1 to 13. The mRNA structure is depicted at the bottom, with single nucleotide mutations (missense variants) indicated. Mutations associated with Chronic Non Spherocytic Hemolytic Anemia (CNSHA) are denoted by red circles. Class II mutations are represented by purple circles, while yellow circles indicate Class III mutations. Class IV mutations are depicted by brown circles, and unidentified reported class mutations are shown as bright green circles.

Almost all mutations in and around this domain cause variants of G6PD deficiency associated with chronic non-spherocytic haemolytic anaemia (class I), and affect both hydrophobic and charge-charge interactions or salt bridges (i.e., weak ionic bonds) (7). All the variants caused by mutations located in this area show a striking reduction in thermal stability in vitro. All point mutations in the G6PD gene, when grouped according to the gradual decrease in conservation of amino acids, show diminishing clinical severity (8).It is noteworthy that many single point mutations have been recorded repeatedly in different parts of the world, suggesting that their origin is unlikely to be from a common ancestor and that they are, therefore, probably new mutations that have arisen independently (9).

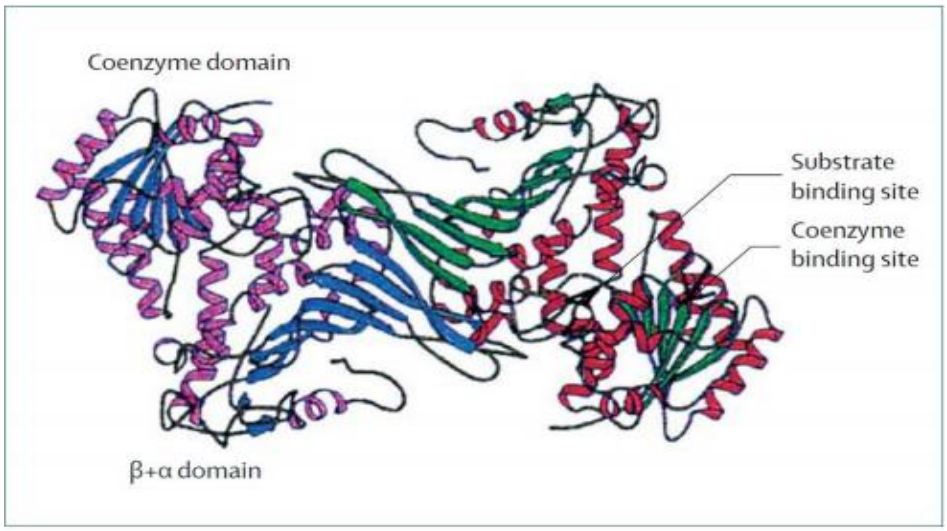
A majority of mutations affecting hydrophobic and charge-charge interactions or salt bridges (i.e., weak ionic bonds) in and around this domain result in forms of G6PD deficiency linked to chronic non-spherocytic hemolytic anemia (class I) (7). In vitro, all the variations resulting from mutations in this region exhibit a marked decrease in heat stability. Based on the progressive reduction in amino acid conservation, all point mutations in the G6PD gene exhibit decreasing clinical severity (9).Notably, several single point mutations have been identified time and time again in various regions of the world, indicating that their genesis is not likely to derive from a common ancestor and that they are most likely novel mutations that developed individually (10).

The G6PD monomer has a molecular weight of approximately 59 kDa and comprises 515 amino acids (10). The N-terminal domain and the β+α domain of the monomer are shown in Figure 1.2. The dinucleotide-binding site is located in the N-terminal domain, while the β+α domain is made up of a nine-stranded antiparallel sheet (11) ;(12). The second section of the molecule contains the dimer interface, which is set up in a barrel

form. The peptide of eight residues that functions as the substrate-binding site is enclosed by a helix that unites the two domains (12). G6PD is formed from 12 introns and 13 exons that together encode a 1545 bp product. The 202 bp area upstream of the G6PD transcription start site contains a TATA box, which regulates the accuracy and frequency of transcription initiation. The promoter region's analysis reveals a high level (70%) of guanine and cytosine content. Multiple transcription factor binding sites may be found in the G6PD promoter region, and these binding sites allow transcription factors to independently and directly control G6PD transcription (8).

Figure 1.2

Three-Dimensional Model of Active G6PD Dimer



Note. From Lancet Glucose-6-phosphate dehydrogenase deficiency by Cappellini and Fiorelli, http://dx.doi.org/10.1016/s0140-6736(08)60073-2 (13)

Deficiency of G6PD can be brought on by a decrease in the total quantity of the enzyme molecules, an alteration in the structure of the enzyme which results in a qualitative change, or occasionally both. According to Au *et al.* (2000) (14), changes in amino acids at different locations have the potential to destabilize the enzyme molecule, which is the major contributing factor to G6PD deficiency.

Additionally, it was discovered that a structural $NADP^+$ molecule is a crucial component for preserving the enzyme's (G6PD) structural integrity. The loss in enzyme stability is also a result of mutations that affect how this structural $NADP^+$ molecule binds to the enzyme (15).

All cells have glucose-6-phosphate dehydrogenase, but the amount varies depending on the tissue (11).The enzyme in healthy red blood cells only utilizes 1% to 2% of its maximal capacity, even in the presence of oxidative stress induced on by methylene blue). G6PD-deficient red blood cells have a significantly reduced reserve of reductive potential, which results in pathological characteristics (14). The variable phenotypic expression of G6PD deficiency was observed after it was recognized as a clinical illness (3).

The World Health Organization (WHO) has provided initial guidelines for identifying G6PD deficiency. Initially, residual enzyme activity and electrophoretic mobility were used to characterize the deficit biochemically. Since then, additional criteria have been used to describe more than 400 biochemical variants of G6PD deficiency. These criteria include physicochemical characteristics like thermo stability, chromatographic behavior, and kinetic variables like Km for various substrates and optimal pH (16).

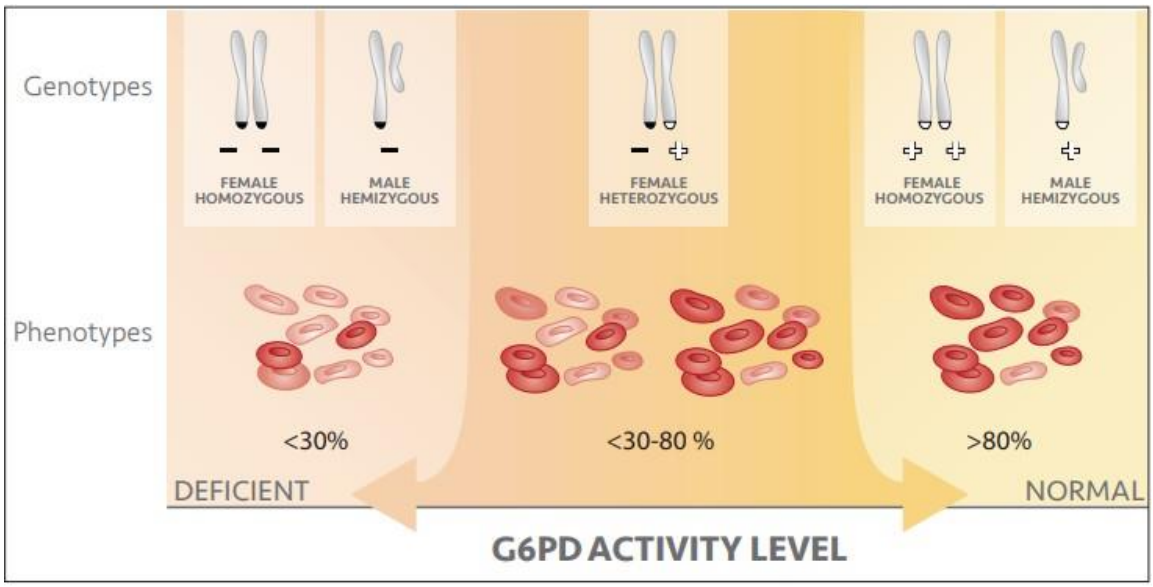
1.2.1 Genetics and G6PD Deficiency Phenotypes

As previously established, G6PD is an X-linked disorder, with two allele copies in women and one in men. Women can therefore be homozygous normal (two G6PD normal alleles), homozygous deficient (two G6PD deficient alleles), or heterozygous for G6PD (one normal allele and one deficient allele), but males can only be hemizygous G6PD normal or hemizygous G6PD deficient. Throughout the lyonization process, which occurs during the development of the female embryo, one or both X chromosomes stay dormant. The outcome is that females who are heterozygous for G6PD have two populations of red blood cells: one population that expresses the enzyme with low activity and another population that expresses the enzyme with normal activity (16).

The total G6PD activity in a heterozygous female is determined by the proportion of defective to normal cell populations. As a result, heterozygous females with intermediate G6PD activity levels ranging from 30% to 80% of normal are possible (Figure 1.3).

Figure 1.3

G6PD Deficiency Genotypes, Phenotypes and Activity Level



Note. Domingoa et al 2018. G6PD Deficiency Genotypes, Phenotypes and Activity Level in Addressing the gender-knowledge gap in glucose-6-phosphate dehydrogenase deficiency: Available from: http://dx.doi.org/10.1093/inthealth/ihy060 (17)

1.2.2 G6PD Deficiency in Heterozygotes

The G6PD level in heterozygous females must be at or below 50% of normal since hemolysis requires at least 50% of the red cells to be defective. Yet, the problems with recent or current hemolysis mentioned for male patients would exist in the case of heterozygous female patients. However, they are frequently prevented by employing a similar strategy, particularly when a cytochemical test is employed (18). In situations of "extreme phenotypes," red blood cells with G6PD deficiency may be so rare that heterozygous G6PD deficiency may only be diagnosed by DNA analysis, for which the underlying mutation must be found or recognized. G6PD-normal and G6PD-deficient red blood cells exist in the blood of heterozygous females, making them epigenetic mosaics. The phenotype of heterozygous females ranges from G6PD normal to G6PD, just as insufficient as that of a hemizygous male. This is because the ratio between the two types has a normal value of (i.e., 50% of each type), but this ratio varies greatly from person to person (19). Occasionally, some of these facts are misinterpreted. Numerous studies show the frequency of G6PD deficiency in a sample of the population that is sex-pooled at the population level. This is unfortunate because the true frequency

of the G6PD-deficient allele(s) in that population will not be known because only a subset of G6PD-deficient heterozygous females will have been classified as such; instead, the frequency of G6PD-deficient males alone, which is identical to the frequency of the corresponding allele, could have been used to precisely determine the frequency of the G6PD-deficient allele. The claims that G6PD deficiency "is more frequent in males" or "more expressed in males" are also frequently made, yet none of these claims is true. Contrarily, it is true that whereas G6PD deficiency is consistently exhibited in homozygous females and G6PD-deficient hemizygous males, the expression and hence the possible degree of hemolysis varies greatly in heterozygous females (18). Unusually, G6PD deficiency is sometimes classified as "X-linked recessive," even in textbooks. This is obviously incorrect, as G6PD deficiency is frequently manifested in heterozygotes, both biochemically and clinically (12).

Heterozygotes for G6PD are one type of mutation associated with CNSHA. Mothers of male patients with this condition frequently have normal G6PD levels, either because the variant in the offspring is a de novo mutation or because the mother is a heterozygote yet phenotypically normal, likely as a result of somatic selection favoring hematopoietic progenitor cells with the normal G6PD allele. A G6PD test, the clinical course, and family history are often sufficient to diagnose disorders linked to G6PD deficiency. The comprehensive database of known human mutations (Human Gene Mutation Database) can be used to find mutations in the G6PD gene in rare circumstances (such as heterozygotes and particularly in the case of CNSHA). Additionally, there is still a chance that new mutations will be found using this approach. Additionally, only molecular analysis can make a definitive diagnosis of a female's (19).

Enzyme stability is decreased by all mutations. (10) The homodimer or homotetramer form of the active G6PD enzyme. Most gene mutations result in alterations to the sequence of amino acids in the enzyme, which reduces stability. Disturbance in protein folding is the suggested cause for this decline in stability. Effective dimerization is disrupted as a result, especially at the dimer interface. It was also discovered that a structural NADP molecule is a necessary component that keeps the enzyme's structural integrity intact. The decline in the stability of the enzyme is also caused by mutations that change how this structural NADP molecule binds (12).

1.3 Function of G6PD

1.3.1 The Pentose Phosphate Pathway

Glucose-6-phosphate undergoes glycolysis in the majority of mammalian tissues to produce pyruvate, which is mostly oxidized via the citric acid cycle before entering the respiration chain and creating ATP. Glucose-6-phosphate, however, can also take on a number of catabolic fates that result in molecules unique to various cell types. The pentose phosphate route, also known as the phosphogluconate pathway or the hexose monophosphate pathway, is crucial in various organs because it catalyzes the conversion of glucose 6-phosphate to pentose phosphates. $NADP^+$ functions as the electron acceptor in this oxidative cycle, producing NADPH (20).

The pentoses are utilized by cells that divide quickly to produce RNA, DNA, and coenzymes such ATP, NADH, $FADH_2$, and coenzyme A. These cells consist of those in the skin, intestinal mucosa, and bone marrow. The electron donor NADPH, which is required for reductive biosynthesis or to lessen the negative effects of oxygen radicals, is the key outcome of the pentose phosphate pathway in other tissues rather than pentoses (20). These tissues depend on the NADPH synthesized through this pathway for their massive synthesis of fatty acids (liver, adipose, lactating mammary gland), as well as their very active production of cholesterol and steroid hormones (adrenal gland, gonads) (21).

Because they are all directly exposed to oxygen, erythrocytes, lens, and corneal cells are vulnerable to the free radicals that oxygen produces, which can have harmful effects. Protein, lipid, and other sensitive molecules can suffer from oxidative damage, but it can be stopped or reversed by maintaining high ratios of reduced to oxidized glutathione and NADPH to $NADP^+$. A genetic defect in G6PD, the enzyme catalyzing the first reaction in the pentose phosphate pathway, might have a damaging impact on one's health because of NADPH's critical role in avoiding erythrocyte oxidative damage (22).

1.3.2 Function of G6PD in the Pentose Phosphate Pathway (PPP)

The energy and phosphoribose sugar required for the synthesis of nucleic acids (RNA and DNA) during cell replication and gene transcription are provided by glucose oxidation, whether it occurs through glycolysis or the Pentose Phosphate Pathway (PPP) (23). Additionally, the PPP provides NADPH, which is crucial for the recycling of

oxidized glutathione and for anabolic activities including the synthesis of fatty acids, as well as for controlling the cellular redox potential. The enzyme glucose-6-phosphate dehydrogenase (G6PD) is the PPP's rate-limiting component. The most common enzymopathy in humans, G6PD deficiency affects about 400 million people globally to varied degrees (24).

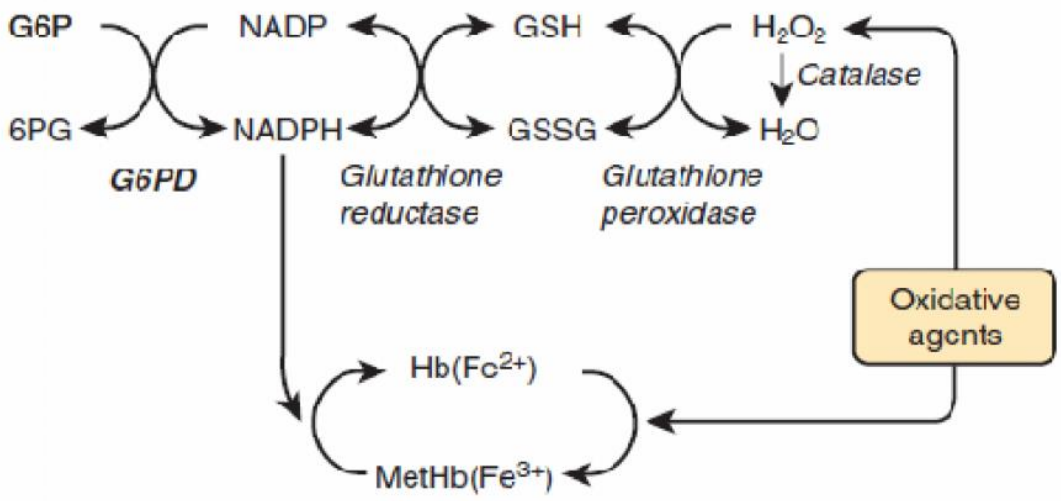
The red cell has several lines of defense against oxidative stress. Hydrogen peroxide (H2O2) is a powerful oxidizing agent arising from superoxide via superoxide dismutase (SOD). Superoxide is a by-product of auto-oxidation of Hb within the red cells, and of the oxidative burst in neutrophils (20). In red cells, H2O2 can be detoxified to H2O by 3 different enzyme-mediated mechanisms. (i) Catalase directly degrades H2O2 to H2O: it has 2 to 4 molecules of NADPH in its structure. (ii) Glutathione peroxidase catalyzes the same reaction coupled to the oxidation of reduced glutathione (GSH): it relies on the NADPH-linked glutathione reductase for regeneration of GSH. (iii) Peroxiredoxin-2 also degrades H2O2 at the expense of its own sulhydryl groups that become disulphides, and can be regenerated by thioredoxin through thioredoxin reductase. all 3 mechanisms depend on NADPH, a steady supply of which can be provided in red cells only by G6PD (23)

In red blood cells, the primary function of G6PD is to synthesize nicotinamide adenine dinucleotide phosphate in its the reduced form (NADPH). NADPH, present in all cells, plays a critical role in protecting proteins and other molecules from oxidative damage (25) (Figure 1.4). This function is especially vital in red blood cells as efficient oxygen transporters, which are susceptible to damage from continuous production of oxygen radicals during methemoglobin formation (26).

The extremely reactive oxygen radicals either spontaneously degrade or undergo transformation into highly harmful hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (SOD). The process of converting H_2O_2 to H_2O is catalyzed by the enzymes glutathione peroxidase (GSHPX) and catalase (27). Both enzymes require NADPH to operate properly. It is a structural part of the enzyme catalase and a substrate for the enzyme glutathione reductase, which restores reduced glutathione (GSH) after its conversion to oxidized glutathione (GSSG) in the reaction catalyzed by glutathione peroxidase (GSHPX (28). As shown in Figure 1.4

Figure 1.4

G6PD and the Glutathione (GSH) Cycle (29)



Source: Bonsu, Beaudet and Sly. 2013 G6PD and the Glutathione (GSH) Cycle,found in Molecular Basis of Glucouse -6-Phosphate Dehydrogenaze Deficiency in Cape Coast, Ghana. Master thesis. In: Scriver. https://koha.mdc-berlin.de/contents/96-00018-3.pdf (29).

1.4 Classification of the G6PD Enzyme Variants

The G6PD enzyme displays a polymorphic structure and is associated with over 80 distinct mutations (30). Based on their clinical presentation and residual enzymatic activity, G6PD variations can be categorized into 5 classes. Depending on where the mutations are located, distinct G6PD variations result in varying degrees of enzyme insufficiency. According to biochemical characterisation, some G6PD variations have an impact on catalytic efficiency, while others have a major impact on protein stability (31). Class II variants like G6PD Canton (Arg459Leu), linked to severe enzyme deficiency, are also found to affect protein stability. Based on the three-dimensional structure, Class I G6PD variants that cause severe enzyme deficiency have amino acid substitutions clustered near the NADP+ binding site or the dimer interface, disrupting the structural stability of G6PD.(32). The consequences of various mutations on G6PD activity are not entirely known, despite the fact that the impact of a single mutation on protein function has been amply documented. Generally speaking, enzyme deficits brought on by double and triple mutations are more severe than those caused by single mutations. About ten times less enzyme activity was present in G6PD-deficient people with the double mutation than in those with the single mutation. Important details about

the activity of the enzyme (efficiency of NADPH synthesis), which represents the enzyme's reaction to oxidative stress, should be revealed by the thorough biochemical characterization of G6PD variations. It is critical to comprehend how various mutations result in variable degrees of enzyme insufficiency. Furthermore, the information helps to clarify the molecular reasons behind the enzyme deficiency seen in G6PD-deficient people. Unfortunately, not every variation of G6PD has been fully described (32).

The most common type of G6PD variation involves missense point mutations, which lead to amino acid substitutions. Large deletions and rearrangements have not been identified, while small in-frame deletions are rare. Mutations that induce the more severe phenotype associated with Chronic Non Spherocytic Hemolytic Anemia (CNSHA) tend to cluster in exons 6, 10, and 13. These exons encode the regions responsible for substrate binding, the dimer interface, and the structural $NADP^+$ site, respectively (30).

230 G6PD variants with known mutations exist at this time. The majority of them are minor in-frame deletions (which result in the loss of one or a few amino acids) or missense mutations (which each cause the replacement of a single amino acid). The result is a qualitatively aberrant G6PD protein with some residual enzyme activity. There are noticeably no frame-shift mutations, which would produce no enzyme activity, and just one nonsense mutation (G6PD Georgia), which would provide the similar effect, has been discovered in a heterozygote. The idea that total loss of G6PD activity is fatal is supported by these data (32).

Certain variants have two cis mutations: the most well-known is G6PD A-, where a codon 126 mutation (N126D), which does not result in G6PD deficiency, coexists with one of three additional mutations (M68V, R227L, L323P). The reason of G6PD insufficiency is the result of the two mutations together. The ideal test for G6PD is to detect an enzyme deficit because every variant that is enzyme-deficient has the potential to induce clinical manifestation. Although algorithms that predict the pathogenicity of variants have gained popularity, they are not as reliable in this case (30).

Among the numerous variations of the G6PD enzyme, the standard form is referred to as G6PD B. It is the most prevalent variant across all population groups studied. Another common variant is G6PD A^+, found in 20-30% of black Africans, which does

not cause hemolysis and exhibits typical catalytic characteristics. G6PD A^+ differs from G6PD B in terms of its faster electrophoretic mobility. Another frequently observed variant, A^-, is associated with primaquine sensitivity in black individuals. It occurs in 10-15% of African Americans as well as in Western and Central Africa, and is the most prevalent form linked to hemolysis (10).

G6PD Mediterranean is the most prevalent abnormal variant seen in individuals of Mediterranean origin. It shares the same electrophoretic mobility as G6PD B but is characterized by diminished catalytic activity and acute hemolysis. Canton G6PD is the most common variation in Asians, and it exhibits similar biochemical characteristics to G6PD Mediterranean (31). G6PD Mediterranean is the model for a more severe enzyme deficit that, although rare, does not result in anemia. Prior to the discovery that leukocyte and platelet contamination was primarily to blame, it was thought that patients with the G6PD Mediterranean variant had a residual 1 or 2 percent of the enzyme in their red blood cells. Given that there is hardly any enzyme activity in red blood cells, it is amazing that red cell lifespans in G6PD Mediterranean individuals are normal or almost normal.The leftover enzyme may have exceptionally advantageous kinetic characteristics, which could explain this (10). The remaining enzyme may have exceptionally favorable kinetic characteristics, which could explain this. This type, as its name suggests, is primarily prevalent in the Mediterranean region, though it can be found elsewhere in the world, such as the Indian subcontinent . (31).

G6PD variations were classified, by the World Health Organization (WHO), into five groups based on the level of enzyme activity as shown on Table 1.1 (32).

Table 1.1

G6PD Variants Classification According to Enzyme Activity

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

Data derived from World Health Organization. 1989. (32)

Class I G6PD-deficient variations include those that induce congenital nonspherocytic hemolytic anemia (CNSHA), but class II or class III G6PD-deficient variations are those that do not, depending on the extent to which red cell enzyme activity is decreased. The line differentiating classes II and III is no longer clear and is likely no longer beneficial. Class IV variations show typical behavior. Since an earlier investigation on G6PD (33) no variants with increased activity have been found, with Class V being designated for them.

While a minority of CNSHA individuals have a rare, sporadic, or severe G6PD deficiency, which corresponds to class I, the majority of G6PD-deficient individuals have mild, simple, or common G6PD deficiency, which corresponds to categories II or III (32). This is due to the fact that most G6PD-deficient individuals remain asymptomatic. A - and Med are common alleles that cause moderate to mild severe deficiency and they belong to class II and III. Patients affected by the A + and B variants fall under class IV and class V. Most people affected by the deficiency fall in Class II and III, like moderate deficiency and have fewer problems

1.5 Clinical Manifestations of G6PD Deficiency

Most G6PD-deficient people are asymptomatic their entire lives and are unaware of their condition. The disease typically presents as acute hemolysis, which typically develops when red blood cells undergo oxidative stress caused on by substances like medicines, infections, or eating fava beans. Life expectancy, quality of life, or activity levels of those with G6PD deficiency do not appear to be impacted. (34). The exact sequence of events after an exogenous trigger factor is present is uncertain. Both of these factors could play a role in the precise mechanism by which enhanced sensitivity to oxidative damage results in hemolysis. In G6PD deficiency, acute hemolysis can have a variety of causes, but it is always clinically evident as fatigue, back pain, anemia, and jaundice (35). The condition is identified by elevated levels of reticulocytosis, lactate dehydrogenase, and unconjugated bilirubin. The most common clinical manifestations of G6PD deficiency present as Neonatal Jaundice, Acute hemolytic anemia (AHA), Favism, Chronic Non Spherocytic Hemolytic Anemia (CNSHA), Drug and infection induced Hemolytic Anemia (13).

1.5.1 Neonatal Jaundice

The most prevalent clinical symptom seen in individuals having G6PD deficiency is neonatal jaundice. According to studies, newborn jaundice affects around one-third of infants with G6PD deficiency (36). If severe neonatal jaundice is left untreated, it can lead to neurodevelopmental abnormalities and kernicterus, a well-documented cause of mortality. In newborns with G6PD deficiency, jaundice develops due to spontaneous hemolysis, hemolysis triggered by certain oxidant drugs and illnesses, or impaired bilirubin glucuronidation in hepatocytes (37).

Bilirubin binds to albumin in the bloodstream and is transported to the liver, where it undergoes enzymatic binding with glucuronic acid to form mono and diglucuronides. These conjugated bilirubin compounds are then excreted into the bile and the gastrointestinal tract (38). In newborns, a significant portion of the conjugated bilirubin in the gut is converted back to unconjugated bilirubin through hydrolysis and reabsorbed into the bloodstream via the enterohepatic circulation. This process places additional strain on the liver. Neonates typically experience some degree of hyperbilirubinemia as they eliminate bilirubin transferred from the placenta. However, if neonatal jaundice, characterized by abnormally high bilirubin levels, persists without treatment, it can lead to chronic bilirubin encephalopathy or kernicterus, which may result in cognitive impairment in the child (15).

Numerous investigations have demonstrated that decreased bilirubin clearance plays a key part in jaundice in infants lacking in G6PD, rather than hemolysis being the main reason (20). Nonetheless, some studies have hypothesized that neonatal jaundice in newborns with G6PD deficiency could occur either as a result of an acute hemolysis or as an amplification of a physiological process, particularly in the presence of oxidants(38). It is significant to remember that the balance between bilirubin production and excretion is represented by the total serum bilirubin levels at any given time. Compared to another neonate with low bilirubin conjugation or excretion ability, a neonate with acute hemolysis leading to high bilirubin generation and an excellent liver elimination ability may not experience substantial symptoms of hyperbilirubinaemia. Indirect hyperbilirubinaemia without hemolysis is caused by a mutation in the promoter area of the uridine diphospho glucuronosyl transferase gene, which has been demonstrated to occur together with G6PD deficiency (16). While some researchers

have suggested that decreased activity of the G6PD enzyme in the liver hepatocytes could result in a conjugation failure, it is also likely that hyperbilirubinaemia in G6PD deficient newborns arises from this source. However, severe newborn hyperbilirubinaemia due to G6PD deficiency may also occur in conjunction with other neonatal jaundice causes, such as mother-child ABO and Rh incompatibility, exacerbating pre-existing jaundice (37).

1.5.2 Acute Hemolytic Anemia (AHA)

Individuals with G6PD deficiency are susceptible to developing acute hemolytic anemia (AHA) when exposed to three main triggers: fava beans, infections, and certain medications. The severity of anemia observed can vary based on the type of oxidant stress and G6PD mutation present, with the Mediterranean variant often producing more severe symptoms than the A- variant (36).

Patients with AHA may have a diagnostic issue that, once treated, may not require any particular therapy; nevertheless, in other circumstances, AHA may be a medical emergency that requires prompt attention. An urgent blood transfusion is necessary in such circumstances and might even save a person's life. Hemodialysis may be recommended if there is acute renal failure (39).

1.5.3 Favism

Individuals with G6PD deficiency are asymptomatic until they are exposed to particular triggers, making identification difficult (28). Ingestion of fava beans, systemic illnesses, or exposure to specific drugs are typical triggers. G6PD deficiency can also lead to the development of chronic non-spherocytic hemolytic anemia (CNSHA), although this is a rare occurrence (40). G6PD deficiency was shown to be associated with a genetic susceptibility to favism, which was initially identified primarily in Greece, Italy, and the Middle East but is currently present in over 30 countries, including North Africa, Thailand, and China (41).

Favism, typically caused by the consumption of fresh beans, exhibits a peak season in April and May, coinciding with the harvest period. However, hemolytic crises can also be triggered by dried, frozen, or pollen forms of fava beans, and breastfeeding newborns may experience hemolysis if their mothers have consumed fava beans (42). The toxic components of fava beans, identified as divicine and isouramil, two pyrimidine

aglycones, can induce metabolic disruptions leading to hemolysis when their levels exceed the capacity of G6PD-deficient cells to generate glutathione (43). While only a small number of G6PD-deficient individuals, particularly those with G6PD Mediterranean and Canton variants in the Middle East and Asia, are sensitive to fava beans, others are rarely affected. The condition primarily affects male children between the ages of one and five, although females with severe enzyme deficiencies can also be affected (44).

Clinical signs of favism include pallor, jaundice, hematuria, and severe hemolytic anemia, which manifest 24-48 hours after the consumption of fava beans. In rare cases, a blood transfusion may be necessary during severe hemolytic episodes (45).

1.5.4 Chronic Nonspherocytic Hemolytic Anemia (CNSHA)

Chronic Non Spherocytic Hemolytic Anemia (CNSHA) is an infrequent condition typically associated with sporadic gene mutations, leading to hemolysis during normal red blood cell metabolism (46). The clinical presentation of CNSHA shares similarities with Hereditary Spherocytosis (HS), including symptoms like jaundice and gallstones, with varying degrees of severity.

Any triggering agents capable of inducing acute or chronic hemolysis in G6PD-deficient individuals can also cause hemolysis in patients with CNSHA. The treatment for this anemia is the same as that given for CNSHA of various aetiologias: monitoring, regular folic acid intake, red cell blood transfusions when necessary, and iron chelators if iron overload occurs. Splenectomy is typically performed in severe patients and can occasionally lead to higher hemoglobin levels, despite the fact that some patients may not respond to it (47). Prenatal diagnosis, genetic counseling, and mutation identification are suggested for severe cases.

1.5.5 Drug Induced Hemolytic Anemia

The diagnosis of G6PD deficiency is often established when hemolysis occurs in patients receiving primaquine, which has also led to the identification of other medications associated with acute hemolysis in G6PD-deficient individuals (48). While most children with G6PD deficiency are usually asymptomatic, certain drugs can cause significant clinical manifestations. However, pinpointing a specific medication in this context can be challenging due to interindividual differences in pharmacokinetics,

where a drug considered safe for one patient may not be safe for another. Additionally, a patient may be taking multiple medications with potential oxidant effects, and hemolysis typically begins within 2 to 3 days of initiating the drug, followed by the development and worsening of anemia from the 7th to the 8th day. If the medication is discontinued, hemoglobin concentrations tend to increase from the 9th to the 10th day (49).

Antibiotics such as Ciprofloxacin, Nalidixic acid, Ofloxacin, and Chloramphenicol, as well as Primaquine (an antimalarial medication), Methylene blue (an antimethemoglobinemic agent), and Dapsone (an antimycobacterial agent), have all been linked to hemolysis (50). Aspirin and acetaminophen are two widely used medications that are generally considered potentially hemolytic but are actually safe to give to people with G6PD deficiency. It is likely true that large aspirin doses induce a modest reduction in the lifespan of red blood cells in these people. However, a number of studies confirm that hemolysis, when it happens, is almost certainly caused by fever and infection in people with common variations of G6PD with deficient activity using aspirin at regular doses (36).

Hemolysis can also happen after exposure to other substances, such as naphthalene, which is frequently consumed or inhaled while using mothballs or herbal remedies like henna. Studies *in vivo* and *in vitro* indicate that, although normal ascorbic acid levels protect against oxidative stress, ascorbic acid in excessive amounts reduces the function and survival of G6PD-deficient RBCs. Furthermore, acute hemolysis occurred in G6PD-deficient individuals receiving high doses of ascorbic acid. By giving an electron to oxidizing radicals at physiological amounts, ascorbic acid serves as an antioxidant. Pharmacologic quantities of ascorbic acid, however, cause damage to G6PD-deficient RBCs by encouraging the generation of H_2O_2 as a byproduct of cycling between the ascorbate radical and the ionized ascorbic acid (51).

1.5.6 Infection Induced Hemolytic Anemia

Infection is thought to be the most common cause of hemolysis in persons with G6PD deficiency. Hepatitis A and B viruses, cytomegalovirus pneumonia, and typhoid fever are among infections that can cause hemolysis (52). Concurrent drug usage, liver function, and age can all have an impact on the severity of hemolysis.

Both hepatitis and hemolysis can elevate total bilirubin levels, which may sometimes lead to diagnostic confusion. Timely transfusions can significantly improve the clinical course of severe hemolysis. Viral hepatitis, in combination with G6PD deficiency, can potentially result in acute renal failure, with contributing factors including renal ischemia-induced acute tubular necrosis and tubular obstruction caused by hemoglobin casts (53). Hemodialysis may be required for certain patients experiencing hemolysis. However, acute renal failure is uncommon in children with this condition (54). After severe trauma, G6PD-deficient individuals may be at higher risk of sepsis and once infected may have a more severe clinical course (52).

1.6 G6PD Deficiency and Malaria

Malaria, an acute febrile illness, is primarily caused by Plasmodium parasites transmitted to humans through Anopheles mosquitoes. As of 2020, approximately half of the world's population faced the risk of malaria (55). There exists a notable geographical association between the prevalence of G6PD deficiency and the endemicity of malaria. It is estimated that around 400 million individuals worldwide, which accounts for approximately 8% of the population residing in malaria-endemic regions, are affected by G6PD deficiency (56).

It has been proposed that blood diseases such as G6PD deficiency may offer protection against some malarial infections. Inadequate antioxidant defenses lead to RBC stress and damage, which makes them vulnerable to phagocytosis-mediated destruction. It has been proposed that RBCs infected with parasites belong to this category and are eliminated by the body, severing the malaria parasites' intra-erythrocytic growth chain. Better protection against P. falciparum is possible when the infected and damaged red blood cells are removed. This is possibly linked to certain red blood cell illnesses such sickle cell, hemoglobin C, and G6PD deficiency (55).

Many studies have found a strong geographic correlation between the prevalence of G6PD deficiency and malaria endemicity, which is similar to the well-known situation with Hb S. Because of this, the two abnormalities frequently coexist, raising the question of whether G6PD deficiency exacerbates sickle cell anemia. The effect is negligible overall. Macrophages recognize G6PD-deficient red blood cells as abnormal early in the Plasmodium falciparum infection process and eliminate them, which

appears to be a very likely defense mechanism. Numerous recent investigations have supported the original theory that heterozygous females, like in classic balanced polymorphisms, have the privilege to defend. G6PD deficiency may provide protection against cerebral malaria but not malaria accompanied by severe anemia, while this conclusion has been contested as potentially resulting from "collider bias." The idea that having some G6PD-deficient red cells (as in heterozygous females) is more protective than having all G6PD-deficient red cells (as in hemizygous men) continues to seem paradoxical. It still requires a strong mechanical explanation (56).

Malaria is often addressed through the utilization of 8-aminoquinoline medications. Among these, drugs like primaquine (PQ) are employed to eliminate dormant liver stage parasites. However, in individuals with G6PD deficiency, these drugs can lead to varying degrees of hemolysis, ranging from mild to severe. It has been determined that the mechanism by which primaquine damages cells is by serving as an electron donor for oxygen, which is bonded to hemoglobin iron in its elemental state. By only supplying one electron to the bonded oxygen, iron in its ferrous state prevents the production of peroxide and free radicals. Primaquine enhances the oxidative process in red blood cells by providing the second electron, which causes oxidative damage (36) and raising membrane ATPase activity. Red blood cells will undergo morphological changes as a result of ATP hydrolysis, which will ultimately result in hemolysis (50).

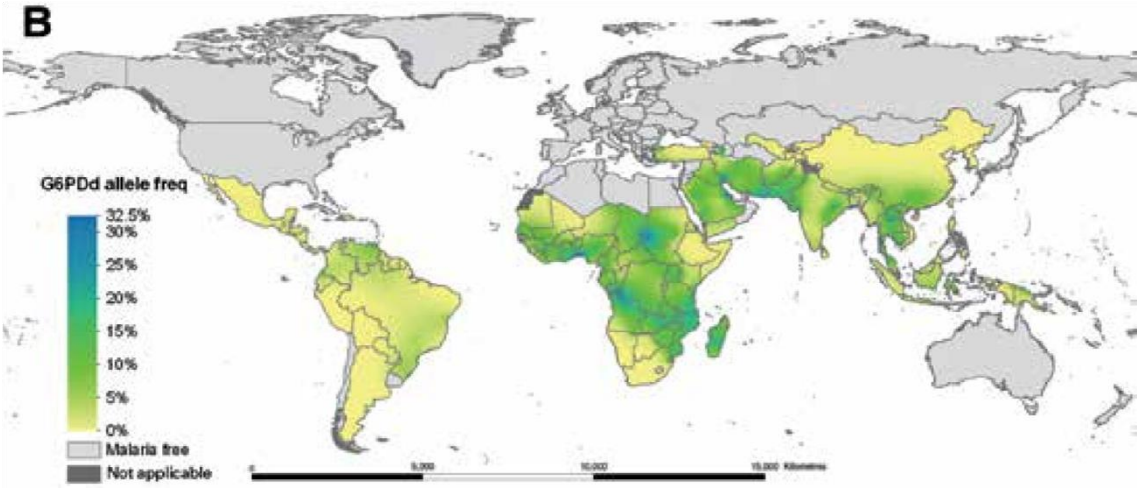
The risk of primaquine-induced hemolysis is influenced by both the dosage administered and the extent of G6PD deficiency (57). The objective of G6PD testing is to ascertain whether a patient can safely receive primaquine. Typically, G6PD activity levels in the blood-cell population of around 30% or higher, in comparison to normal values, are considered to carry an acceptable risk when using therapeutic doses of primaquine (55).

1.6.1 Estimated G6PD-Defeicient Population by WHO Region

G6PD deficiency is a condition characterized by an estimated allele frequency of 8.0% in countries where malaria is prevalent. The highest frequencies of G6PD deficiency alleles have been observed in sub-Saharan Africa and the Arabian Peninsula, reaching a peak of 32.5% (Figure 1.5).

Figure 1.5

Estimated G6PD Deficiency Allele Frequencies



Note: Howes et al 2013. Estimated G6PD Deficiency Allele Frequencies ,found in Spatial distribution of G6PD deficiency variants across malaria-endemicAvailable from: http://dx.doi.org/10.1186/1475-2875-12-418 (58)

In general, central Asia and South-East Asia exhibit lower allele frequencies, rarely surpassing 20%. However, the majority of G6PD-deficient individuals come from Asian countries due to their larger population sizes. The Americas have the lowest estimated allele frequencies, with G6PD deficiency being virtually absent in Argentina, Bolivia, Costa Rica, northern Mexico, and Peru (Table 1.2) (59).

Table 1.2

Estimated G6PD-Deficient Population Across WHO Regions

% of G6PD Allele Frequency	WHO Region
12.1	Africa
2.6	Americas
9.0	Eastern Mediterranean
2.9	Europe
5.8	South-East Asia
9.2	Western Pacific
8.0	World

1.7 Diagnosis of G6PD Deficiency

To prevent hemolytic crises, effective management of G6PD deficiency necessitates the use of straightforward and accurate enzyme testing. The diagnosis of G6PD deficiency requires direct confirmation of reduced enzyme activity in red blood cells, which can be achieved through either a screening test or a quantitative assay. The G6PD enzyme test

measures the rate of NADPH generation from NADP+ in red cells, and it can be performed using a sequestrene (EDTA) or heparinized blood sample (48).

The identification of G6PD deficiency in individuals is influenced by various genetic variations, the presence of heterozygosity in the disorder, and elevated enzyme levels in young red blood cells (60) .Timing is crucial for reliable test results in order to prevent the masking of an enzyme deficiency. For instance, individuals experiencing acute hemolysis who are tested for G6PD deficiency may yield falsely negative results. This can occur because older erythrocytes, which exhibit higher enzyme deficiency, have already undergone hemolysis, while young erythrocytes and reticulocytes retain normal or near-normal enzyme activity (46).

Many different disease processes commonly exhibit clinical symptoms, which is analogous to the clinical manifestation of G6PD deficiency, which is primarily characterized by hemolytic anemia. Therefore, the following illnesses need to be considered in the differential diagnosis: Spherocytosis that is inherited, Sickle cell anemia, Thalassemia, acquired autoimmune hemolytic anemia, Pyruvate kinase deficiency, hemolytic anemia caused by cold antibodies, and abnormalities of bilirubin conjugation (36).

1.7.1 Screening Tests for the Detection of G6PD Deficiency

For examining a significant number of samples, screening tests can be quite beneficial. They are also appropriate for diagnostic reasons in stable individuals, but not in patients with other medical disorders or in the post-hemolytic phase (18). In addition, it is not expected that they will be able to detect all heterozygotes.

The ideal screening test shouldn't give "false-negative" findings (i.e., it shouldn't mistakenly label a G6PD-deficient person as normal), but it could sometimes give "false-positive" results (i.e., a G6PD-normal subject might be mistakenly labeled as having G6PD deficiency) (61). Ideally, the spectrophotometric test for each patient identified as G6PD-deficient by screening should be confirmed.

1.7.1.1 Fluorescent Spot test (FS test)

The Fluorescent Spot test, widely used for semi-quantitative analysis, proved being highly beneficial in field studies requiring rapid screening of a large number of patients (62). In this test, known as the FS test, NADPH produced by G6PD present in a lysate of blood cells emits fluorescence when exposed to long-wave UV light. G6PD deficiency, which hampers the production of sufficient NADPH, leads to the absence of fluorescence. The fluorescence intensity is categorized into two groups: normal activity (bright fluorescence) and deficiency (no fluorescence) (63). The FS test offers simplicity and sensitivity, although its drawback lies in the low cutoff point, making it less effective in identifying female heterozygotes (64).

1.7.1.2 Methemoglobin Reduction Test

The test employed is a qualitative screening method that indirectly detects the concentration of NADPH by measuring the levels of reduced methemoglobin generated through NADPH oxidation (50). In this procedure, hemoglobin is converted to methemoglobin by sodium nitrite. Methemoglobin persists in the absence of methylene blue, but when samples are incubated with methylene blue, the pentose phosphate pathway is activated in individuals with normal G6PD levels. During the incubation stage, the methemoglobin is reduced. However, in G6PD deficient patients, the pentose phosphate pathway is blocked, preventing this reduction (65).

Sequence-specific oligonucleotide probes (SSOPs) have been found to be specific and comparable to the PCR restriction fragment length polymorphisms (RFLP) approach in terms of both sensitivity and specificity for Single Nucleotide Polymorphisms (SNPs) assessed by ELISA. These tests can differentiate between G6PD-deficient and normal people; however, they are not quantitatively accurate. G6PD activity quantitation by the spectrophotometric assay in steady state is the ideal follow-up test for a conclusive diagnosis where a screening test indicates deficiency or is doubtful. Hemizygous deficient males and homozygous deficient females can be identified with a G6PD activity of about 30% of normal (63). The rate of NADPH formation in µmol per min per gm Hb is measured by a spectrophotometer at a wavelength of 340 nm at 30°C. Quantitative measurements are carried out by adding a precise amount of hemolyzate to an assay mixture containing G6PD and NADP. The assay is expressed as G6PD U/RBC

or U/g Hb, and the blood sample used for the test must also be used to evaluate the Hb concentration or RBC count (64).

Some other qualitative screening tests are also available including the cresyl blue dye decolorization tests. In which this tests are based on staining the Red Blood Cells thus the G6PD deficient RBCs will satin poorly and can be identified microscopically(66).

1.7.2 Quantitative G6PD Assay

The quantitative spectrophotometric analysis of the rate of NADPH production from NADP is used to estimate enzyme activity and make diagnosis of G6PD deficiency. A known quantity of hemolysate is added to an assay mixture, which includes a co-factor (NADP) and a substrate (glucose-6-phosphate), and then the rate of NADPH production is subsequently measured spectrophotometrically (63).

There are many commercial test kits for this test, which work on a similar principal. Blood can be drawn into ethylenediamine tetra-acetic acid (EDTA), citrate phosphate dextrose (CPD), acid citrate dextrose (ACD), and lithium heparin to be used in the G6PD assay. However, it should be noted that some of the enzyme deficient variants lose their activity rapidly, making deficiency appear more severe than it should. The level of enzyme activity increases in reticulocytes and decreases with red cell aging.

1.7.3 Polymerase Chain Reaction (PCR)

A technique for amplified hybridization that uses enzymatic synthesis to create millions of identical copies of the target DNA. The test DNA sample, which is made up of lysed cells or tissue that has been enzymatically digested with RNAse and proteinases before being extracted, is present in the test reaction mixture (66). Two oligonucleotide primers (probes) matching opposing strands of DNA are used to amplify the intervening segment of the target DNA more than a million times using a thermostable DNA polymerase and nucleotide triphosphates after the target DNA is heated to denature it and the strands are separated. At least 15-30 times are required to complete the process of generating DNA from primers in successive cycles while heating the chain to separate it in between cycles. The amplified target DNA sequences, also called as amplicons, are analyzed using directly labeled probes, Southern blots, or gel electrophoresis (67).

Since DNA samples are far more stable than the enzyme in blood samples and only require a very little sample volume to make a diagnosis, they may be used for prenatal diagnosis and family research, which is the major benefit of this sort of technique. The additional benefit of utilizing PCR to identify G6PD deficiency is how fast, simple, and sensitive it is. It is not influenced by blood transfusions, reticulocytosis with a normal enzyme test level, or the ease with which heterozygous females may be found with this method. However, because of its high cost, the absence of necessary technical skills, and the ease with which samples can get contaminated, PCR has limits in settings with low resources (66).

1.8 Age of Red Cells and Selective Hemolysis

The decline of G6PD with the aging of red blood cells means that any condition associated with an increase in reticulocyte count will lead to elevated G6PD activity (68). Consequently, if an individual has genetically normal G6PD, hemolysis will cause red cell G6PD activity to rise over the range of normal. This will effectively rule out G6PD deficiency, thus, it has no effect on the diagnosis. However, red cell G6PD may now be elevated to the point of being close to or even within the normal range if the subject is genetically G6PD deficient. As a result, the patient may be falsely labeled as G6PD normal (although the level may have been low at the beginning of the attack). Therefore, after a hemolytic attack, there is a risk of misdiagnosis because older cells have been destroyed and young cells have been rapidly released into the peripheral circulation by the marrow reaction (68). Even though the reticulocyte count is normal, the mean red cell age can be much younger than normal since reticulocytes grow into morphologically "mature" erythrocytes within 1 to 2 days. It should be noted that the reticulocyte count is not a sensitive measure of mean red cell age, despite the fact that it provides an informative warning to prevent this kind of mistake (18).

1.9 G6PD Deficiency in Diabetes, cancer and CVD

Hemolysis is thought to be a side effect of diabetic ketoacidosis (DKA) in people with G6PD deficiencies. One possibility is that G6PD-deficient RBCs are more susceptible to oxidative stress because diabetics have lower blood GSH levels (69). However, another study found that DKA was not a substantial contributor to hemolysis in G6PD-deficient individuals since the majority of these cases also had potential drug or infection causes (70). Significantly, hemolysis frequently occurs in DKA patients only

after blood sugar levels have been brought into the euglycemic range (71). Keeping his blood glucose in the high-normal range prevented further hemolysis in one diabetic patient who experienced repeated bouts of hemolysis with each hospital admission, coinciding with iatrogenic hypoglycemia after receiving insulin injection (70). During hyperglycemia, there is an excess of substrate (particularly, glucose-6-phosphate), allowing for NADPH production even with the reduced G6PD activity in G6PD-deficient individuals. This could be the reason for the stimulation of hemolysis after the return to normoglycemia. When glucose levels return to normal or fall, hemolysis is brought on by the quick decline in the production of NADPH that is brought on by the abrupt drop in glucose. In G6PD-deficient people with DKA, it is crucial to carefully reduce high blood glucose levels and to keep an eye out for hemolysis (72).

Affected people with G6PD deficiency may be more likely to develop diabetes and cardiovascular disease, according to a recent meta-analysis and epidemiological studies. G6PD deficiency is primarily responsible for changing redox homeostasis and the development of Cardio vascular disease (CVD), which predisposes the body to faulty vasodilation, as demonstrated by a number of G6PD deficiency models. Human hemolysis and G6PD deficiency have a well-established relationship, and G6PD's antioxidant properties highlight how it guards against the onset of numerous other diseases, including as diabetes and cardiovascular disease. The primary cause of death and disability in people with diabetes mellitus is still cardiovascular disease (CVD). Heart failure and atherosclerosis are caused by mechanisms that are made worse by diabetes (73). By managing G6PD deficiency with small-molecule activators or GSH precursors to prevent excessive oxidative stress, early intervention can lower the risk of vascular dysfunction and the incidence of CVD.Similarly, screening and counseling for G6PD deficiency in females may close the gender knowledge gap and have an impact on the health of newborns (74).

Research has demonstrated that G6PD expression in tumor cells is elevated when compared to normal cells, and that this expression is linked to the overall prognosis of individuals with tumors. Increased G6PD activity has also been shown in numerous studies to be associated with a number of cancer types, including melanoma, bladder cancer, endometrial carcinoma, prostate cancer, kidney cancer, stomach cancer, cholangiocarcinoma, colon adenocarcinoma, lung cancer, cervical cancer, ovarian

carcinoma, hepatocellular carcinoma (HCC), glioma, pancreatic cancer, and dysplasia(75).

Cancer cells typically prefer PPP because it generates large amounts of NADPH, which is essential for their rapid growth. Redox signaling abnormalities resulting from G6PD deficiency impact cell survival and apoptosis and have been linked to the development and course of various cancer types. In Northern Sardinians, a study examining the impact of G6PD deficiency on several cancer types revealed that the lack of G6PD was negatively correlated with hepatocellular carcinoma, resulting in a 55% reduction in risk irrespective of age and gender. After controlling for age, sex, smoking history, diabetes, and socioeconomic position, upper and lower endoscopy samples from G6PD deficient individuals compared to controls demonstrated a decreased risk for endodermal, gastric, and colorectal cancer. However, the chance of acquiring ectodermal/mesodermal malignancies, which include lung, breast, prostate, and hematological cancers, did not seem to be affected by G6PD deficiency (57).

1.10 Management of G6PD Deficiency

The main method of managing G6PD deficiency focuses on avoiding substances that can cause oxidative stress, such as fava beans and specific medications, in order to prevent hemolysis. However, for this approach to be successful, patients must be knowledgeable about their deficiency, either through experiencing a previous episode of hemolysis or participating in a screening program. It is worth noting that acute hemolysis in individuals with G6PD deficiency is typically short-lived and does not necessitate special care. This is because in healthy individuals, reticulocytes and younger red blood cells typically retain approximately 10% of residual enzyme activity similar to individuals with normal G6PD (70).

In some cases, special transfusions of red blood cells are required for children experiencing acute hemolysis that results in severe anemia. When it comes to neonatal jaundice caused by G6PD deficiency, the treatment approach is similar to that of neonatal jaundice caused by other factors. While splenomegaly can occasionally occur in patients with congenital non-spherocytic hemolytic anemia, splenectomy is typically not beneficial in these cases (36).Due to a G6PD deficiency, gallstones may develop as a consequence of hemolysis. There have been reports of prenatal G6PD deficiency diagnoses, however this method is dubious given the low death and morbidity rates

associated with G6PD deficiency (61). Gene therapy is still a possibility for severe cases of the deficit that are unresponsive to conventional therapies. The specific treatment depends on the concentration of bilirubin in the blood. If the concentration of unconjugated bilirubin is close to or exceeds 150 mol/L, patients are typically given phototherapy to prevent neurological damage. However, if the concentration surpasses 300 mol/L, a blood transfusion may be necessary (76).

The best way to control G6PD deficiency is to stay away from oxidative stressors and the factors that cause them (70). Healthcare professionals should first determine the presence and severity of G6PD deficiency and then get a complete patient history, including information about over-the-counter medication use and dietary practices. Patient education is essential, especially for those with a more severe form of deficiency and a lower level of enzyme activity. This teaching should focus on avoiding triggers and understanding the signs and symptoms of hemolytic anemia. Complications may also be avoided with dietary and lifestyle changes. Oxidative stress markers are raised by excessive alcohol consumption, tobacco use, physical inactivity, and an imbalanced or inadequate diet. Already G6PD-deficient erythrocytes are more vulnerable to oxidative stress, which raises the risk of hemolysis(5). For G6PD deficiency, avoiding oxidative stresses and the factors that cause them is the best course of treatment. Following the diagnosis and assessment of G6PD deficiency, medical practitioners should get a complete medical history, including information about over-the-counter medicine use and dietary practices. Patient education is essential, especially for those with a more severe form of deficiency and lower levels of enzyme activity. This teaching should focus on avoiding triggers and understanding the signs and symptoms of hemolytic anemia. Modifications to diet and lifestyle may also aid in avoiding problems. Oxidative stress markers are elevated by excessive alcohol consumption, tobacco use, physical inactivity, and an imbalanced or inadequate diet.. Already-deficient erythrocytes in G6PD are more vulnerable to oxidative stress, which raises the risk of hemolysis(5).

1.11 Literature Review

The deficiency of G6PD is a public health concern in many countries; the highest prevalence is reported in Africa, southern Europe, the Middle East, Southeast Asia, and Mediterranean countries. Migration and resettlement have an impact on the distribution of this disorder (41). Many studies were performed to estimate the prevalence of G6PD. The deficiency has been reported with prevalence rates ranging from less than 1% in Japan and Northern European to 58% in Kurdish Jews (77) , America (3.4%), Europe (3.9%), the Pacific (2.9%), sub-Saharan Africa (7.5%), the Middle East (6.0%) and Asia (4.7%) (10).

In 2019, a study carried out in Cameroonian, West Africa among 1001 blood donors reported that the prevalence of G6PD deficiency was 7.9 % (78). A study in China showed that the overall prevalence of G6PD deficiency in China was 2.1% at the national level. The top six common mutations were c.1388 G>A, c.1376 G>T, c.95 A>G, c.392 G>T, c.871 G>A and c.1024 C>T, accounting for more than 90% of G6PD deficient alleles. (79).

Shanthaladevi (80) reported that the incidence of G6PD deficiency in India was 0.8 percent in a study of 2005 healthy blood donor samples, another study conducted in India discovered that the Mediterranean variant is more common than the other varieties (81).

A study in the Saudi population screened 2100 healthy male donors for the presence of G6PD, and 100 were found to be deficient (4.76%). Molecular characterization revealed that 6% of the 100 people with G6PD deficiency had the A376G mutation and 2% had the G202A mutation (82).

Another study in the Eastern Province of Saudi Arabia that focused on molecular characterization of G6PD deficiency found that the G6PD Mediterranean mutation is the most common (84%) followed by G6PD A^- (5.8 %) (83). In Jeddah (84), it was reported that the most common variant is Mediterranean with (89.1%), then Aures (10.0%) and G6PD Chatham (0.9%). One more study in Al-Hassa and AlQatif areas of Saudi Arabia showed G6PD Mediterranean as the most frequent variation, with incidence of 45.9% and 36.5% for these two regions, respectively (85).

A study performed among Kurdish populations in Northern Iraq reported a frequency of G6PD deficiency of 10.9%, which is higher than the neighboring Iranian Kurdish population, which reported a frequency of 5.3% (86). The study also found that G6PD Mediterranean and Chatham mutations comprise the vast majority of the deficient variants among Kurdish population (87).

Another study conducted in Jordan found that the frequency of G6PD deficiency was 8.5% in the Jordan Valley and 3.2% in the Amman region. The same research found six different G6PD mutations, with the G6PD Mediterranean mutation (c.563C>T) accounting for 53.3% of the total mutations and being the most common, and the G6PD A^- mutation being more prevalent in Jordan Valley, while other G6PD mutations were found in Amman with a low incidence (88).

In Turkey, a study reported that the frequency of this G6PD deficiency varied between 0.5-11.4% depending upon geographical areas, and revealed that the enzyme deficiency was associated with Mediterranean type of G6PD deficiency B^- (563T) (80%), A^-(376G/202A) type of G6PD deficiency (4%) and G6PD Chatham (2%) (89).

In Qatar, Malik *et al* (90) determined G6PD variants in Qatari's population among 6045 participants and found that the most frequent G6PD-causing variants were p.Ser188Phe (G6PD Mediterranean), p.Asn126Asp (G6PD A^+), p.Val68Met (G6PD Asahi), p.Ala335Thr (G6PD Chatham), and p.Ile48Thr (G6PD Aures) with allele frequencies of 0.0563, 0.0194, 0.00785, 0.0050, and 0.00380, respectively (90).

In 2014, Osman *et al.* studied the mutation that caused G6PD deficiency in Egyptian Favism patients. A total of 100 unrelated Egyptian patients were included in the study, which found that the Mediterranean mutation is the most common mutation in 87.7% of the patients (91).

In Gaza Strip, Sirdah (92) studied the molecular heterogeneity of G6PD deficiency. The study that included 80 Palestinian children with hemolysis, revealed three different G6PD mutations, known as G6PD Mediterranean, G6PD A-, and G6PD Cairo. The two other variations (G6PD Chatham and G6PD Aures), found in Arab and Middle Eastern countries, were not present in the population under study. In addition, the authors described a new mutation known as G6PD Gaza (c.536 G>A; p.Ser179Asn), which was identified in a female as a heterozygous genotype.

1.12 Objectives

To date, there have been no investigations conducted in the West Bank region of Palestine to assess the prevalence of G6PD deficiency. Hence, the purpose of this study is to conduct the initial examination focusing on both the prevalence and molecular characteristics of G6PD deficiency among asymptomatic Palestinian blood donors. The specific objectives of this study were as follows:

1. Assess the prevalence of G6PD deficiency in a random sample of healthy blood bank donors in northern West Bank, Palestine using the Methemoglobin (methylene blue dye) reduction test.
2. Validate the obtained results by determining the G6PD enzyme activity in the samples that exhibit G6PD deficiency as indicated by the Methemoglobin reduction test.
3. Investigate the molecular basis of G6PD deficiency and characterize the specific deficient variants among the donors who have confirmed G6PD deficiency.

1.13 Hypothesis

Prior to this study, no research had been conducted on the prevalence of G6PD deficiency in Palestine. Therefore, the hypothesis for the prevalence of this enzyme deficiency in Palestine, specifically in the northern West Bank, was estimated to be between 2% and 10%. This range was based on earlier studies conducted in neighboring countries. Despite the widespread occurrence of G6PD deficiency among Palestinians, there is limited available information regarding its distribution in the West Bank.

Chapter Two

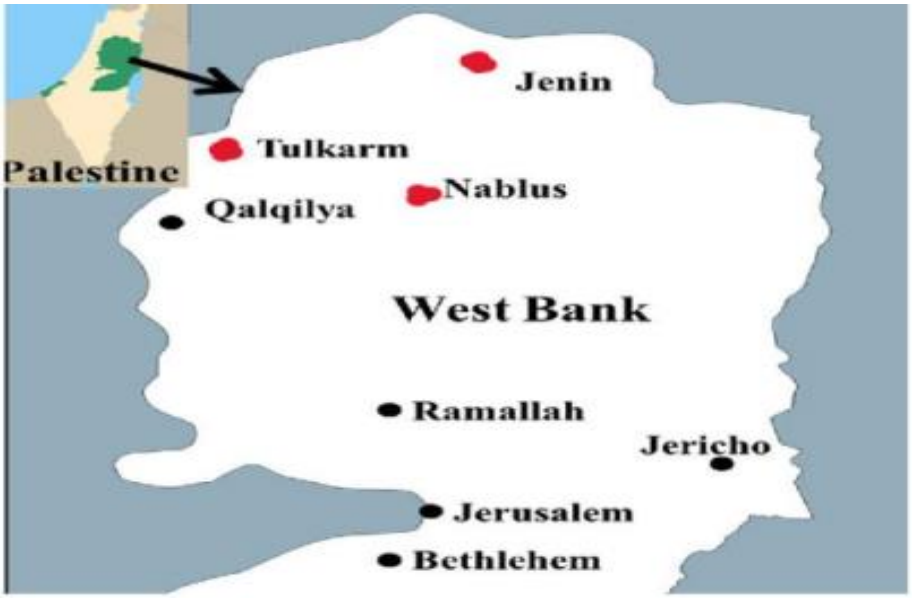
Materials and Methods

2.1 Study Area

This was a multicenter cross-sectional study, that was conducted with the objective of screening for the prevalence of G6PD deficiency among asymptomatic healthy blood donors, in three Governmental hospitals of three major cities (Nablus, Jenin and Tulkarm) (Figure 2.1) in the Northern district of the West Bank, Palestine. The routine work and molecular testing were carried out in the research laboratory, Depart. Of Biology and Biotechnology, An-Najah National university, Palestine.

Figure 2.1

Geographic Distribution of Sample Collection in Northern Westbank, Palestine



2.2 Ethical Approval and Consideration

The study was approved by the Palestinian Ministry of Health (MOH) and the Board or Ethics Committee at An-Najah National University, Nablus, Palestine. The principles of Helsinki Declaration were implemented. Written informed consent was not obtained for this study. All the information collected from the donors was used for scientific research only. Neither identifying information nor samples drawn beyond the standard volume were obtained for this study.

2.3 Sample Collection and Storage

This study was conducted in the period from September 2020 to January 2021. Ten (10 ml) of peripheral blood samples was collected in two tripotassium ethylene-diaminetetraacetate (EDTA K3) tubes, one for routine work and the other for molecular analysis.

Blood samples were taken in the EDTA K3 tubes and were kept at 4°C later on for genomic DNA extraction. Sample collection was performed by a well-trained technician at the 3 hospital centers, preserved in ice and transported at the same day to An-Najah research laboratory for further analysis.

2.3.1 The Inclusion Criteria

Healthy blood male donors, aging 18-40 years, mean 25 ± 5. Body weight > 50kgm, Hemoglobin > 12.5 gm/dl, temperature 37°C and Systolic blood pressure =120-140 mmHg, and Diastolic blood pressure = 80-90 mmHg.

2.3.2 Exclusion Criteria

Donors whose blood was rejected for transfusion due to limitations in age, weight, hemoglobin level, or any other physiological condition and /or increased risk of transfusable infection or malignancies were not included in the study.

2.4 Biochemical Tests

2.4.1 The Methemoglobin Reduction Test

The Methemoglobin Reduction Test (MRtest) was carried out on all blood samples within less than 24 hours from collection. The MRtest involves oxidation of hemoglobin (Hb) to methemoglobin (Meth Hb) by sodium nitrite. Methylene blue dye will result in the reduction of methemoglobin to hemoglobin through oxidative pathway. This reaction will cause a stimulation in the pentose phosphate pathway and the subsequent activation of methemoglobin reductase enzyme. The deficiency or complete absence of the G6PD enzyme activity is determined by the methemoglobin unchanged brown-color following the addition of methylene blue dye (93).

Reagents and Procedure:

1. Normal Saline
2. 0.28M glucose solution
3. 0.18M sodium nitrite solution
4. 0.0004M methylene blue chloride solution.

About 0.5 ml of blood was drawn into three tubes marked as, positive control, negative control and a test (unknown) tube. To the test tube marked as (unknown test), 25µl of sodium nitrite solution and 25µl of methylene blue dye solution were added and mixed well. The negative control tube contains only the blood sample with no additional reagents, while the positive control contains Sodium nitrite solution beside the blood sample (Table 2.1.). Following an incubation period of about 90-120 minutes, 0.5 ml from the solution of each of the three tubes, was transferred to a new clean test tube and diluted with 5ml of distilled water. The color of the unknown test tube was visually compared to the color of positive and negative controls. A clear red color result was interrupted as negative, while a brown one color indicates a positive result with G-6-PD deficiency.

Table 2.1

The Methemoglobin Reduction Test Reagents

Sample	Test	Positive control	Negative control
Blood	0.5ml	0.5ml	0.5ml
Sodium nitrite solution	0.025ml	0.025ml	……..
Methylene blue	0.025ml	…….	……..

2.4.2 Biochemical Measurement of G-6-PD Enzymatic Activity

All blood samples that showed a positive MRtest were further subjected to G6PD enzymatic activity spectrophotometrically at a wavelength of 340nm using biochemical commercial kit (Randox Ltd. Laboratories, Antrim, England), The reaction rate, and therefore the rate of increase in absorbance at 340 nm, usually increases for the first few minutes, then becomes linear and finally slows down as the substrate is used up according to manufacturer instructions. According to the test manufacturer instructions, the absorbance limit of G6PD enzyme activity relies on the reduction of $NADP^+$ product.

$$\text{G6PD} + \text{NADP}^{+} \xrightarrow{\text{G6PDH}} \text{Gluconate-6-P} + \text{NADPH} + \text{H}^{+} \quad \{1\}$$

Briefly, 200µl of blood was washed with 2ml normal saline solution (NaCl 0.9%) and centrifuged at 3000rpm for 10 minutes. Supernatant was discarded with repeated washing for 3 times. The sample was suspended in 500µl digitonin and left to stand for 15 minutes at 4°C. The mixture was then centrifuged and the activity was measured spectrophotometrically at 340nm wavelength. The absorbance was measured for three times after 1, 2 and 3 minutes, respectively. The following equation was used to calculate the G6PD enzyme activity:

mU/erythrocytes per ml of blood =33650* ΔA 340 nm/min. {2}

To calculate G6PD activity as mU/gm of hemoglobin:

= (mU.Erythrocytes per ml × 100) / (Hemoglobin (Hb g/dl) × 1000)

To convert the unit ml to dl, the factor 100 was used.

To convert mU to U, the factor 1000 was used.

Hemoglobin (Hb g/dl) = Hb concentration was determined for each sample.

Reference values = (6.97 to 20.5) U/g Hb (37°C).

2.5 Molecular Analysis

2.5.1 Blood Collection and Genomic DNA Extraction

Three (3µl) of EDTA K3 blood were drawn from each donor, and genomic DNA was extracted using (Nucleospin, Duren/Germany, MACHERY nAGEL) Blood DNA extraction mini kit protocol, according to manufacturer's recommendations and kept at −20 °C until analyzed. Briefly, the procedure was as follow: The whole blood was centrifuged for 15 minutes at 1500 rpm to separate the Buffy coat for maximal DNA yield. About 200-µL blood Buffy coat sample was added to 30µL protease K, into sterile eppendorff tube. 200 µL of B3 buffer was added to the mixture, and vortexed for 2 minutes vigorously. The mixture was incubated for 30 minutes at 70 ^{o}C with vortex each 10 minutes. 210 µL of absolute ethanol (96%) was added to the mixture and mixed well, the lysate was incubated for 5 minutes at room temperature (15–25°C). The lysate was then transfered into the Nucleospin MinElute column and centrifuged for 1 minute

at 11000 rpm. After that the MinElute column was washed twice with the provided washing buffers BW and B5 respectivily, and centrifuged for 1 minute at 110000 rpm. Finally, DNA was eluted into a sterile eppendorff tube with 70µL of preheated Buffer BE provided in the kit. The DNA was kept at -20 °C until analyzed.

2.5.2 DNA Quantification and Purity Assessment

The concentrations and quality (260/280) of DNA samples were analyzed spectrophotometrically by a Nano Drop 2000 analyzer (IMPLEN, Germany). About 1µl of Buffer BE was used as a blank and then 1µl of DNA was checked. The ratio 260/280 was calculated to show the amount of DNA to protein which should be > 1.6 to be consider satisfactory for analysis.

2.5.3 Polymerase Chain Reaction

Genotyping was performed for all samples using polymerase chain reaction (PCR). Genomic DNA was amplified using the lyophilized master mix kit (PROMEGS, MADISON, WI USA). The master mix kit consists of hot-start Taq DNA polymerase, $MgSO_4$, dNTPS and a loading dye buffer. The primers used in this study were described earlier by (89), as shown in (Table 2.2).

PCR primer sequences are indicated from 5′ to 3′ end. Primer sequences were checked for specificity using NCBI Primer-BLAST and UCSC In-Silico PCR (http://www. genome.ucsc.edu). Bio-Rad Thermal Cycler (T100™, Foster City, California, USA) was used for DNA amplification.

Table 2.2

PCR Primer Sequences used for Amplification of G6PD Exons

Primer	Sequence 5` ➔ 3`	Amplicon size (bp)
Exons	F: CAG CGG CAG CGG GTA TG	1123
1+2	R: GGC CCT GCA ACA ATT AGT TGG	
Exons	F: CAC CAA GGG TGG AGG ATG ATG	1076
3+4+5	R: AGA GTG GTG GGA GCA CTG	
Exons	F: CTG GGA GGG CGT CTG AAT G	633
6+7	R: GCT CTG CCA CCC TGT GC	
Exons	F: GCC CTT GAA CCA GGT GAA CA	853
8+9	R: TCC AGT GCC CGC ACA CAG	
Exons	F: CAC TGG TCC ACA CCC TGA GA	956
10+11+12+13	R: GTG CAG GTG AGG TCA AT	

The PCR protocol was as follows: a total volume of 50µL containing, 25µl Master mix, 1µl forward primer (10µM), 1µl reverse primer (10µM), 21µl nuclease-free water and 2µl Genomic extracted DNA.

PCR cycling conditions were: An initial denaturation step at 94°C for 5 minutes, and 35 cycles of the following: denaturation 94°C for 40 sec, annealing 57°C for 60 sec, and extension 72°C for 60 sec. A final extension step of 72°C for 5 min to complete the elongation processes.

The PCR products were separated by electrophoresis on a 1.7% agarose gel and visualized by Gel Red Biotium USA staining.

2.5.4 Agarose Gel Electrophoresis

Amplified PCR products were analyzed in 1.7% agarose (Sigma, Deisenhofen, Germany) in 1x TBA buffer (Promega, Madison, USA). Agarose gel electrophoresis was performed using horizontal electrophoresis unit (MIDI Horizontal "HU13" SCIE-PLAS, Ltd, England) at 85V for 90 minutes. PCR amplicons were visualized using ultraviolet transilluminator documentation system (Uvitec, Cambridge, UK) and compared with 100bp DNA ladder (Ready to use "RTU" Gene direx, Inc. USA. Cat no. DM001-R500) (0.5µg/5µl/well).

2.5.5 DNA Purification

Purification of PCR amplicon products to remove contaminated nucleotides, non-reactive primers and primer dimers (Brisco et al.), was performed using the DNA clean up commercial kit (NucleoSpin Gel and PCR clean up, MACHEREY-NAGEL, DUREN, Germany) following manufacturer instructions. Briefly, one volume of sample was mixed with 2 volumes of NTI buffer in the DNA binding step. The mixture was transferred to the nucleospin column, centrifuged at 11000rpm for 30 sec. After that the nucleospin column was washed twice with the provided washing NT3 buffer, followed by drying of the spin column to prevent enzymatic reaction inhibition by residual ethanol. Finally, DNA was eluted into a sterile eppendorff tube using NE buffer provided in the kit, to be used later on for DNA sequencing.

2.5.6 DNA Sequencing

The PCR products of G-6-PD samples were cleaned up by mixing 5µL of PCR product with 1µL of clean up reagent Eppic FAST (A&A biotechnology) and incubated for 15 mins at 37℃.

Sangers sequencing was performed using the BigDye™ Direct Cycle Sequencing Kit (Thermo Fisher scientific, USA) and run in the applied Biosystem 3500 Genetic Analyzer. Nucleotide variations were examined using *Finch TV* chromatogram viewer. DNA sequencing was performed at the Arab American University research laboratories in Ramallah. The sequence results were first analyzed visually and then using the suitable BLAST Bioinformatics tool.

2.6 Materials

Table 2.3

List of Materials and Instruments used in the Study

Chemicals and kits	
Item	Manufacturer/country
EDTA tubes	Greiner bio-one. UK
Sodium nitrite + glucose	Sigma-Aldrich
Methylene blue chloride solution	Sigma-Aldrich
Gel Red	Biotium,USA
G6PD kit	Randox Laboratories, Ltd., Antrim, UK
DNA extraction kits from blood	Nucleospin Blood, Genomic DNA from Blood). Duren, Germany, MACHEREY-NAGEL.
lyophilized PCR master mix	PROMEGS, Madison. WI USA
PCR primers	Metabion, Germany
100 bp DNA leadder marker	Gene direx, Inc.US Cat No-DM001-R500
DNA cleanup kit	nucleoSpin Gel and PCR Clean up, MACHEREY-NAGEL, Duren, Germany
Tris-Borate EDTA Buffer (5x)	SIGMA –USA
Agarose	Sigma-Aldrich
Machines and instruments	
Item	Manufacturer/country
Spectrophotometer	Thermo Scientific Evolution 160 UV-VIS v8.01 Spectrophotometer. US
Thermal cycler	Biometra- An Analytica Jena Company – Germany
Nano-drop 2000 spectrophotometer	Genova Nano, Bibby Scientific Ltd ,UK
Eppendorf ThermoMixer	Eppendorf ThermoMixer® F - Eppendorf International.US
Gel electrophoresis unit	MIDI Horixontal "HU13"SCIE-PLAS,LTD, England

Chapter Three

Results

3.1 Study Population

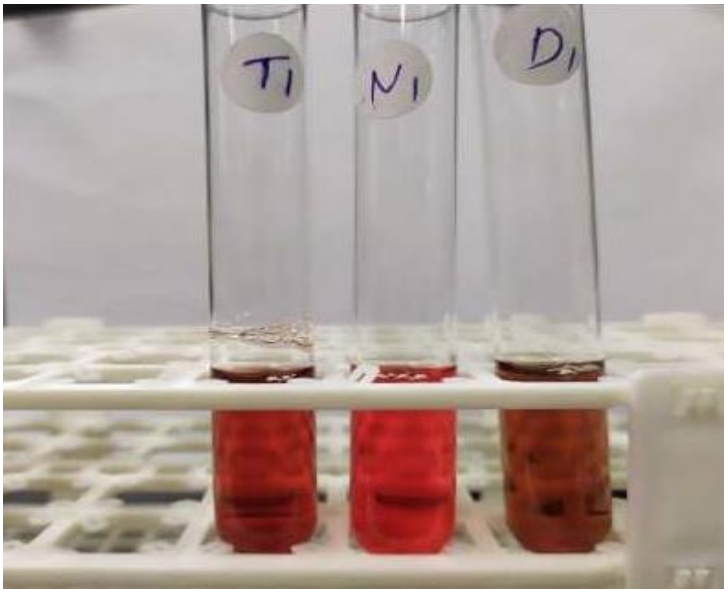
A total of 1380 blood samples from healthy male donors having age ranging between 18 to 40 years were collected for the study from three cities in northern West Bank: Tulkarem, Nablus and Jenin (455 samples from Tulkarem, 505 samples from Nablus and 420 samples from Jenin). Since criteria of blood donors usually exclude the females from blood donation, females were not considered in this study.

3.2 Characteristics of G6PD Deficiency Status

According to biochemical screening results of the qualitative methemoglobin reduction test (MRtest), it was found that from the1380 samples, 1348 (97.7%) persons had normal G6PD enzyme activity with negative MRtest results, while 32 (2.3%) persons gave MRtest positive result, most probably indicating G6PD enzyme deficiency. Samples of negative and positive MRtest results are shown in (Figure 3.1) and (Figure 3.2), respectively.

Figure 3.1

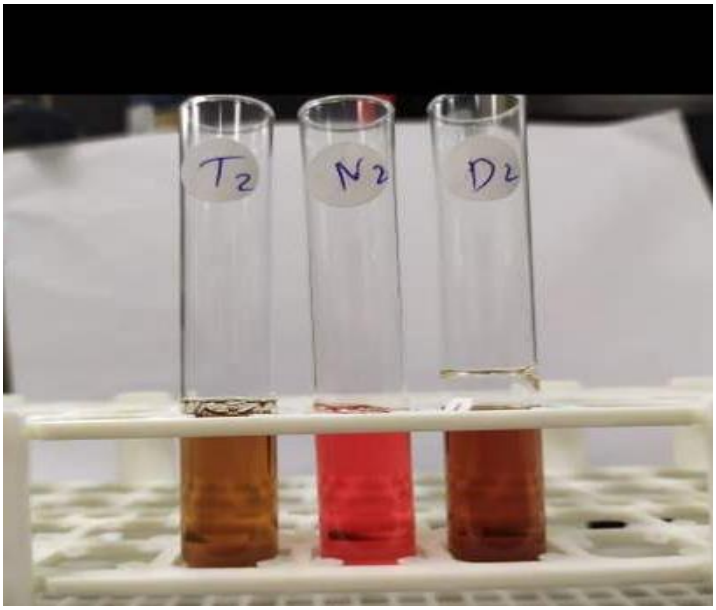
Negative MRtest of Normal G6PD Subjects



When the test solution (T) has a clear red colour, matching with the normal reference tube (N) then the sample has normal G6PD activity

Figure 3.2

Positive MRtest of Deficient G6PD Subjects



When the solution in test tube T has a brown colour matching with the positive reference tube D, it implies that there is reduced G6PD activity which is typical in full expression of the enzymopathy in hemizygous males

The positive results obtained by the MRtest (32 samples), indicating G6PDH deficiency, have been confirmed quantitatively using a semi-automated G6PD reagent kit to measure the G6PD enzyme activity values that were between 0.11 to 6.5U/gHb for the 32 samples, which are all below the normal range (7.0-20 U/gHb). Enzyme activity results and available information of the 32 samples are shown on (Table 3.1).

The mean age of the 32 G6PD deficient participants of this study was (25.2 ±5) years. In addition, the average hemoglobin concentration was (14.5 ± 0.8) g/dl, and the average red blood cells (RBCs) concentration was (4.5 ± 0.4) x 10^9. The mean hemoglobin and RBCs values of the normal donor population and the G6PD-deficient donor's population were compared, and it was found that the difference in the mean hemoglobin and RBCs values was not statistically significant.

The distribution of G6PD deficient cases in the 3 cities (Table 3.2) was 9/505 (1.78%) in Nablus, then 11/455 (2.4%) in Tulkarem, and finally12/420 (2.86%) in Jenin with the

highest incidence of G6PD deficiency (figure A.1 in Appendex A). Therefore, the prevalence of G6PD deficiency observed in this study was 2.3% (32/1380).

Table 3.1

Enzyme Activity, Results and Available Information of the 32 Samples

Sample No.	City	Age (Years)	G6PD Activity (U/gHb)	Hb (g/dl)	RBCs x 10^9	ABO Group	Rh	G6PD Variants
1	Tulkarm	23	4.6	14.6	4.4	A	+ve	A-
2	Tulkarm	21	1.8	15.1	5.0	A	+ve	Med
3	Jenin	21	0.65	13.8	4.15	B	+ve	Med
4	Tulkarm	25	1.6	14.7	4.23	B	+ve	Med
5	Jenin	22	0.8	13.5	4.71	O	+ve	Med
6	Jenin	29	2.5	14.7	4.45	O	+ve	Asahi
7	Tulkarm	19	3.7	14.5	5.22	A	+ve	A-
8	Tulkarm	19	0.64	13.5	4.31	B	+ve	Med
9	Jenin	23	4.1	16.7	5.4	O	+ve	A-
10	Nablus	23	2.4	15.8	4.52	B	+ve	Asahi
11	Nablus	21	0.18	14.1	4.15	A	+ve	Med
12	Jenin	20	2	15.3	4.0	A	+ve	Med
13	Nablus	30	6.5	16	5.03	B	+ve	ND
14	Jenin	24	5.2	15.2	4.30	O	+ve	A-
15	Jenin	30	1.77	15.5	4.6	B	+ve	Med
16	Nablus	30	6.1	15.7	4.83	B	+ve	ND
17	Nablus	32	0.11	14	4.75	A	+ve	Med
18	Jenin	26	0.5	15.7	4.33	B	+ve	Med
19	Tulkarm	21	0.94	15.8	5.0	A	+ve	Med
20	Tulkarm	20	3.3	14.7	4.18	B	+ve	Asahi
21	Jenin	24	1.89	15.5	4.5	B	+ve	Med
22	Tulkarm	28	1.2	13.8	4.18	A	+ve	Med
23	Tulkarm	33	4.7	15.4	4.8	O	+ve	A-
24	Jenin	28	3.8	14.7	5.0	A	+ve	A-
25	Jenin	25	0.44	14.3	4.3	B	+ve	Med
26	Jenin	27	5.5	15.5	5.0	B	+ve	ND
27	Nablus	25	0.42	14.4	4.5	A	+ve	Med
28	Nablus	29	5.11	14.8	4.66	B	+ve	A-
29	Tulkarm	26	2.1	13.4	4.0	O	+ve	Med
30	Tulkarm	37	2.3	15	4.33	O	-ve	Med
31	Nablus	35	6.5	17	5.15	B	+ve	ND
32	Nablus	30	5.88	14.5	4.8	B	+ve	ND

Table 3.2

Distribution of G6PD Deficiency among Cities

City	No. of G6PD deficient samples	Total No. of samples	G6PD deficiency incidence rate
Nablus	9	505	1.78%
Tulkarem	11	455	2.41%
Jenin	12	420	2.85%
Total	32	1380	2.3%

In evaluation of ABO blood groups among the G6PD deficient samples, the distribution was as follows: B had the highest incidence of 14/32 (43.7%), followed by A blood group 10/32 (31.3%), and O blood group 8/32 (25%). However, none of the 32 G6PD deficient samples of this study had AB blood group (Table 3.3).

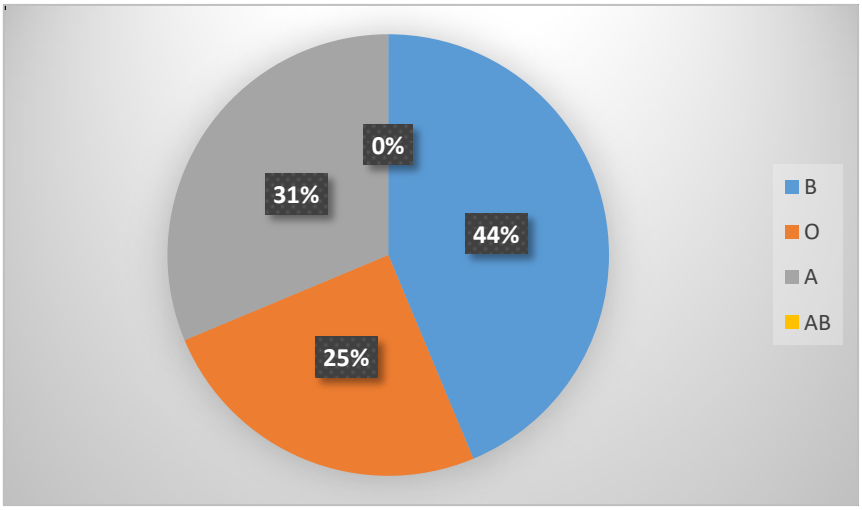
Table 3.3

ABO Blood Group Distribution among Deficient Samples

ABO Blood Group	No. of G6PD Deficient Samples	%
A	10	31.3
B	14	43.7
AB	0	-----
O	8	25.0
Total	32	

Figure 3.3

ABO Blood Group Distribution in G6PD Deficient Samples

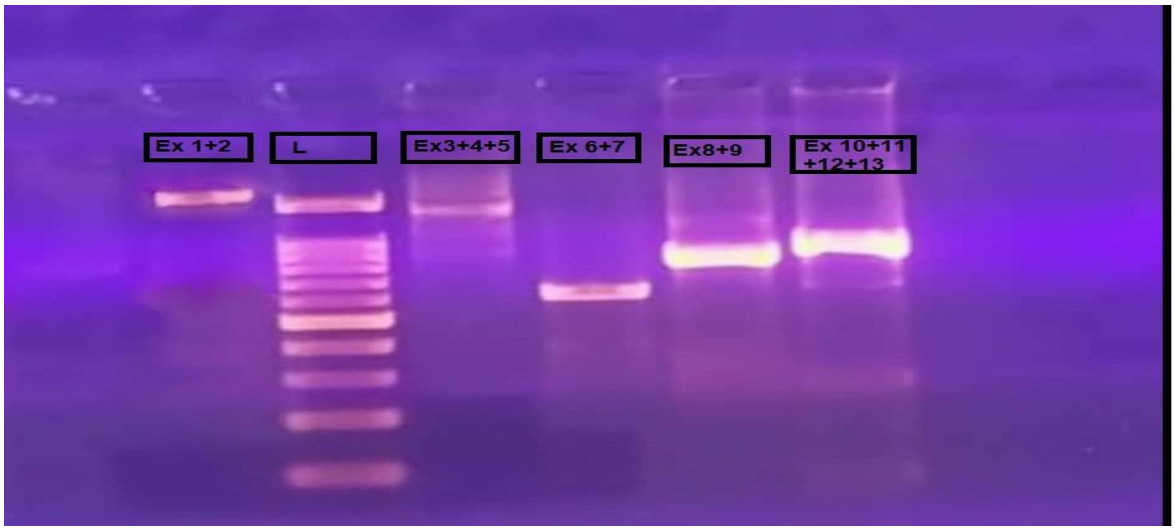


3.3 PCR and DNA Sequencing

The PCR technique was employed to amplify the 13 exons of the G6PD gene utilizing five sets of primers, as specified in Table (2.2). Subsequently, the resulting five amplicons, which spanned the 13 exons of the G6PD gene, underwent agarose gel electrophoresis. Figure 3.5 illustrates a representative agarose gel displaying these amplicons. Following gel electrophoresis, the five amplicons were extracted and purified from the agarose gels for subsequent DNA sequencing. Standard Sanger DNA sequencing was employed to sequence the PCR amplicons. The DNA sequence data obtained were initially analyzed visually, and subsequently, the BLAST bioinformatics tool was utilized for further analysis.

Figure 3.4

Representative Agarose Gel Electrophoresis (1.7%) of the Different Amplicon Sizes of G6PD Exons



3.4 Detection of G6PD Mutations

In the cohort of 32 participants included in this study, a total of three variants were identified in the G6PD gene. The exonic variations observed in this study are as follows: c.563 C>T, c.376 A>G, and c.202 G>A (refer to Table 3.4).

All three exonic variants detected in the study were classified as missense mutations, resulting in the presence of three distinct G6PD variants (see Table 3.5). G6PD Mediterranean was found to be the most prevalent variant, accounting for 54.2% of the cases, followed by G6PD A- and G6PD Asahi with frequencies of 16.7% and 8.3%,

respectively. The three genetic variants identified in the G6PD gene among the participants of this study are shown in Table 3.4.

In this study, the most commonly observed genetic change within the coding region was identified as c.563 C>T (G6PD Mediterranean) in exon 6. This alteration caused a substitution of the amino acid serine (TCC) with phenylalanine (TTC) at codon 188, which is a distinct characteristic of the Mediterranean mutation (figure A.2 , figure A.3 in appendix A). Out of the total of 32 participants, the majority (17 individuals) carried the G6PD Mediterranean genetic variation, representing a prevalence rate of 53.1%.

Table 3.4

The Three Genetic Variants Identified in the G6PD Gene among the Participants of this Study

Variant ID	Chr: bp	cDNA change	a.a. change	Exon #	SNP type
rs5030868	X:154534419	c.563 C>T	p.Ser188Phe	Exon 6	Non-synonymous
rs1050829	X:154535277	c.376 A>G	p.Asn126Asp	Exon 5	Non-synonymous
rs1050828	X:154536002	c.202 G>A	p.Val68Met	Exon 4	Non-synonymous

The second most frequently observed mutation was the c.367 A>G (G6PD A-) variation located in exon 5 at position 376. This variation led to a change in the amino acid from asparagine (AAT) to aspartate (GAT) at position 126. Among the 32 cases, seven individuals (21.9%) were found to possess this mutation (Table 3.5).

Lastly, the c.202 G>A genetic variation (G6PD Asahi), located in the coding region of exon 4, caused a substitution from a GTG codon to an ATG codon, leading to an amino acid change from valine to methionine at position 68 (Table 3.4). Among the 32 participants, three individuals (9.4%) exhibited this genetic variation.

Table 3.5

Frequency of G6PD Variants among the Participants of this Study

G6PD variant		Frequency, n (%)
G6PD Med	p.Ser188Phe	17 (53.1%)
G6PD A-	p.Asn126Asp	7 (21.9%)
G6PD Asahi	p.Val68Met	3 (9.4%)
Undetermined	--	5 (15.6%)
Total		32 (100%)

3.5 Genotype- Phenotype Correlations

Phenotypically, individuals with G6PD Mediterranean exhibit an enzyme activity below 10%, aligning with its classification as class II. In contrast, G6PD A- patients display an enzyme activity ranging from 10% to 60%, consistent with its classification as class III. Patients with the Mediterranean variants demonstrated lower G6PD enzyme activity compared to those with G6PD A and Asahi variants.

The genetic cause of G6PD deficiency could not be determined in five subjects, accounting for 15.6% of the participants. This limitation arose due to financial constraints, preventing sequencing testing in all 32 samples. To overcome this, we focused on conducting sequencing specifically in exon 6 for all 32 samples (figure A.4 in Appendix A). Previous local and regional reports have consistently highlighted the Med c.563 C>T mutation as the most prevalent. For the remaining exonic variations, random selection was performed, with particular attention given to exons 4 and 5, which are associated with the second most prevalent G6PD mutations reported in the literature. This strategic approach yielded successful detection of the majority of mutations within our sample.

Chapter Four

Discussion, Conclusion and Recommendations

4.1 Discussion

This study represents the first investigation conducted to assess the prevalence of G6PD deficiency in the West Bank population among asymptomatic healthy blood donors. After conducting biochemical screening using the methemoglobin reduction test, it was observed that out of a total of 1380 healthy blood donors, 32 individuals (2.3%) had G6PD deficiency, while 1348 individuals (97.7%) exhibited normal G6PD levels. Among the regions studied, the highest percentage of G6PD deficiency was found in Jenin, where 12 cases (2.86%) were identified among 420 donors. Tulkarem had 11 cases (2.4%) out of 455 donors, while the lowest incidence was observed in Nablus, with 9 cases (1.78%) among 505 donors.

Furthermore, the most common ABO blood group among the G6PD-deficient blood donors was the B blood group. This finding aligns with a study conducted by Pant et al. (94) and other studies conducted in India among healthy blood donors. The B blood group was found to be the most prevalent among G6PD-deficient blood donors, with a frequency of 42.8%. It was followed by the A, O, and AB blood groups,with frequencies of 28.6%, 20%, and 8.6%, respectively (95).

The prevalence of G6PD deficiency in the present study, at 2.3%, is comparatively lower when compared to similar studies conducted in neighboring countries. For instance, Alharbi and Ali Khan reported a prevalence of 4.7% in the Saudi population (82), while in Jordan, the prevalence ranged from 3.2% to 8.5% depending on the region (88). Kurdish populations in Northern Iraq exhibited a frequency of 10.9% (84). In contrast, Shanthaladevi *et al* reported an incidence of G6PD deficiency of 0.8% in India (80), which is lower than our findings. Additionally, in China, the incidence of G6PD deficiency was 2.1%, which is relatively similar to the prevalence observed in our study.

Within the scope of this study, three missense mutations were identified. Among them, the G6PD Mediterranean variant (c.563 C>T) exhibited a wide distribution, being present in 17 out of 32 deficient donors, resulting in an allelic frequency of 53.1%. The significant prevalence of this variant suggests that it is the primary mutation responsible

for the majority of cases of enzymopathy among Palestinians. Notably, all samples carrying the Mediterranean variant displayed an enzyme activity of less than 10%. According to the classification of G6PD variants by the World Health Organization (WHO), G6PD Mediterranean is categorized under class II variants.

G6PD Mediterranean is distributed across Africa, Asia, the Middle East, and various Mediterranean regions. Turkey, for instance, exhibits a frequency of 80% for the G6PD Mediterranean variant (89), while Jordan reports a frequency of 53.3% (88). Saudi Arabia shows rates of 45.9% and 36.5% in different regions (85) also this variant was report in Gaza (92). In this study, the prevalence of G6PD Mediterranean (53.1%) aligns with the findings observed in other Arab populations within the region.

The second variant identified in this study is the A- variant (c.376 A>G) detected in exon 5. It was found in 7 out of 32 deficient samples, representing a frequency of 21.9%. In a study conducted in Jordan, G6PD A- was reported to have a percentage of 19% (96), which is similar to the percentage observed in our study. Another study conducted in Gaza showed a higher percentage of G6PD A- among their deficient study populations, reaching 28.5% (92). This variant was also detected in Turkey, but with a lower incidence of 4% (86). Furthermore, Saudi Arabia exhibited a frequency of 5.8% (84), which is lower than what was observed in our study. According to the World Health Organization (WHO) classification of G6PD variants, G6PD A- is classified under class III variants.

The Asahi variant (c.202 G>A) was identified in exon 4 and was detected in 3 out of 32 deficient samples, representing a frequency of 9.4%. This variant is characterized by a change in an amino acid from valine to methionine. Previous reports from Qatar indicated allele frequencies of 0.00785 for this mutation, and a Jordanian study reported a percentage of 3.6% (88). The enzymatic activity associated with this variant ranges from 10% to 60%, which classifies it as a Class III variant according to the classification provided by the World Health Organization (WHO).

This study did not detect other variants commonly observed in neighboring Arab and Middle Eastern countries, such as G6PD Chatham and G6PD Auresc. These variants have been reported in Qatar (90), Iraq (87), and Iran (86), but they were not found in the participants of this study.

4.2 Limitations

- The study does possess certain limitations, such as the collection of samples during the COVID-19 pandemic, which resulted in a restricted number of samples available for this study.
- Financial problems.

4.3 Recommendations

Firstly, future studies should aim to gather data from a broader range of cities in Palestine to comprehensively determine the spectrum of G6PD deficient variants.

The prevalence of G6PD deficiency at 2.3% among asymptomatic blood donors in this study, despite its relatively low incidence, should be regarded with concern. Therefore, further investigations are recommended to screen for G6PD deficiency in healthy blood donors. The methemoglobin reduction (MR) test, which demonstrated concordant results with quantitative methods for identifying deficient G6PD values, can be utilized as a cost-effective and convenient screening tool. Integrating the MR test into comprehensive medical checkups for healthy blood donors would contribute to increasing the number of safe and voluntary blood donations.

Additionally, it is imperative to conduct additional research to explore the relationship between ABO blood groups and G6PD deficiency.

Given the extensive molecular diversity of G6PD deficient variants observed in Palestine, further studies and research are essential to identify prevalent G6PD variants. These investigations will facilitate the development of targeted and rapid molecular assays specifically designed for screening purposes.

4.4 Conclusion

This study provides the first assessment of G-6-PD deficiency prevalence among asymptomatic, healthy blood donors in the northern of West Bank, revealing a prevalence rate of 2.3%. Additionally, the study explores the molecular diversity of G6PD variants. The most frequently observed G6PD variant in this study is Mediterranean (c.563 C>T), accounting for 53.1% of cases. Following that, G6PD A- (c.376 A>G) is detected at a frequency of 21.9%, and G6PD Asahi (c.202 G>A) at 9.4%. Phenotypically, all deficient samples with identified mutations display enzyme

activity levels consistent with their classification as either class II or class III, also the most common ABO blood group that was detect among G6PD deficient donor was B. This study is the first study in north West Bank about the prevalence of G6PD among asymptomatic individual

List of Abbreviations

Abbreviation	Meaning
ROS	Reactive Oxygen Species
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
G6PD	Glucose-6-Phosphate Dehydrogenase
GSH	Reduced Glutathione
WHO	World Health Organization
PPP	Pentose Phosphate Pathway
SOD	Superoxide Dismutase
H_2O_2	Hydrogen Peroxide
TBE	Tris-Borate-EDTA
AHA	Acute Hemolytic Anemia
CNSHA	Chronic Non Spherocytic Hemolytic Anemia
CVD	Cardio Vascular Disease
AIVHA	Acute Intravascular Hemolytic Anemia
CPD	Citrate Phosphate Dextrose
ACD	Acid Citrate Dextrose

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Appendices

Appendix A

Figures of Study

Figure A.1

Distribution of G6PD Deficiency in the Cities of the Study Area

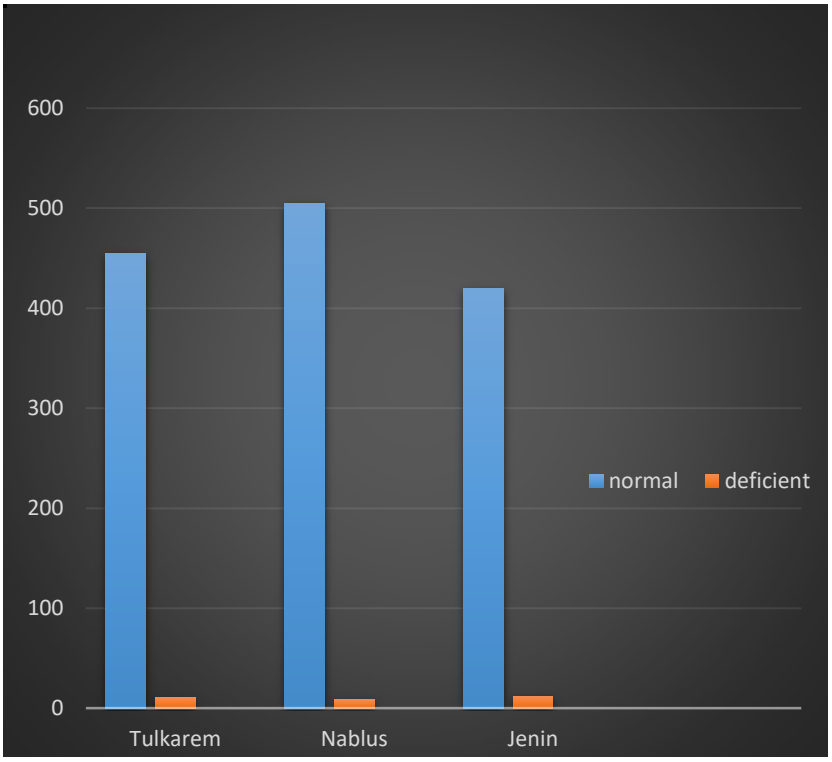


Figure A.2

Sequence Result of the PCR Product for Exon 6 in a Normal Control for Mediterranean Mutation..The Arrow Indicates C (Cytosine) in a Normal Control for Mediterranean Mutation (563 C-T)

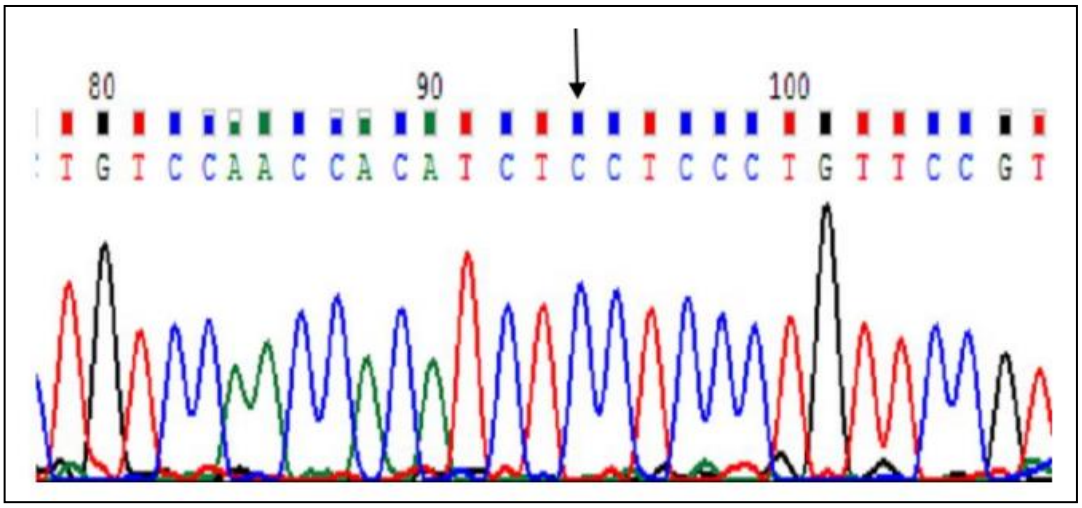


Figure A.3

Sequence Result of the PCR Product for Exon 6 in a Male Participant. The Arrow Indicates C (Cytosine) Nucleotide Substituted by T (Thymine) in Mediterranean Mutation 563C-T

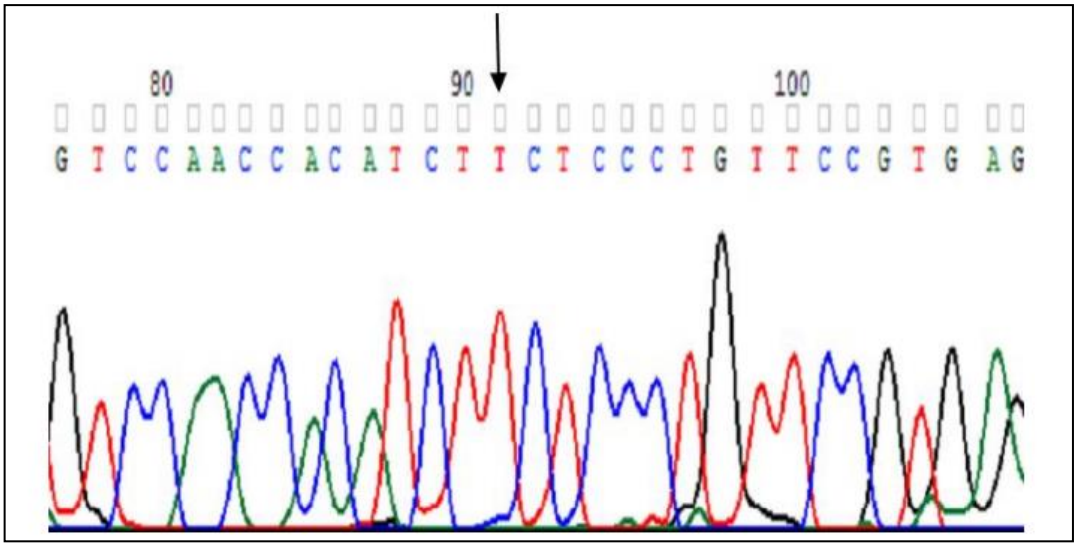
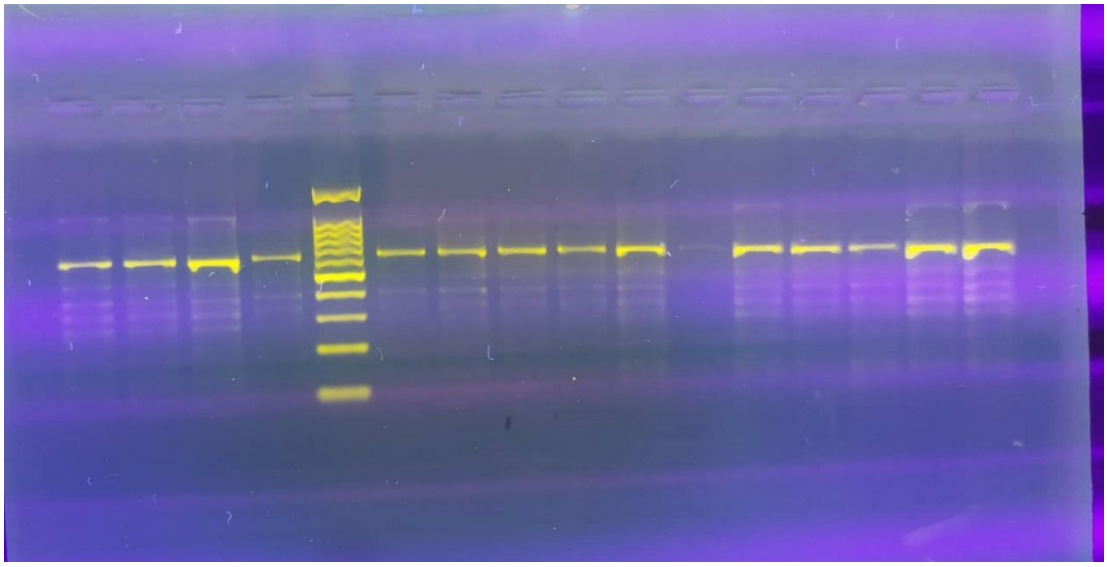


Figure A.4

Agarose Gel Electrophoresis for Exon 6 for all Samples



جـامعـــة النجاح الوطنية

كليـــة الدراســـات العليـــا

الانتشار والتوصيف الجزيئي لنقص انزيم نازعة الهيدروجين من الجلوكوز 6 فوسفات لدى الفلسطينيين الاصحاء في شمال الضفة الغربية

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قدمت هذه الرسالة استكمالاً لمتطلبات الحصول على درجة الماجستير في برنامج العلوم الحياتية، من كلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس– فلسطين.

2023

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الملخص

يعد نقص انزيم نازعه هيدروجين من الجلوكوز 6 فوسفات احد الاضطرابات الاكثر شيوعا من مجموعة الامراض الإنزيمية، اذ يعاني 10٪ من سكان العالم من هذا النقص الإنزيمي، ويقدر عدد المصابين بأكثر من 400 مليون شخص حول العالم, يؤدي عوز هذا الانزيم الى فقر الدم الانحلالي عقب تناول بعض الأدوية او الأغذية مثل الفول او التعرض لإصابة فيروسيه.

تهدف هذه الدراسة الى معرفه مدى انتشار نقص انزيم G6PD لدى الفلسطينيين الاصحاء في شمال الضفة الغربية، وتحديد نشاط الانزيم في الحالات التي اظهرت النقص كذلك التعرف على الانماط الوراثية الأكثر شيوعا بين الفلسطينيين في الضفة الغربية.

تم جمع 1380 عينه من الاشخاص الأصحاء المتبرعين في الدم في مستشفيات وزاره الصحة في طولكرم، نابلس وجنين، وفحص نقص انزيم G6PD بواسطه اختبار اختزال الميتهيموغلبين وتحديد نشاط الانزيم في العينات المصابة، ثم استخدام تسلسل قواعد الحمض النووي لتشخيص العينات لطفرات G6PD.

كانت نسبه انتشار عوز الانزيم في الضفة الغربية 2.3٪ ونسبة النشاط الإنزيمي للمصابين تراوحت بين (6.5–0.11) وهي اقل من النسبة الطبيعية (7–20) وهو ما يتسق مع تصنيف الطفرات ضمن الطبقة II و III، تم الكشف عن 3 متغيرات في جين G6PD وهي c.563 C>T, c.376 A>G and c.202 G>A، حيث كانت G6PD البحر الابيض المتوسط (c.563 C>T) المتغيرة الاكثر شيوعا بنسبة 53٪ونسبه نشاط انزيمي اقل من10٪وهو ما يتسق مع تصنيفها ضمن الطبقة الثانية، تليها G6PD A-وAsahi بنسبه 21.9 % و 9.4% على التوالي ونسبه النشاط الانزيمي بين 10٪–60٪ وتم تصنيفها ضمن الطبقة الثالثة.

في الختام هذه الدراسة هي الأولى في الضفة الغربية لانتشار نقص انزيم G6PD وهدفت لمعرفة نسبه الانتشار لدى الاشخاص الأصحاء في شمال الضفة الغربية والتي كانت بنسبة 2.3٪ وكانت طفره حمى البحر الابيض المتوسط هي الأكثر شيوعاً.

الكلمات المفتاحية: عوز نازعه هيدروجين الجلوغوز 6 فوسفات، اختبار اختزال الميتهيموغلبين، المتغيرات الجينية، نشاط الانزيم.