# MOLECULAR BASIS OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY IN CAPE COAST, GHANA

**by**

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## DECLARATION

I hereby declare that except for references to other people's work, which have been duly acknowledged, this thesis is the result of my own research. Neither all nor parts of this thesis have been presented for another degree elsewhere.

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**ABSTRACT**

Glucose-6-phosphate dehydrogenase (G6PD) is a cytoplasmic enzyme that is essential for a cell's capacity to withstand oxidant stress. G6PD-deficiency is the commonest enzymopathy of humans with a worldwide distribution. The geographical correlation of its distribution with the historical endemicity of malaria suggests that the defect has risen in frequency through natural selection by malaria. This study was carried out to ascertain the molecular basis of G6PD-deficiency in Cape Coast in the Central Region of Ghana. Two hundred (200) unrelated persons (82 males and 118 females), all visiting the Out Patients Department (OPD) of the Central Regional Hospital, Cape Coast, were screened for G6PD-deficiency using the methaemoglobin reduction test. Genomic DNA from 38 G6PD-deficient persons (18 males and 20 females) and 12 randomly selected individuals were subjected to molecular analysis for the presence of two known mutations that appear to be most frequent in Sub-Saharan Africa; the G202A or G6PD A- and A376G or G6PD A variants, and the C563T Mediterranean variant, using appropriate restriction enzyme digestion of fragments amplified by polymerase chain reaction. G6PD-deficiency was found to have a prevalence of 19.0%. Molecular analysis of the mutations underlying the deficiency in the subjects revealed a predominance of the *G6PD* A allele, accounting for 86.8% of allelic frequency. G6PD A- had a rather low prevalence with allelic frequency of 7.9% out of the 38 chromosomes tested. All the samples analyzed were negative for the Mediterranean mutation, however, three individuals who tested positive for the enzymopathy at the biochemical level were found to be negative for the three mutations at the molecular level. Findings from the genetic analysis contradict reports in other works that implicate G6PD A- as the predominant underlying mutant for G6PD-deficiency in Sub-Saharan Africa and suggests that the enzymopathy in Cape Coast is mostly due to the A376G mutation. Furthermore, it is possible that G6PD-deficient persons may bear other mutations not screened for or as yet uncharacterized at the molecular level among Ghanaians.

## DEDICATION

This work is dedicated to my wife, Mrs. Faustina Osei Mensah Bonsu for her love, unflinching support and encouragement.

## ACKNOWLEDGEMENTS

To him who kept me from falling and presented me before his glorious presence without fault and with great joy, The Almighty God, be glory and honour.

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Finally, I am very grateful to my family and all my friends for their warmth and support throughout my studies.

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| | |
|---|---|
| AHA | Acute haemolytic anaemia |
| ATP | Adenosine Triphospate |
| CNSHA | Chronic Non-Spherocytic Haemolytic Anaemia |
| DNA | Deoxyribonucleic Acid |
| dNTP | Deoxyribonucleotide Triphosphate |
| EDTA | Ethylenediamine tetra-acetic acid |
| ESCs | Embryonic Stem Cells |
| FADH2 | Flavin adenine dinucleotide (reduced form) |
| G6P | Glucose-6-phosphate |
| G6PD | Glucose-6-phosphate dehydrogenase |
| GSH | Glutathione (reduced form) |
| GSHPX | Glutathione peroxidase |
| GSSG | Glutathione |
| Hb | Haemoglobin |
| Ibid | *Ibidem* (Latin: meaning the same place) |

| | |
|---|---|
| MetHb | Methaemoglobin |
| MRT | Methaemoglobin Reduction Test |
| NADH | Nicotinamide adenine dinucleotide (reduced form) |
| NADP | Nicotinamide Adenine dinucleotide Phosphate |
| NADPH | (Reduced) Nicotinamide Adenine dinucleotide Phosphate |
| NNJ | Neonatal Jaundice |
| OPD | Out-Patients' Department |
| PCR | Polymerase Chain Reaction |
| PPP | Pentose Phosphate Pathway |
| RBCs | Red Blood Cells |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| SOD | Superoxide dismutase |
| TBE | Tris-Borate EDTA |
| W.H.O | World Health Organization |

# CHAPTER ONE

## 1.0 General Introduction

## 1.1 Introduction

The non-nucleated erythrocyte is unique among human cell types, in that the plasma membrane, its only structural component, accounts for all of its diverse antigenic, transport, and mechanical characteristics (Mohandas and Gallagher, 2008). The mature red cell is well adapted to its role in oxygen transport. Devoid of nuclei and cytoplasmic organelles, its metabolism is simplified, with no capacity for protein or lipid synthesis and oxidative phosphorylation. As a result, the red cell is totally dependent on glycolysis, the catabolism of plasma glucose generating high-energy phosphates, principally ATP and reducing equivalents in the form of glutathione (GSH) and pyridine nucleotides (NADH and NADPH) ( Arya *et al*., 1995). Disorders in the glycolytic pathway can therefore predispose humans to red cell disorders.

Inherited red cell disorders with altered membrane and cell function can be broadly classified as either due to mutations in various membrane or skeletal proteins resulting in conditions such as hereditary spherocytosis, elliptocytosis, ovalocytosis, etc., or as a result of secondary effects on the membrane resulting from mutations in certain genes, for instance sickle cell disease and glucose-6-phosphate dehydrogenase (G6PD) deficiency among others.

Glucose-6-phosphate dehydrogenase is the key regulatory enzyme of the pentose phosphate pathway and is considered to have an important “housekeeping” function.

The housekeeping function of the enzyme is best exemplified by G6PD-deficiencysyndrome, a disease that manifest in the red blood cell (Notaro *et al*., 2000). As the catalyst for the initial step in the pentose phosphate oxidative pathway, this enzyme occupies a key position in red cell metabolism. The pentose phosphate pathway results in the reduction of nicotinamide adenine dinucleotide phosphate (NADP) to NADPH. Because the red cell has no citric acid cycle, it possess no other means for reducing this co-enzyme; however NADPH is required by the erythrocyte to maintain glutathione in reduced state (GSH), and possibly, to serve other functions (Cappellini and Fiorelli, 2008).Glutathione, in turn, is required to maintain intact sulphydryl (-SH) groups within the erythrocyte and perhaps on the erythrocyte cell surface. Furthermore, it plays an important role in the catabolism of hydrogen peroxide in the erythrocyte. In G6PD-deficient individuals, the NADPH production is diminished and detoxification of hydrogen peroxide is inhibited. Lipid peroxidation leading to breakdown of erythrocyte membranes and oxidation of proteins and DNA results in cellular damage (Luzzatto and Poggi, 2008).

It was recognized early in the studies of primaquine-induced haemolysis that susceptible subjects were also sensitive not only to other 8-aminoquinoline antimalarial drugs but also to other drugs and toxic substances, fava (broad) beans and certain stresses, including infection and diabetic acidosis (Mason *et al*., 2007). The exact mechanisms of haemolysis, however, are not yet known (*ibid*).

An estimated 400 million people are affected by glucose-6-phosphate dehydrogenase deficiency worldwide and the geographic distribution of G6PD-deficiency is instructive (Beutler and Vulliamy, 2002). Frequencies as high as 25% occur in tropical Africa, parts

of the Middle East, and Southeast Asia, areas where malaria is most prevalent. In addition to such epidemiological observations, *in vitro* studies show that growth of *Plasmodium falciparum* is inhibited in G6PD-deficient erythrocytes (Beutler and Vulliamy, 2002). The parasite is very sensitive to oxidative damage and is killed by a level of oxidative stress that is tolerable to a G6PD-deficient human host. Because the advantage of resistance to malaria balances the disadvantage of lowered resistance to oxidative damage, natural selection sustains the G6PD-deficient genotype in human populations where malaria is prevalent (Marla *et al*; 2009). Under overwhelming oxidative stress, caused by drugs, herbicides, or divicine found in fava beans, G6PD deficiencies cause serious medical problems (*ibid*). Most G6PD-deficient individuals are asymptomatic; however, the combination of G6PD-deficiency and certain environmental factors may produce the clinical manifestations such as abnormal breakdown of the red cell of the newborns, causing neonatal jaundice, which is characterized by yellow pigment seen on the skin and eye (Akanni *et al*, 2010). Acute haemolytic anaemia and chronic haemolytic anaemia are other clinical manifestations of G6PD-deficiency (Mason *et al*., 2007).

The human G6PD monomer is a 515 amino acid protein encoded by a human X-linked gene located in the telomeric region of the long arm of the X-chromosome (Xq28) (Kletzien *et al*., 1994). The gene spans about 18.5 kb and its first exon corresponds to a non-coding region while the remaining 12 exons range from 12 to 236bp (*ibid*). Mutations in the *G6PD* gene represent one of the most common human genetic abnormalities affecting people of all races world-wide (Arya *et al*., 1995). Variants of G6PD have been identified by biochemical analysis and only a small proportion of this

heterogeneity has been confirmed at the DNA level by identification of mutations in the G6PD coding sequence and comparison of the gene encoding variant enzymes with the sequence of the normal G6PD gene (Beutler, 1992). Almost all mutations of G6PD affect the coding sequence of the gene. The vast majority are single base substitutions, leading to amino acid change. A few of these occur as a second mutation, most frequently in combination with the polymorphic mutation that distinguishes the non-deficient variant G6PD A, from the wild type enzyme G6PD B (*ibid*). The only deletions seen in the coding sequence are small and in-frame (Mason *et al*., 2007). The absence of large deletions of frame shift mutations suggests that a total lack of G6PD is incompatible with life (Mehta *et al*, 2000).

In sub-Saharan Africa, three most common G6PD electrophoretic variants are well known. These include: G6PD B, which has normal enzyme activity (60 to 80% frequency range) and is not associated with haemolysis, G6PD A, which has 85% normal enzyme activity (15 to 40% frequency range) but not associated with haemolysis though enzyme activity is slightly reduced, and G6PD A-, which has 12% normal enzyme activity (0 to 25% frequency range) and can be associated with haemolysis (Daoud *et al.*, 2008).In Ghana, as far back 1989, the incidence of G6PD deficiency disease was known to be 15 – 25% (W.H.O. Working Group, 1989). Current data on the prevalence of the enzymopathy in Ghana is not yet available. Apart from electrophorectically characterized variants for a Ghanaian population (Owusu and Opare-Mante, 1972), there is no data on the mutations that cause G6PD deficiency disease among Ghanaians.

## 1.2 Problem Statement

Although G6PD deficiency forms part of the general health screening in many countries, this enzymopathy has received little attention in Ghana. G6PD A-, the commonest African mutation, has been assumed to be the major variant associated with the defect in Ghana (Owusu and Opare-Mante, 1972), a view based on works in other Sub-Saharan countries (Ademowo and Falusi, 2002). However, the precise mutations causing the enzymopathy in the country have remained unknown.

## 1.3 Study Hypothesis

It is hypothesized that glucose-6-phosphate dehydrogenase (G6PD) variant A-, is not the predominant variant associated with G6PD deficiency in the Cape Coast metropolis.

## 1.4 General Objective

The aim of this study is to determine the mutations causing glucose-6-phosphate dehydrogenase deficiency in Cape Coast in the Central Region of Ghana.

## 1.5 Specific Objectives

1. To estimate the prevalence of G6PD-deficiency among persons reporting at the Out Patients Department of the Central Regional Hospital in Cape Coast.
2. To characterize the specific mutations underlying G6PD-deficiency among patients in the Cape Coast metropolis.
3. To ascertain whether other mutations, apart from the A- variant, are the underlying factors of G6PD deficiency in Ghana.

## 1.6 Justification

To date, there is no documented community-based estimation of the frequency of G6PD-deficiency in Ghana because screening for common mutations is rarely a routine of the healthcare process in the country. Furthermore, people who report to various health facilities with malaria cases are prescribed anti-malarial treatment without being tested for G6PD deficiency, a situation that may be fatal in people with lethal variants of the enzymopathy, should the wrong drugs be dispensed.In the absence of any empirical data, determining the prevalence of glucose-6-phosphate dehydrogenase deficiency and characterization of the deficient alleles among Ghanaians is therefore, a matter of public-health importance.

# CHAPTER TWO

## 2.0 Literature Review

### 2.1 Background

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme that catalyses the first reaction in the pentose phosphate pathway (PPP), providing reducing power to all cells in the form of NADPH (reduced form of nicotinamide adenine dinucleotide phosphate). The latter enables cells to counterbalance oxidative stress that can be triggered by several oxidant agents and to preserve the reduced form of glutathione (GSH). Since red blood cells do not contain mitochondria, the PPP is their only source of NADPH and therefore defense against oxidative damage is dependent on G6PD (Luzzatto *et al*, 2001). Glucose-6-phosphate dehydrogenase deficiency is an X-linked, hereditary genetic defect caused by mutations in the *G6PD* gene, resulting in protein variants with different levels of enzyme activity, that are associated with a wide range of biochemical and clinical phenotypes. The most common clinical manifestations are neonatal jaundice and acute haemolytic anaemia, which in most patients is triggered by an exogenous agent (*ibid*).

The highest frequencies of the defect are detected in Africa, Asia, the Mediterranean region, and in the Middle East; owing to recent migrations, however, the disorder is also found in North and South America and in northern European countries (Beutler, 1996). A pathological disorder linked to ingestion of fava beans (*Vicia faba*), later identified as G6PD deficiency, has been recognized for centuries (*ibid*). The Greek philosopher and mathematician, Pythagoras, forbade his followers from eating fava beans, possibly

because of their pathological effects (Luzzatto *et al*, 2001). A clinical picture of the favism was drawn in the early 20$^{th}$ century (Drousiotou *et al*., 2004). However, because the response to fava bean ingestion is inconsistent, popular theories on the pathogenesis of favism were related to toxic effects or allergy (*ibid*). It is now known that G6PD-deficiency is the most common human enzyme defect, present in more than 400 million people worldwide (WHO, 1989; Mason, 1996; Nkhoma *et al*.,2009) and the frequency of mutations varies greatly among different populations (Luzzatto *et al*, 2001). G6PD A- is the most prevalent mutation in Afro-Americans with a gene frequency of 11% (*ibid*) and is the only form of the enzymopathy that is widespread in Africa (Ademowo and Falusi, 2002). G6PD Mediterranean is a more severe enzymopathy and commonly found in populations living in or originating from around the Mediterranean Sea.

**2.2 The Pentose Phosphate Pathway**

In most animal tissues, the major catabolic fate of glucose-6-phosphate is glycolytic breakdown to pyruvate, much of which is then oxidized via the citric acid cycle, and then the respiration chain ultimately leading to the formation of ATP. Glucose-6-phosphate does have other catabolic fates, however, which lead to specialized products needed by the cell. Of particular importance in some tissues is the oxidation of glucose-6-phosphate to pentose phosphates by the pentose phosphate pathway (also called the phosphogluconate pathway or the hexose monophosphate pathway (Figure 2.0) (Chen *et al*., 1991). In this oxidative pathway, $NADP^+$ is the electron acceptor, yielding NADPH. Rapidly dividing cells, such as those of bone marrow, skin, and intestinal mucosa, use the pentoses to make RNA, DNA, and such coenzymes as ATP, NADH, $FADH_2$, and coenzyme A. In other tissues, the essential product of the pentose phosphate pathway is

not the pentoses but the electron donor NADPH, needed for reductive biosynthesis or to counter the damaging effects of oxygen radicals (Chen *et al*., 1991).

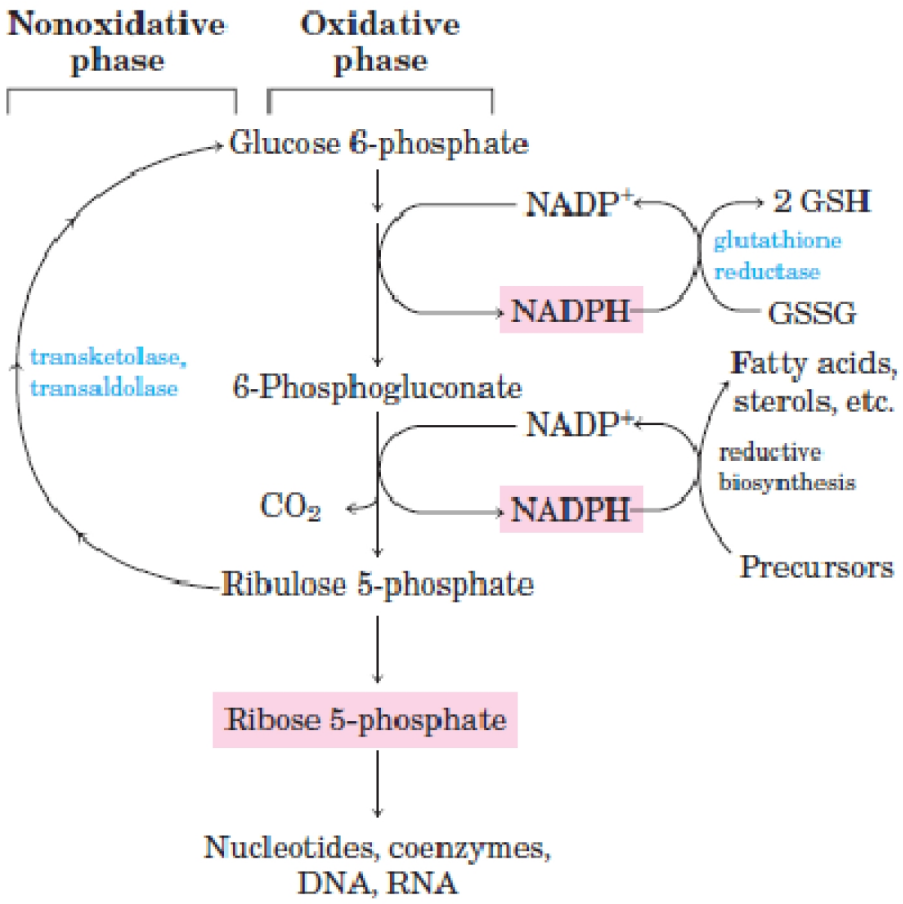


**Figure 2.0**: General scheme of the pentose phosphate pathway (Luzzatto *et al*., 2001)
*$NADP^+$ the electron acceptor yields NADPH in the oxidative phase, used to reduce glutathione, (GSSG) to GSH and to support reductive biosynthesis. The other product of the oxidative phase is ribose 5-phosphate, which serves as precursor for nucleotides, coenzymes, and nucleic acids. In cells that are not using ribose 5-phosphate for biosynthesis, the non-oxidative phase recycles six molecules of the pentose into five molecules of the hexose glucose 6-phosphate, allowing continued production of NADPH and converting glucose 6-phosphate (in six cycles) to $CO_2$.*

Tissues that carry out extensive fatty acid synthesis (liver, adipose, lactating mammary gland) or very active synthesis of cholesterol and steroid hormones (liver, adrenal gland, gonads) require the NADPH provided by this pathway (Finkel and Holbrook, 2000). Erythrocytes and the cells of the lens and cornea are directly exposed to oxygen and thus, to the damaging effect of free radicals generated by oxygen. By maintaining a reducing atmosphere (a high ratio of NADPH to $NADP^+$ and a high ratio of reduced to oxidized glutathione), they can prevent or undo oxidative damage to proteins, lipids, and other sensitive molecules. In erythrocytes, the NADPH produced by the pentose phosphate pathway is so important in preventing oxidative damage that a genetic defect in glucose 6-phosphate dehydrogenase, the first enzyme of the pathway, can have serious medical consequences (Kletzien *et al*., 1994).

### 2.2.1 Pentose Phosphates and NADPH Production

The first reaction of the pentose phosphate pathway (Figure 2.0) is the oxidation of glucose-6-phosphate by G6PDto form 6-phosphoglucono-δ-lactone, an intramolecular ester. $NADP^+$ is the electron acceptor, and the overall equilibrium lies far in the direction of NADPH formation. The lactone is hydrolyzed to the free acid 6-phosphogluconate by a specific lactonase**,** then 6-phosphogluconate undergoes oxidation and decarboxylation by 6-phosphogluconate dehydrogenase to form the ketopentose ribulose 5-phosphate. This reaction generates a second molecule of NADPH. Phosphopentose isomerase converts ribulose 5-phosphate to its aldose isomer, ribose 5-phosphate. In some tissues, the pentose phosphate pathway ends at this point, and its overall equation is:

Glucose-6-phosphate + $2NADP^+ + H_2O \rightarrow$ ribose 5-phosphate + $CO_2$ + 2NADPH + $2H^+$ (Ursini *et al*., 1990).

The net result is the production of NADPH, a reductant for biosynthetic reactions, and ribose 5-phosphate, a precursor for nucleotide synthesis. In tissues (such as erythrocytes) that require primarily NADPH, the pentose phosphates produced in the oxidative phase of the pathway are recycled into glucose 6-phosphate in the non-oxidative phase allowing the oxidative reactions to continue. The reactions of the non-oxidative part of the pentose phosphate pathway are readily reversible and thus provide a means of converting hexose phosphates to pentose phosphates (Ursini *et al*., 1990).

### 2.2.2 Glucose-6-phosphate dehydrogenase in Red Cell Metabolism

In metabolic maps, G6PD is commonly referred to as the first enzyme of the hexose monophosphate shunt, or the pentose phosphate pathway (Fig. 2.0). However, recent research has revealed that the main role of G6PD in red blood cells is not glucose utilization; rather, it is production of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) (Berg *et al*., 2002). NADPH has a crucial role in preventing oxidative damage to proteins and to other molecules in all cells (Figure. 2.1) (*ibid*). This role is particularly crucial in red cells because being efficient oxygen carriers, they have a built-in danger of damage by oxygen radicals generated continuously in the course of methaemoglobin formation (*ibid*). The highly reactive oxygen radicals either decay spontaneously or are converted by superoxide dismutase (SOD) to hydrogen peroxide ($H_2O_2$), which is still highly toxic. Detoxification of $H_2O_2$ to $H_2O$ is effected by catalase and by glutathione peroxidase (GSHPX) (Gaetani *et al*.,

1994). NADPH is crucial for the function of both enzymes; it is a structural component of catalase (*ibid*), and it is required as a substrate by glutathione reductase, which regenerates GSH when it has been oxidized to GSSG by GSHPX (Figure 2.1).

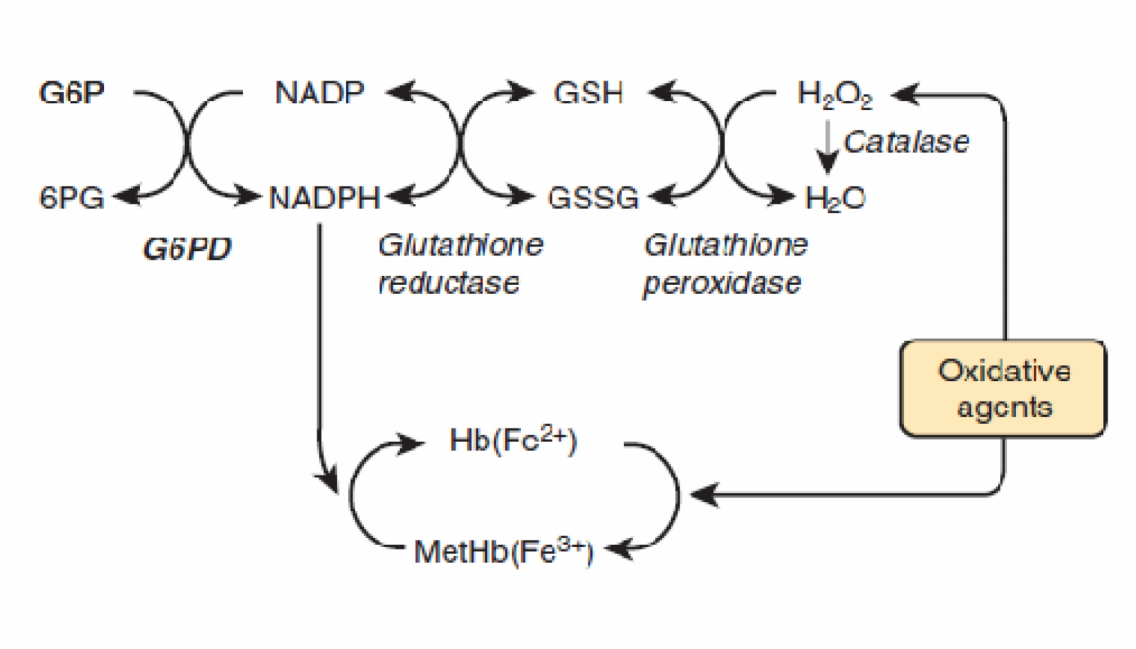


**Figure 2.1**:G6PD and the glutathione (GSH) cycle (Luzzatto *et al*., 2001)
*$NADP^+$ the electron acceptor yields NADPH in the oxidative phase, used to reduce glutathione, (GSSG) to GSH and to support reductive biosynthesis. The other product of the oxidative phase is ribose 5-phosphate, which serves as precursor for nucleotides, coenzymes, and nucleic acids. In cells that are not using ribose 5-phosphate for biosynthesis, the non-oxidative phase recycles six molecules of the pentose into five molecules of the hexose glucose 6-phosphate, allowing continued production of NADPH and converting glucose 6-phosphate (in six cycles) to $CO_2$.*

G6PD-deficient red cells are highly vulnerable to oxidative damage, even though G6PD deficiency is never complete in humans (Mason *et al*., 1995). When complete G6PD deficiency was produced in mouse embryonic stem cells (ESCs) by targeted inactivation

of the G6PD gene (Pandolfi *et al*., 1995), the G6PD-null cells thus obtained were viable, but they formed colonies only in a low-oxygen environment; even so, they had an impaired capacity to form erythroid colonies (Paglialunga *et al*., 2004). When G6PD-null ESCs were injected into mouse blastocysts, chimeric embryos were obtained, and germ line transmission was achieved; however, only female heterozygous mice were obtained because hemizygous male embryos died (Longo *et al*., 2002). Thus, a G6PD-null mutation is an embryonically lethal condition.

### 2.3 Structure of G6PD

The monomer of G6PD consists of 515 amino acids, with a molecular weight of about 59 kDa. A model of the three-dimensional structure of G6PD (Figure 2.2), was published in 1996 (Naylor *et al.*, 1996) and subsequently the crystal structure of human G6PD has been elucidated in 2000 (Au *et al*, 2000). The enzyme is active as a tetramer or dimer, in a pH-dependent equilibrium. Every monomer consists of two domains: the N-terminal domain (amino acids 27–200), with a β-α-β dinucleotide binding site (amino acids 38–44); and a second, larger, β+α domain, consisting of an antiparallel nine-stranded sheet (Au *et al.*, 2000). The two domains are linked by an α-helix, containing the totally conserved eight-residue peptide that acts as the substrate binding site (amino acids 198–206) (Mason, 1996).The structure, at 3 A (0·3 nm) resolution, reveals an $NADP^+$ (a coenzyme) molecule in every subunit of the tetramer, distant from the active site but close to the dimer interface (Au *et al.*, 2000). Stability of the active quaternary structures is crucial for normal G6PD activity.

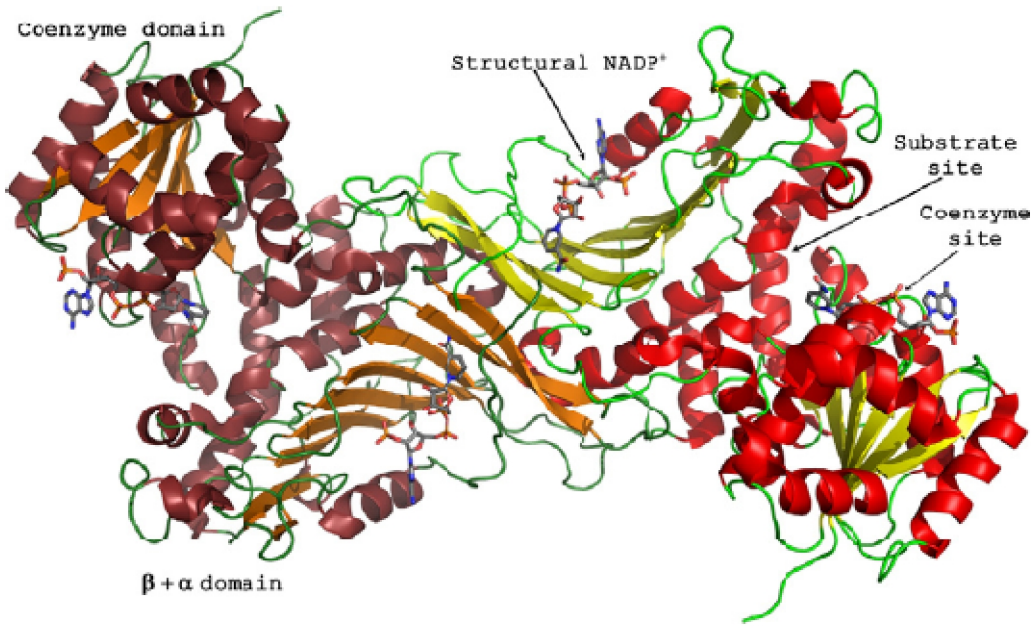


**Figure 2.2*:*** Three-dimensional model of active G6PD dimer (Mason *et al*., 2007).
*The human G6PD dimer: showing the domain structure, the active site and the position of the bound structural NADP molecule. One subunit is in red and green while the other is in shades of brown.*

Glucose-6-phosphate dehydrogenase is present in all cells; however, its concentration varies in different tissues (Mason, 1996).In healthy red blood cells, the enzyme operates at only 1–2% of its maximum potential (even under oxidative stress generated by methylene blue): a large reserve of reductive potential exists, which is substantially decreased in G6PD-deficient red-blood cells, leading to pathophysiological features (Au *et al.*, 2000). After G6PD deficiency was established as a clinical disorder, its phenotypic expression was noted to be heterogeneous (Beutler, 1990).

The World Health Organization (W.H.O) made initial recommendations for the characterization of G6PD deficiency, which have subsequently been updated (Betke *et al.*, 1967; W.H.O Working Group, 1989). Initially, the deficiency was characterized biochemically, by measuring residual enzyme activity and electrophoretic mobility. More than 400 biochemical variants of G6PD deficiency have since been defined according to other criteria, including physicochemical properties like thermostability, chromatographic behaviour, and kinetic variables such as Km for different substrates and optimum pH (Beutler, 1984).

The G6PD deficiency can be caused by a reduction in the number of enzyme molecules, a structural difference in the enzyme causing a qualitative change, or both. Examination of G6PD variants shows that, in most cases, G6PD deficiency is due to enzyme instability, implying that amino acid substitutions in different locations can destabilize the enzyme molecule (Au *et al.*, 2000).

**2.4 G6PD Terminology and Variants Classification**

G6PD is the accepted abbreviation for the enzyme glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49); the G6PD gene is designated *Gd* (Beutler, 1990). The terms *G6PD normal* and *G6PD deficient* are used to designate the phenotypes; G6PD(+) and G6PD(−) are used to designate the phenotypes of individual cells. Because *Gd* is X-linked, males can be only normal hemizygotes (*Gd*+) or deficient hemizygotes (*Gd*−); females can be normal homozygotes (*Gd*+/*Gd*+), deficient homozygotes (*Gd*−/*Gd*−), or heterozygotes (*Gd*+/ *Gd*−). The phenotype of the latter group is often referred to as "intermediate" because their overall red cell G6PD level usually lies in between the

normal and the deficient range. Consequently, G6PD deficiency should not be regarded as a recessive but rather as a co-dominant trait. According to a classification introduced in 1966 (Table 2.0), G6PD-deficient variants that result in congenital nonspherocytic haemolytic anemia (CNSHA) are designated class I; G6PD-deficient variants that do not result in CNSHA are designated class II or class III, depending on the severity of the reduction in enzyme activity in red cells. The separation between class II and class III is blurred and probably no longer useful. Class IV variants are those with normal activity. Class V was reserved for variants with increased activity, but after an initial report on G6PD Hektoen (Dern *et al*., 1969), none has been found. In practice, because the majority of G6PD-deficient persons are mostly asymptomatic, their G6PD deficiency is referred to as mild, simple, or common (corresponding to class II or III); the minority of persons who have CNSHA are referred to as having rare, sporadic, or severe G6PD deficiency (corresponding to class I). When a diagnostic test for G6PD is carried out, the phrase "positive result" is sometimes used to indicate that the test has shown G6PD deficiency; this has caused confusion; tests should therefore be correctly interpreted as haven shown either a *normal* result or a *deficient* result (W.H.O, 2010).

**Table 2.0 Heterogeneity and Clinical Expression of Glucose-6-Phosphate dehydrogenase deficiency**

| Class* | Clinical Manifestation | G6PD Activity (% of Normal) | Number of Known Mutants Alleles** | Example | Comments |
|---|---|---|---|---|---|
| IV | None | >85 | 2 | A, B | G6PD is the normal "wild type |
| II + III | Asymptomatic in the steady state, but risk for NNJ, AHA, favism | <30 | 75 | Med, A-, Orissa, Mahidol, Canton, Vanua Lava, Seattle | Most of these variants are known to be polymorphic |
| I | NNJ (severe), CNSHA, acute exacerbations | <10 in most cases | 61 | Sunderland, Nara, Guadalajara | Never polymorphic; but same mutation can recur |

**The separation between class II and class III is blurred and, in the authors' opinion, no longer useful.*

***Variants in class II and III can be regarded as having a mild phenotype confined to acute episodes; variants in class I have a severe phenotype with chronic illness*.

*AHA, acute hemolytic anemia*; *CNSHA, congenital nonspherocytic hemolytic anemia. NNJ, neonatal jaundice* (Luzzatto, 2006)

## 2.5 Genetics and molecular basis of G6PD deficiency

The *G6PD* gene is located at the telomeric region of the long arm of the X chromosome (band Xq28), close to the genes for haemophilia A, congenital dyskeratosis, and colour blindness (Figure 2.3) (Trask *et al*., 1991). It consists of 13 exons and 12 introns,

spanning nearly 20 kb in total (Table 2.0); it encodes 515 amino acids, and a GC-rich (more than 70%) promoter region. The 5' untranslated portion of the mRNA corresponds to exon I and part of exon II; the initiation codon is in exon II (Chen *et al*., 1991). In the promoter region, there are several binding sites for the transcription factor SP1—GGCGGG and CCGCCC sequences similar to those in other housekeeping gene promoters (Toniolo *et al*., 1991). Wild type *G6PD* is referred to as *G6PD B*.

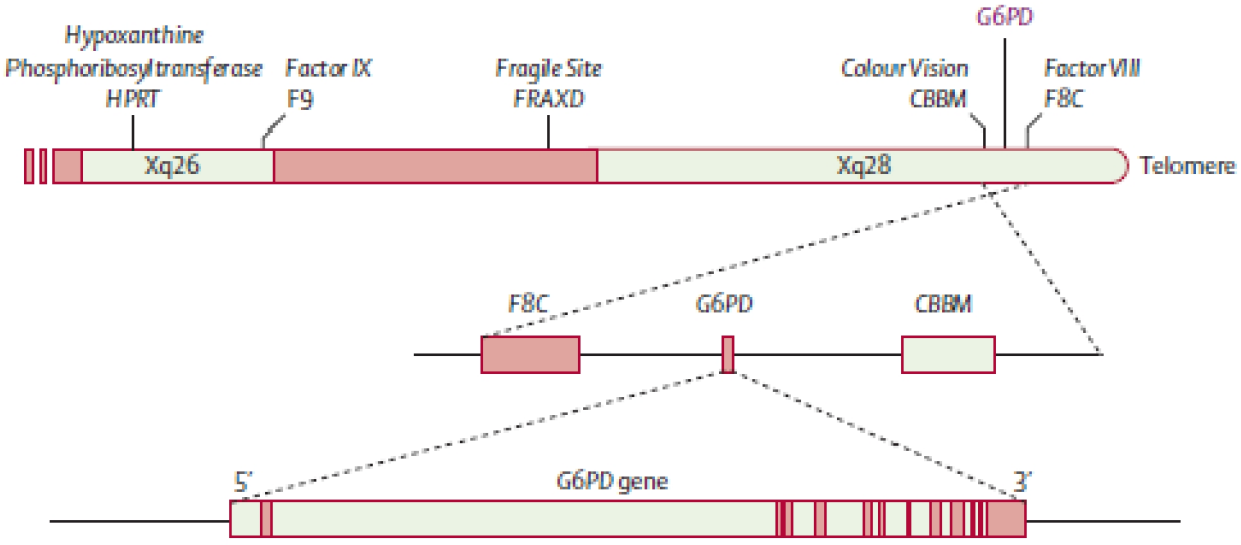


**Figure 2.3:** Location of *G6PD* gene on X chromosome (Beutler and Vulliamy, 2002).

All mutations of the *G6PD* gene that result in enzyme deficiency affect the coding sequence (Figure 2.4) (Vulliamy *et al*., 1997). The promoter region of *G6PD gene* has been characterized extensively, by band shift assays, also known as electrophoretic mobility shift assays, and systematic mutagenesis (Franze *et al*., 1998); however, no mutations have yet been reported in the human promoter region, although findings from

a mouse model have shown that mutations of GC boxes can affect transcriptional activity greatly (Philippe *et al*., 1994).

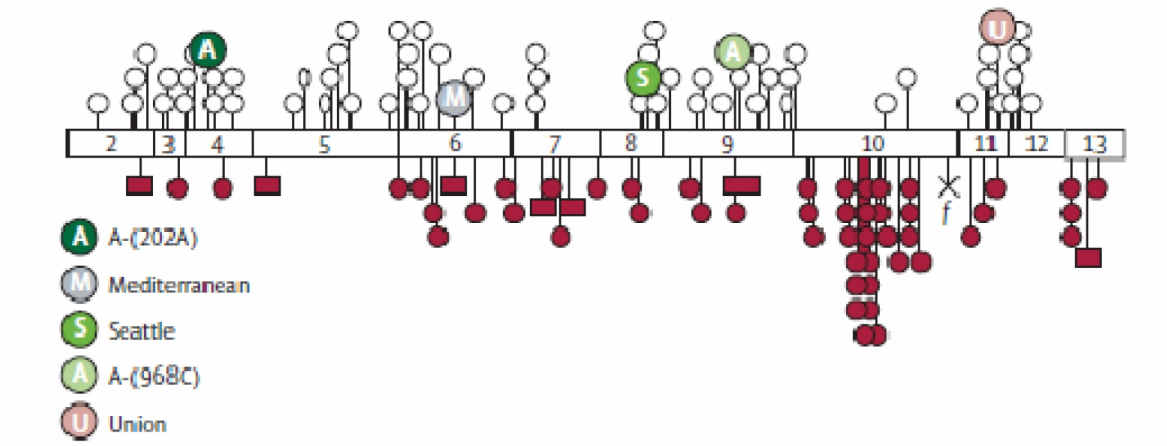


**Figure 2.4:** Most common mutations along coding sequence of *G6PD* gene (Luzzatto *et al*., 2001).

*Exons are shown as open numbered boxes. Open circles are mutations causing classes II and III variants. Filled circles represent sporadic mutations giving rise to severe variants (class I). Open ellipses are mutations causing class IV variants. Filled squares = small deletion. Cross = a nonsense mutation. f = a splice site mutation. 202A and 968C are the two sites of base substitution in G6PD-A.*

Point mutations are spread throughout the entire coding region; a cluster of mutations that cause a severe phenotype (class I, CNSHA) occurs in exons 10 and 11 (amino acids 380–430, close to the dimer interface) (Philippe *et al*., 1994). Analysis of the three-dimensional model of human G6PD enzyme, obtained from the crystallized protein, (Naylor *et al*., 1996; Au *et al*., 2000) has indicated that the $NADP^+$ binding site is located in a part of the enzyme close to the N terminus, with the highly conserved amino acid (in 23 species) Arg72 playing a direct part in coenzyme binding (Scopes *et al*.,

1998). The cluster of mutations around exons 10 and 11 designates the subunit interface, which interacts with other important residues located elsewhere but which is brought close to this domain by protein folding.

As mentioned above, 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) (Scopes *et al*., 1998). 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 (Cheng *et al*., 1999).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 (Hirono *et al*., 1997; Vulliamy *et al*., 1998).

In most cases, the mutations cause G6PD deficiency by decreasing the *in vivo* stability of the protein: thus, the physiological decrease in G6PD activity that takes place with red cell aging is greatly accelerated. In some cases, an amino acid replacement can also affect the catalytic function of the enzyme (Mason *et al*, 1999). Variants of G6PD can be referred to, mainly by their amino acid mutations. For instance, G6PD Mediterranean Ser188Phe [or 188 Ser →Phe] has a phenylalanine residue at position 188 instead of the normal serine residue. Also G6PD A- Val68Met [or 68 Val → Met], the commonest African mutation, has methionine at position 68 instead of the normal valine residue. The sporadic variant Tsukui Ser188del causes CNSHA, and has serine residue deleted at

position 188.A more strictly correct and unambiguous notation is to refer to the mutations by their base (nucleotide) change at the genomic or cDNA level. For example, the A- 202 G→ A mutation has an adenine at position 202 instead of the original guanine. The Mediterranean variant has thymine substituted for cytosine at position 563 [Med 563 C →T] (Mason *et al*., 2007).

Apart from mutations that lead to enzyme deficiency, several polymorphic sites in introns have been identified, enabling the definition of G6PD haplotypes (Vulliamy *et al*., 1991; Maestrini *et al*., 1992). These haplotypes have been used in an attempt to understand the evolutionary history of the *G6PD* gene. Looking at linkage disequilibrium in haplotypes themselves, and with coding sequence polymorphisms, dating of the most common mutations and estimation of the timeframe of malaria selection has been possible (Luzzatto, 2006).

The inheritance of G6PD deficiency shows a typical X-linked pattern, which was identified through favism having a higher incidence in males than in females, long before G6PD deficiency was identified as the cause (Beutler, 1996). Males are hemizygous for the *G6PD* gene and can, therefore, have normal gene expression or be G6PD-deficient. Females, who have two copies of the *G6PD* gene on each X chromosome, can have normal gene expression, be heterozygous or homozygous. Heterozygous females are genetic mosaics as a result of X-chromosome inactivation; in any cell, one X chromosome is inactive, but different cells randomly inactivate one chromosome or the other, and the abnormal cells of a heterozygous female can be as deficient for G6PD as those of a G6PD-deficient male: therefore, such females can be susceptible to the same pathophysiological phenotype (Beutler, 1996). Although

heterozygous women, on average, have less severe clinical manifestations than G6PD-deficient males, some develop severe acute haemolytic anaemia (Lim *et al*., 2005).

**Table 2.1 Molecular characteristics of *G6PD* gene**

| | **Number** |
|---|---|
| **DNA** | |
| Size of gene | 18.5kb |
| Total number of exons | 13 |
| Introns | 12 |
| **mRNA** | |
| Size in nucleotides | 2269 |
| 5' untranslated region* | 69 |
| Coding region* | 1545 |
| 3' untranslated region* | 655 |
| **Protein** | |
| Amino acids | 515 |
| Molecular weight | 59.265 kDa |
| Subunits per molecule of active enzyme | 2 or 4 |
| Molecules of tightly bound NADP per subunit | 1 |

* Number of nucleotides (Mason *et al*., 1999)

## 2.6 Epidemiology of G6PD-deficiency and malaria selection

Deficient *G6PD* alleles are distributed worldwide; a conservative estimate is that at least 400 million people carry a mutation in the *G6PD* gene causing deficiency (figure 2.5). The highest prevalence is reported in Africa, southern Europe, the Middle East, Southeast Asia, and the central and southern Pacific islands; however, because of fairly recent migration, deficient alleles are nowadays quite prevalent in North and South America and in parts of northern Europe (Frank, 2005). For any given population, definition of the quantitative contribution of each allele to the overall prevalence of G6PD deficiency is still difficult, since epidemiological studies based on enzyme activity screening have been imprecise and have not extended to global coverage.

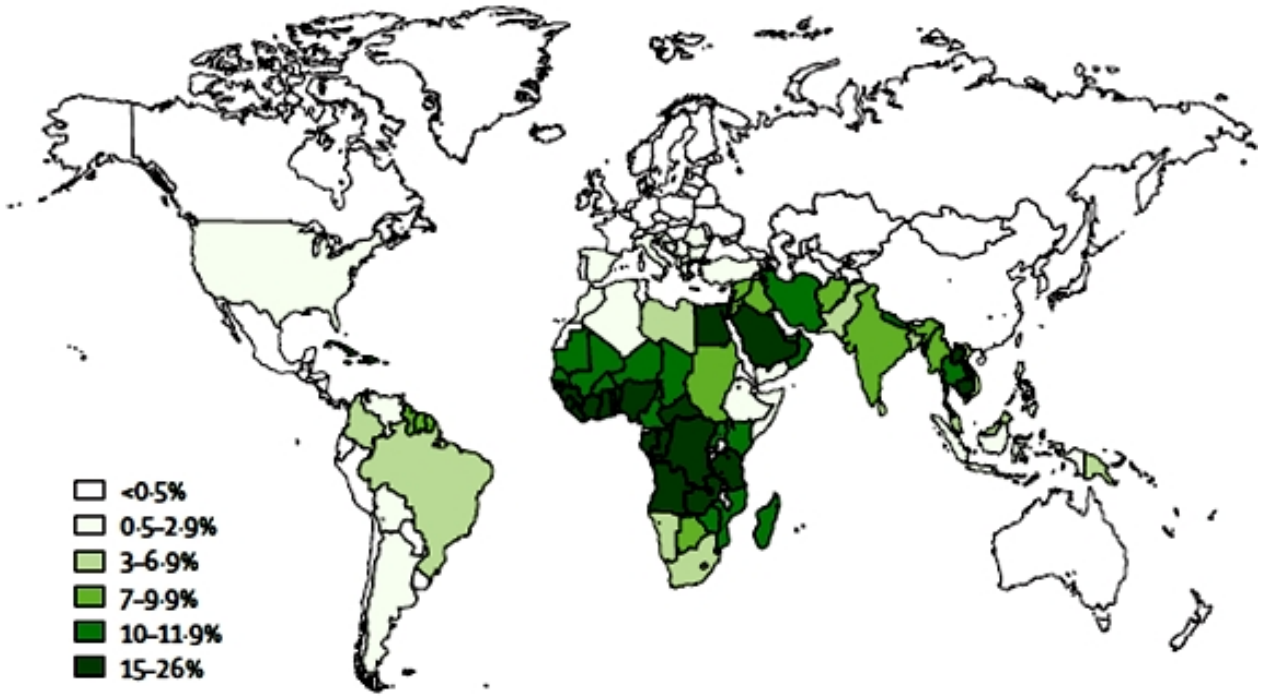


**Figure 2.5**: World map distribution of G6PD deficiency (WHO working group, 1989)

In recent years, molecular analysis has been used to map the prevalence of G6PD deficiency. The worldwide distribution of malaria is said to be remarkably similar to that of mutated *G6PD* alleles (Luzzatto, 1995), making the malaria hypothesis of G6PD deficiency - that G6PD deficiency is protective against malaria (Ruwende *et al*., 1995) -a generally well-accepted notion. Two apparent exceptions to the geographical rule - southern Europe and North America - have clear explanations. Malaria was only eradicated in southern Europe during the 20th century; in North America, the defect is confined to immigrants from regions where malaria is found, and the descendants of these immigrants (Greene, 1993). Findings of early studies designed to assess the connection between malaria and G6PD deficiency were somewhat contradictory (Oo *et al*., 1995). Ruwende and colleagues in 1995 noted that the *G6PD* A– allele is associated with a reduction in the risk of severe *Plasmodium falciparum* malaria, for female heterozygotes and male hemizygotes (46% and 58%, respectively).

Evidence of protection against malaria also comes from *in vitro* work in which parasites were cultured in red-blood cells with different G6PD genotypes. Several groups, comparing the growth of parasites in *G6PD* A– and *G6PD* Mediterranean mutated red-blood cells with that in healthy cells, showed that parasite growth is slowest in G6PD-deficient cells (Cappadoro *et al*., 1998). Intracellular schizogenesis, rather than invasion, is affected in G6PD-deficient red blood cells, in which oxidative injury of the parasite can happen (Clark and Hunt, 1981). Luzzatto and co-workers showed that red blood cells with normal G6PD activity, taken from *G6PD* A– heterozygous females had 2–80 times more parasitic growth than G6PD-deficient red-blood cells (Luzzatto *et al*., 2001). G6PD-deficient red-blood cells infected with parasites undergo phagocytosis by

macrophages at an earlier stage of parasite maturation than do normal red blood cells with parasitic infection, which could be a further protective mechanism against malaria (Cappadoro *et al*., 1998).

Several polymorphic alleles can be found in most areas of high, G6PD-deficiency prevalence. Tropical regions of Africa are one exception, where the variant *G6PD* A– accounts for about 90% of G6PD deficiency. *G6PD* A– is also frequent in North and South America, in the West Indies, and in areas where people of African origin are present. Moreover, *G6PD* A– is quite prevalent in Italy (Martinez di Montemuros *et al*., 1997), the Canary Islands (Pinto *et al.*, 1996), Spain and Portugal, and in the Middle East, including Iran, Egypt, and Lebanon (Beutler *et al*., 1989).

The second most common variant is *G6PD* Mediterranean, which is present in all countries surrounding the Mediterranean Sea, (Luzzatto *et al*., 2001) although it is also widespread in the Middle East, including Israel, (Karimi *et al*., 2003) where it accounts for almost all G6PD deficiency in Kurdish Jews (Oppenheim *et al*., 1993), India, and Indonesia. In several populations, such as the countries around the Persian Gulf, *G6PD* A– and *G6PD* Mediterranean coexist at polymorphic frequencies (Bayoumi *et al*., 1996). Other polymorphic variants are the Seattle and Union variants, which have been reported in Southern Italy, Sardinia (Fiorelli *et al*., 1989), Greece, the Canary Islands (Cabrera *et al*., 1996), Algeria, Germany, and Ireland. *G6PD* Union has also been reported in China, (Perng *et al.,* 1992).

## 2.7 Laboratory Diagnosis of G6PD Deficiency

Since the clinical picture of favism and other forms of acute haemolytic anaemia associated with G6PD deficiency is characteristic, the final diagnosis must rely on direct demonstration of decreased activity of this enzyme in red cells (Au *et al*., 2000). In neonatal jaundice (NNJ) and chronic nonspherocytic haemolytic anaemia (CNSHA), the differential diagnosis is much wider, and therefore this test is even more important. Fortunately, the enzyme assay is very easy, and numerous "screening tests" can be used as substitutes if a spectrophotometer is not available. However, a number of potential pitfalls and sources of error must be understood, and the use of commercial kits is not a substitute for such understanding (Kotaka *et al*., 2005). The value and limitations of the regular quantitative assays are discussed below with a mention of the use of alternatives methods.

### 2.7.1 Test for G6PD deficiency

G6PD can be assayed by the classic spectrophotometric method (Kotaka *et al*., 2005), which directly measures the rate of formation of NADPH through its characteristic absorption peak in the near ultraviolet spectrum at 340 nm. Red cell activity is expressed in international units (micromoles of NADPH produced per minute) per gram of haemoglobin; therefore, it is best to assay the enzyme activity and the haemoglobin concentration in the same haemolysate and ratio calculated. Because G6PD activity is much higher in leukocytes (particularly in granulocytes) than in erythrocytes, for accurate measurements, it is essential to remove all leukocytes by the Ficoll-Hypaque method or by filtration through cellulose powder (Morelli *et al*., 1981) rather than by the

traditional approach of separating the buffy coat; however, for the purpose of clinical diagnosis of G6PD deficiency, this is not strictly necessary (Kotaka *et al*., 2005).

In normal red cells the range of G6PD activity, measured at 30 °C, is 7 to 10 IU/g of haemoglobin (Luzzatto, 2006). Several "screening tests" for G6PD deficiency are useful and reliable provided that they are run properly and their limitations are understood. The most popular are the dye decolourization tests (Motulsky and Campbell-Kraut, 1961), the methaemoglobin reduction test (Beutler, 1966) and the fluorescence spot test (Brewer *et al.*, 1962). Recently, a formazan-based screening test has been developed (Tantular and Kawamoto, 2003) and field-tested (Jalloh *et al*, 2004). All these methods are semi-quantitative, and are meant to classify a sample simply as "normal" or "deficient."

Screening tests are especially useful for testing large numbers of samples. They are also perfectly adequate for diagnostic purposes in patients who are in the steady state but not for patients in the post-haemolytic period or with other complications (Jalloh *et al*, 2004); in addition, they cannot be expected to identify all heterozygotes. Finally, an ideal screening test ought not to give "false-negative" results (i.e., it should not misclassify a G6PD-deficient subject as normal), but it can be allowed to give a few "false-positive" results (i.e., a G6PD-normal subject might be misclassified as being G6PD deficient) (Tantular and Kawamoto, 2003). Ideally, every patient found to be G6PD-deficient by screening should be confirmed by the spectrophotometric assay (*ibid*).

### 2.7.2 The Effect of Red Cell Age and Selective Haemolysis

Because G6PD decreases gradually as red cells age, any condition associated with reticulocytosis will entail an increase in G6PD activity (Kotaka *et al*., 2005). This means that if a subject is genetically G6PD normal, in the course of haemolysis, red cell G6PD activity will now be above the normal range. This does not affect the diagnosis because G6PD deficiency will be correctly ruled out. However, if the subject is genetically G6PD deficient, red cell G6PD may now be raised to the extent of being near or even within the normal range, and the patient might therefore be misclassified as being G6PD normal (even though at the onset of the attack the level may have been low) (Jalloh *et al*, 2004).

Thus, after a haemolytic attack two circumstances concur to cause a risk of misdiagnosis: first, the older cells have been destroyed selectively; second, the marrow response has caused a sudden release of young cells into the peripheral blood (Kotaka *et al*., 2005). Although the reticulocyte count is a good warning to avoid this mistake, it must be realized that because reticulocytes turn into morphologically "mature" erythrocytes within 1 to 2 days, their count is not a sensitive index of mean red cell age; in other words, mean red cell age may be significantly younger than normal even when the reticulocyte count is normal (Beutler, 2001).

Several ways have been suggested to circumvent these problems. First, a G6PD level in the low-normal range in the presence of reticulocytosis is always suspicious, because it suggests that the patient is actually G6PD-deficient (Jalloh *et al*, 2004). Second, if the patient is suffering or is recovering from acute haemolytic anaemia, the suspicion

generated from the finding above can be simply kept in store for a few weeks, when the situation will be evolving toward the steady state, and a repeat test will prove whether the patient is indeed G6PD-deficient (Kotaka *et al*., 2005). Also, if either the urgency of some clinical decision or academic curiosity demands a more prompt solution of the problem, the presence of severely G6PD-deficient red cells can be demonstrated either by enzyme assay of the oldest cells (fractionated by sedimentation) or by a cytochemical method (Luzzatto and Poggi, 2008).

### 2.7.3 G6PD Deficiency in Heterozygotes

For haemolysis to be clinically significant in heterozygous females, at least 50% of the red cells must be deficient, and therefore the G6PD level will be about 50% of normal or less. This level of deficiency can be diagnosed by a quantitative test; however, the problems associated with current or recent haemolysis outlined for male patients will be compounded in the case of heterozygous females, and they can usually be overcome by a similar approach, particularly with the use of a cytochemical test (Jalloh *et al*., 2004). In cases of "extreme phenotypes, the G6PD-deficient red cells may be so few that the only way to identify heterozygous G6PD deficiency will be by DNA analysis, for which the underlying mutation must be known or identified for the purpose (*ibid*). A special situation involves heterozygotes for G6PD variants associated with CNSHA. According to Herschel and Beutler (2001), the mothers of (male) patients with this condition are often G6PD normal, either because the variant in the offspring is due to a *de novo* mutation or because the mother is a heterozygote but is phenotypically normal, presumably because somatic selection has favored the haematopoietic progenitor cells with the normal G6PD allele.

In the vast majority of cases, the family history, the clinical course, and a G6PD assay are sufficient to establish the diagnosis of conditions associated with G6PD deficiency. In special cases (e.g. heterozygotes) and particularly in the case of CNSHA, identification of mutations in the G6PD gene can be carried out, with reference to the large numbers of mutations already known (Human Gene Mutation Database, http://www.hgmd.cf.ac.uk/ac/gene.php?gene=G6PD, retrieved 13/09/2012), also in this, way new mutations are still likely to be discovered.

More so, molecular analysis is the only method by which a definitive diagnosis can be made of a female's status. Complete biochemical characterization of G6PD enzyme is needed only for definition of a new variant, as recommended by W.H.O, although inter-laboratory variations have resulted in the new variants being identified in error (Cappellini *et al*., 1996).

The development of simple molecular methods of diagnosis such as polymerase chain reaction (PCR), direct sequencing, denaturing gradient gel electrophoresis, which allow detection of specific mutations, have enabled population screening, family studies and prenatal diagnosis in rare, very severe cases (Mason, 1996). The most common mutations (Mediterranean, A–, Seattle, and Union) can be rapidly detected by restriction enzyme analysis, after PCR amplification of the appropriate *G6PD* exon (Martinez di Montemuros *et al*., 1997).

In practical terms, testing for G6PD deficiency should be considered when an acute haemolytic reaction triggered by exposure to a known oxidative drug, infection, or ingestion of fava beans happens, either in children or in adults, particularly if they are of

African, Mediterranean, or Asian descent. Moreover, members (especially males) of families in which jaundice, splenomegaly, or cholelithiasis are recurrent should be tested for G6PD deficiency (Fiorelli *et al*., 2000). Newborn babies with severe neonatal jaundice, particularly those of Mediterranean or African ancestry, are quite likely to have G6PD deficiency (Mason, 1996).

### 2.8 Clinical Manifestations and Significance

Most G6PD-deficient individuals are asymptomatic throughout their life, and unaware of their status. The illness generally manifests as acute haemolysis, which usually arises when red blood cells undergo oxidative stress triggered by agents such as drugs, infection, or the ingestion of fava beans. G6PD deficiency does not seem to affect life expectancy, quality of life, or the activity of affected individuals (Cocco *et al*., 1998), and the deficiency usually presents as drug-induced or infection-induced acute haemolytic anaemia, favism, neonatal jaundice, or chronic non-spherocytic haemolytic anaemia. Several clinical disorders, such as diabetes (*ibid*) and myocardial infarction (Lee *et al*., 1996), and strenuous physical exercise (Ninfali and Bresolin, 1995), have been reported to precipitate haemolysis in G6PD-deficient individuals; however, coexisting infection or oxidant drug exposure can be the underlying cause in these instances.

The precise mechanism by which increased sensitivity to oxidative damage leads to haemolysis is not fully known; furthermore, the exact sequence of events, once an exogenous trigger factor is present is also unknown. Whatever the cause of the acute haemolysis in G6PD deficiency, it is characterized clinically by fatigue, back pain,

anaemia, and jaundice (Edwards, 2002). Increased unconjugated bilirubin, lactate dehydrogenase, and reticulocytosis are markers of the disorder.

### 2.8.1 Drug-Induced Haemolytic Anaemia

The discovery of G6PD deficiency, as noted earlier, was a coincidence of an investigation into antimalarial-induced haemolysis in patients who had received primaquine (Beutler, 1996). Subsequently, several drugs have been linked to acute haemolysis in G6PD-deficient individuals (Table 2.0) (Luzzatto and Poggi, 2008). It has been established that primaquine's mode of cell damage is by acting as an electron donor to oxygen which is bound in the elemental form to haemoglobin iron. The iron in the ferrous state can provide only one electron to the bound oxygen therefore avoiding the formation of free radicals and peroxide. Primaquine cause oxidative damage in the red blood cell by donating the second electron which accelerates the oxidative process (Beutler, 1996) and increasing the activity of membrane ATPase. The hydrolysis of ATP will induce morphological changes in red blood cells and eventually, haemolysis.

It is often difficult to establish whether a specific drug directly causes haemolytic crisis in G6PD-deficient patients because an agent deemed to be safe for some G6PD-deficient individuals is not necessarily safe for all patients - not least because pharmacokinetics can vary between individuals. Also, drugs with potential oxidant effects are sometimes administered to patients with an underlying clinical condition (such as infection) that could lead to haemolysis. Moreover, patients often take more than one type of medication. Lastly, haemolysis in G6PD deficiency is a self-limiting process and, therefore does not always produce clinically significant anaemia and reticulocytosis

(Cheng*et al*., 1999). Usually, safe alternative agents are available that doctors should be aware of. If no alternatives exist, treatment decisions are based on clinical judgment of risk. *In vitro* tests to establish the likelihood of haemolysis have been developed, but are not yet available in clinical practice (Luzzatto *et al.*, 2001).

**Table 2.2: Drugs and chemicals associated with substantial haemolysis in patients with G6PD deficiency**

| | Definite association | Possible association | Doubtful association |
|---|---|---|---|
| Antimalarial | Primaquine | Chloroquine<br>Pamaquine | Mepacrine<br>Quinine |
| Sulfonamides | Sulfanilamide<br>Sulfacetamide<br>Sulfapyridine<br>Sulfamethoxazole | Sulfadimidin<br>Sulfasazine<br>Glibenclamide | Aldesulfone<br>Sulfadiazine<br>Sulfafurazoles |
| Sulfones | Dapsone | ---------- | ---------- |
| Nitrofurantoin | Naturofurantoin | ---------- | ---------- |
| Antipyretic/ Analgesic | Acetanilide | Aspirin | Paracetamol<br>Phenacetin |
| Other drugs | Nalidixic acid<br>Niridazole<br>Methylthionium<br>Phenazopyridine<br>Co-trimoxazole | Ciprofloxacin<br>Chloramphenicol<br>Vitamin K analogues<br>Ascorbic acid<br>Mesalazine | Aminosalicyclic acid<br>Doxorubicin<br>Probenecid<br>Dimercapol |
| Other Chemicals | Naphthalene<br>2, 4, 6-trinitrotoluene | Acalypha indica extract | |

(Luzzatto and Poggi, 2008)

Clinically-detectable haemolysis and jaundice typically arise within 24–72 hours of drug dosing. Dark urine due to haemoglobinuria is a characteristic sign (Luzzatto *et al*., 2006). Anaemia worsens until days 7–8 and after drug cessation, haemoglobin concentrations begin to recover after 8–10 days. Heinz bodies (denatured haemoglobin precipitates) in peripheral red blood cells, detected by methyl violet staining, are a typical finding.

### 2.8.2 Infection-Induced Haemolytic Anaemia

The most typical cause of haemolysis in people with G6PD deficiency is probably infection. Hepatitis viruses A and B, cytomegalovirus (Siddiqui and Khan, 1998), pneumonia, and typhoid fever are all notable causes (Luzzatto, 1995).The severity of haemolysis can be affected by many factors, including concomitant drug administration, liver function, and age. The total bilirubin concentration can be increased by hepatitis as well as haemolysis, which is a potential source of diagnostic error when haemolysis is precipitated by hepatitis (Choremis *et al*., 1996). In severe haemolysis, prompt transfusions can substantially and rapidly improve the clinical course. Acute renal failure is a serious potential complication of viral hepatitis and concomitant G6PD deficiency; pathogenetic factors include acute tubular necrosis due to renal ischaemia, and tubular obstruction by haemoglobin casts. Some patients with haemolysis need haemodialysis (Selroos, 1992). This complication (acute renal failure) is however rare in children (*ibid*).

### 2.8.3 Favism

Ingestion of fava beans (*Vicia faba*) has long been known to be able to cause haemolysis, the phenomenon so-called favism. Apart from the Mediterranean countries where it was originally noted, the disease is also found in the Middle East, the Far East and North Africa where there is widespread growth and consumption of fava beans (Belsey, 1973). Favism is now widely believed to be most frequently associated with the Mediterranean variant of G6PD deficiency. Not all G6PD-deficient individuals undergo favism after ingestion of fava beans, and even the same individual can have an unpredictable response, suggesting that several factors affect development of the disorder, including the health of the patient and the amount of fava beans ingested (Meloni *et al*., 1983). Favism can develop after ingestion of dried or frozen beans, but is particularly likely to occur after eating fresh beans; the disorder is most frequent in the period when beans are harvested (Arese and De Flora, 1990).

Fava beans are rich in two glycosidic compounds, vicine and convicine, which account for nearly 2% of the dry weight. Upon ingestion, the glycosides are hydrolysed enzymatically to form pyrimidine aglycones, divicine and isouramil respectively. The proposed mechanism for the cause of favism is that these new compounds - divicine, isouramil, and convicine, the toxic constituents of fava beans - then undergo redox cycling (Arese and De Flora, 1990). This increases the activity of the hexose monophosphate shunt and in the process, deplete reduced glutathione (GSH), leading to the formation of free radicals and hydrogen peroxide, which facilitates haemolysis in G6PD-deficient patients(*ibid*).Breastfed babies whose mothers have eaten fava beans are also at risk for haemolysis (Schiliro *et al*., 1979). Favism presents as acute haemolytic

anaemia, usually 24 hours after ingestion. Haemoglobinuria is more severe than that caused by haemolytic crises triggered by drugs or infection, although bilirubin concentrations are lower. Anaemia is generally acute and severe, leading to acute renal failure in some patients, due either to ischaemia or to precipitation of haemoglobin casts. The oxidative damage that takes place in patients with favism causes a series of changes to erythrocytes, leading to rapid clearance of these cells from the circulation. For this reason, haemolytic events in patients with favism can be either intravascular or extravascular (i.e., in the spleen) (Fisher *et al*., 1985). A patient undergoing a severe haemolytic attack can require a blood transfusion. Prevention campaigns in areas with high prevalence of G6PD deficiency, through neonatal screening and health education, have greatly reduced the incidence of favism (Meloni *et al*., 1992).

### 2.8.4 Neonatal Jaundice

Bilirubin from the catabolism of haem is bound to albumin in the circulation and transported to the liver where it combines enzymatically with glucuronic acid producing mono and diglucuronides, which are excreted into the bile and the gut (Maisels, 2006). The conjugation reaction is catalyzed by uridine diphosphate glucuronosyltransferase. In newborns much of the conjugated bilirubin in the gut is hydrolysed back to unconjugated bilirubin and reabsorbed into the bloodstream via the enterohepatic circulation, placing further stress on the liver. In the neonate, there is always a degree of hyperbilirubinaemia, as it has to clear the bilirubin that was previously passed through the placenta. Early or late neonatal jaundice, defined as inappropriately high bilirubin levels for age and weight of the newborn, can occur and if untreated, can lead to chronic

bilirubin encephalopathy or kernicterus, which can leave the child with mental retardation (Mason *et al*., 2007).

Data from a series of studies suggest that about a third of all male newborn babies with neonatal jaundice have G6PD deficiency; however, the deficiency is less common in female neonates with jaundice (Corchia *et al*., 1995). Jaundice is usually evident by 1–4 days of age, similar to physiological jaundice, but is seen at a later time than in blood group alloimmunisation (i.e., rhesus incompatibility). Kernicterus, although rare, can produce permanent neurological damage if not promptly managed (Johnson and Bhutani, 2002; Sgro *et al*., 2006).

Glucose-6-phosphate dehydrogenase deficiency and neonatal jaundice vary widely in their frequency and severity in different populations. Genetic,(Kaplan *et al*., 1996) cultural (Seidman *et al*., 1995), and environmental factors such as maternal exposure to oxidant drugs (*ibid*), herbal remedies, or the effect of naphthalene (camphor) balls that are sometimes used to preserve baby's clothes (Valaes*et al*., 1963) can contribute to these differences. Neonatal jaundice is more typical and more severe in premature infants with G6PD deficiency than in babies born within the normal gestation period (Lopez and Cooperman, 1971). Haemolysis does not seem to contribute as much as impaired bilirubin conjugation and clearance by the liver (Kaplan *et al*., 1996). G6PD-deficient newborn babies who also inherit a mutation of the uridine-diphosphate-glucuronosyltransferase 1 (*UGT1A1*) gene promoter, which causes Gilbert's syndrome, a mild benign liver condition (usually caused by homozygousity for a common polymorphism in the *UGT1A1* promoter), are particularly at risk for neonatal jaundice

(Nicolaidou *et al*., 2005). Treatment of neonatal jaundice in G6PD deficiency follows the same standards of care as that provided for other causes.

To prevent hyperbilirubinaemia and kernicterus in newborns, screening of newborns from populations with a high prevalence of G6PD deficiency has been proposed. However, where screening for the enzymopathy is not undertaken routinely, assessment of neonates should be done in those who develop hyperbilirubinaemia (bilirubin concentrations greater than the 95th percentile [150μmol/L]) within the first 24 hours of life, or in those with a history of neonatal jaundice in siblings (American Academy of Pediatrics, 2004).

### 2.8.5 Congenital Non-Spherocytic Haemolytic Anaemia

Congenital nonspherocytic hemolytic anaemia (CNSHA) of varying severity occurs in males with the rare sporadic G6PD variants (Fiorelli *et al*., 2000) that show very low levels of enzyme activity in red cells and occasionally in other tissues. CNSHA most likely arises when the red cell cannot maintain a sufficient supply of NADPH in the steady state, to prevent the building up of oxidizing agents generated during normal metabolism, either from the oxidation and reduction of haem or from the process of phagocytosis. Subsequently oxidizing agents cause red cell membrane damage and haemolysis. As in other hereditary haemolytic anaemias patients present with neonatal jaundice, increased frequency of gallstones and an enlarged spleen and severe forms may involve lifetime dependence on blood transfusions.

Patients with G6PD-related CNSHA are also subject to acute attacks of haemolysis precipitated by the same agents as people with the common polymorphic variants.

However acute haemolysis in G6PD CNSHA patients can be caused by a greater number of drugs and at lower doses than in those with the polymorphic variants (Associazone Italiana Favismo, deficit di G6PD: www.favism.org).Diagnosis of this complication is based on clinical findings; the disorder is usually suspected during infancy or childhood. Many patients with CNSHA caused by G6PD deficiency have a history of severe neonatal jaundice, chronic anaemia exacerbated by oxidative stress that typically requires blood transfusions, reticulocytosis, gallstones, and splenomegaly (Luzzatto *et al*., 2001). Concentrations of bilirubin and lactate dehydrogenase are raised and, unlike in the acute haemolytic anaemia described above, haemolysis is mainly extravascular.

Management of this anaemia does not differ from that provided for CNSHA of other aetiology: surveillance and regular folic acid, red cell blood transfusions when needed, and iron chelators if iron overload develops. Although patients may not respond to splenectomy, this is generally performed in the severe patients and sometimes results in higher haemoglobin levels (Alfinito *et al*., 1994). In severe forms, identification of the mutation, genetic counseling and prenatal diagnosis are proposed.

### 2.9 Management of G6PD deficiency

The most effective management strategy for G6PD deficiency is to prevent haemolysis, by avoiding oxidative stressors (such as drugs and fava beans). This approach, however, requires the patient to be aware of their deficiency, as a result of a previous haemolytic episode or a screening programme. Incidentally, acute haemolysis in G6PD-deficient individuals is usually short-lived, and does not need specific treatment since normally, reticulocytes and younger erythrocytes retain about 10% of residual enzyme activity

comparable to normal G6PD. In rare cases (usually children), acute haemolysis leading to severe anaemia can require transfusions of red blood cells. Neonatal jaundice caused by G6PD deficiency is treated in the same way as neonatal jaundice of other causes. Some controversy still exists about treatment in relation to bilirubin concentrations (Newman and Maisels, 1992).Usually, when the concentration of unconjugated bilirubin approaches or exceeds 150 μmol/L, patients are given phototherapy to prevent neurological damage; at higher concentrations (>300 μmol/L), a blood transfusion may be necessary. Patients with congenital non-spherocytic haemolytic anaemia sometimes have a well-compensated anaemia that does not require blood transfusions; however, these individuals need to be monitored, because any exacerbating event (such as infection or ingestion of an oxidant drug) can severely worsen the degree of anaemia. Very rarely however, congenital non-spherocytic haemolytic anaemia is transfusion-dependent, so an iron-chelation treatment has to be administered.

Antioxidants such as vitamin E and Selenium seem to have some effect in patients with chronic haemolysis, but no consistent data to support this strategy are available (Nicolaidou *et al*, 2005). Patients with congenital non-spherocytic haemolytic anaemia sometimes develop splenomegaly, but do not usually benefit from splenectomy (Beutler, 1996).Gallstones are a possible complication of haemolysis due to G6PD deficiency (*ibid*). The prenatal diagnosis of G6PD deficiency has been reported, although this approach is questionable when the low mortality and morbidity of G6PD deficiency are considered (Beutler *et al*., 1992). For severe cases of the deficiency, which are refractory to other treatments, gene therapy remains a matter for consideration.

### 3.0 G6PD-deficiency in Ghana

In treatment of malaria, G6PD-deficiency has been implicated as the cause of major problems. Dapsone, used in combination therapy for the treatment of *Plasmodium falciparum* (W.H.O, 1989), and primaquine, used to eliminate the hypnozoite reservoirs of *Plasmodium vivax* and *Plasmodium ovale*, can induce serious haemolytic events (W.H.O, 2006; Fanello *et al*. 2008). According to the Ghana National Drugs Programme (2004), pregnant women who have glucose-6-phosphatase dehydrogenase deficiency or are allergic to sulpha-containing medicines should avoid sulphadoxine-pyrimethamine. Treatment with anti-malarial drugs should therefore be preceded by screening for G6PD-deficiency to prevent haemolytic anaemia (*ibid*).

The striking similarity between the areas where G6PD-deficiency is common and *Plasmodium falciparum* malaria is endemic provides circumstantial evidence that G6PD deficiency confers resistance against malaria (Ruwende and Hill, 1998; Tripathy and Reddy, 2007).

Mockenhaupt *et al.,* (2003) studied 530 pregnant women presenting for routine antenatal care at the Presbyterian Hospital in Agogo, Ghana, to ascertain the prevalence of *Plasmodium falciparum* infection in G6PD-deficient individuals. *P. falciparum* prevalence was found to be highest in G6PD normal women, lower in subjects with heterozygous and lowest in individuals with homozygous G6PD deficiency.G6PD status was assessed in 142 patients visiting the Central Regional Hospital in Cape Coast, Ghana, the prevalence of *P. falciparum* infection was 98.6% in G6PD non-deficient individuals, 1.4% in partially-deficient patients and 0% in fully-deficient individuals,

suggesting that the enzymopathy may play a role in prevention of malaria (Adinortey *et al*., 2011). Regarding the use of sulphadoxine - pyrimethamine for Intermittent Preventive Treatment (IPT) , results from three sentinel sites monitoring the G6PD prevalence in pregnant women, showed full G6PD prevalence rate of 2.9% and partial G6PD prevalence rate of 17.7% (Anti-Malaria Drug Policy for Ghana, 2009).

Identification of mutations that cause some pathology is of interest in understanding the nature of the genetic disease and also in obtaining information concerning the corresponding function of the normal gene in the population. However, regarding G6PD deficiency, there is no empirical data on the precise mutations underlying the enzymopathy among Ghanaians since all previous studies have been defined along the prevalence and correlation of the defect with *P. falciparum* infection.

# CHAPTER THREE

## 3.0 MATERIALS AND METHOD

### 3.1 Study site

The study was conducted in Cape Coast in the Central Region of Ghana. The Central Region occupies an area of 9,826 square kilometres or 4.1% of Ghana's land area (http://ghanadistricts.com/region/?r=3&sa=92, retrieved 15/12/2012). It shares common boundaries with Western Region on the west, Ashanti and Eastern Regions on the north, and Greater Accra Region on the east. On the south is the 168-kilometre length Atlantic Ocean (Gulf of Guinea) coastline (http://en.wikipedia.org/wiki/Central_Region_%28Ghana%29, retrieved 15/12/2012). The region has a population of 2,107,209 with a growth rate of 2.7% per annum. It is also the second most densely populated in the country, with a population density of 214 persons per square kilometer (Ghana Statistical Services, 2010).Samples were collected from patients visiting the Out Patient's Department (OPD) of the Central Regional Hospital. This hospital serves as referral health facility and is located in Cape Coast metropolis, the administrative capital of the region.

### 3.2 Ethical consideration

The study was approved by the Committee on Human Research, Publications and Ethics (CHRPE) of the Kwame Nkrumah University of Science and Technology and Komfo Anokye Teaching Hospital, Kumasi. Each volunteer signed or thumb-printed an informed written consent after the study had been explained to them in a language they

understand. All protocols followed were in line with the ethical standards of the Ghana Ministry of Health.

### 3.3 Sample Collection and Preparation

Blood samples were obtained (between June, 2011 and January, 2012) from 200 Ghanaians most of them traders, fishermen and students, who reside in the neighbouring communities in Cape Coast. About 5ml of blood samples were taken from each consenting voluntary participant by an experienced phlebotomist in a standard venipuncture into EDTA vacutainer tubes and labeled with unique identification codes. Biochemical screening of samples for G6PD deficiency was performed at a laboratory at the haematology unit of the hospital. Samples that tested positive for the enzymopathy during screening, were blotted on Whatman® filter papers (Whatman International Limited, England), dried, sealed in plastic envelopes and stored at -20$^{o}$C awaiting DNA extraction.

### 3.4 Laboratory Analysis

#### 3.4.1 Biochemical Characterization (G6PD Screening): G6PD Assay

The semi-quantitative assay of G6PD activity, namely the methaemoglobin reduction test, was done according to W.H.O recommendations (Betke *et al*., 1967, Luzzatto, 2006). The principle of this screening procedure consists of oxidation of haemoglobin (Hb) to methaemoglobin (MetHb) by sodium nitrite and subsequent enzymatic reconversion to haemoglobin in the presence of methylene blue; this occurs by stimulation of the pentose phosphate pathway and activation of NADPH

methaemoglobin reductase. Thus, in the presence of methylene blue, methaemoglobin is reduced primarily through the oxidative pathway, and the rate of reduction is therefore proportional to the G6PD activity of the cell.

Clean test tubes were arranged and labeled Test, Normal, and Deficient together with patient unique ID. Into each of the tubes labeled Test, 50 µl of sodium-nitrite-glucose and 50 µl of methylene blue reagents were dispensed. To the tubes labeled Deficient, only 50 µl sodium-nitrite-glucose solution was dispensed and to those labeled Normal, no reagent was dispensed. 1.0 ml of the blood sample was then dispensed into all the tubes and mixed after which they were corked with cotton wool and incubated at 37 °C for 3 h.

At the end of the incubation, 3 clean test tubes were arranged and labeled as before (T, N, D), 10 ml of distilled water was dispense into each of the tubes and 0.1 ml of the respective incubated sample was transferred into each of the tubes accordingly and mixed gently. Colour observation and comparison of the three tubes were done to interpret the results as described by Cheesbrough (2005) and explained below:

**A.** 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 3.1).

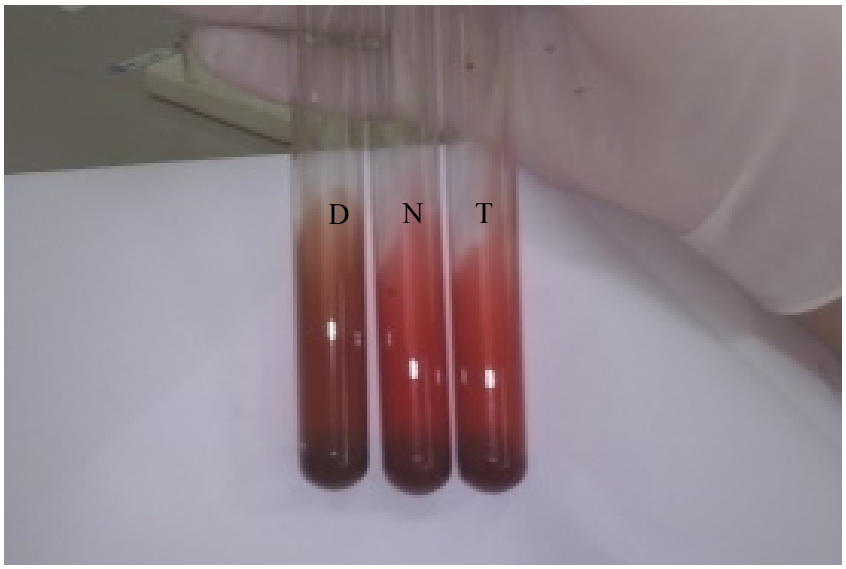


**Figure 3.1**: Sample showing normal G6PD activity. *D = deficient; N = normal and T = Test*

**B**. 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 and homozygous females (figure 3.2).

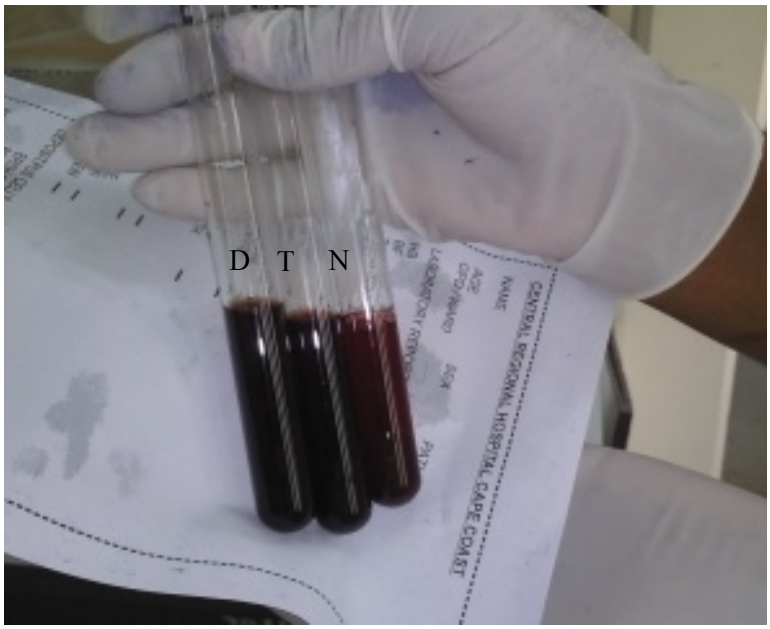


**Figure 3.2**: Sample showing full G6PD-deficiency in a hemizygous male. *D = deficient; N = normal and T = Test*

**C**. When the solution in test tube T has intermediate colour as compared to the positive reference tube D and the normal reference tube N, a partial defect in G6PD activity is present which is mostly observed in heterozygous females (figure 3.3).

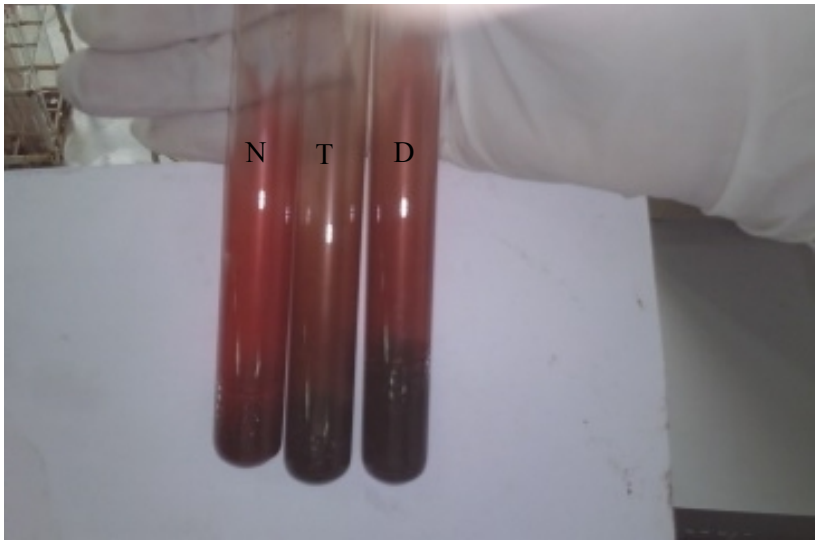


**Figure 3.3**: Sample showing partial G6PD-defect. *D = deficient; N = normal and T = Test*

### 3.5 Molecular Characterization

#### 3.5.1 DNA Extraction

Genomic DNA was extracted from dried blood spots spotted on Whatman filter paper using a modification of the frozen blood protocol of the HiYield™ Genomic DNA Mini Kit (Real Genomics, Real Biotech Corporation, USA).

Filter paper discs (6 x 10 mm diameter portion of each blood spot) were punched directly into 1.5 ml micro-centrifuge tubes containing 200 µl phosphate buffered saline

(PBS). 20 µl of Proteinase K (10 mg/ml) was added and the mixture incubated at 60 $^{o}$C in a water bath for 30 min. The mixture was centrifuged briefly to remove drops from the lid. 200 µl of Genomics Biotech (GB) Buffer was added to the tube, mixed by pulse vortexing and incubated at 85 $^{o}$C for 10 min until the sample lysate was clear. The tubes were inverted every 2 min during the incubation.

An amount of 200 µl of absolute ethanol was then added to the sample lysate and mixed immediately by vortexing for 10 s. Any precipitate formed during this step was broken up by pipetting, followed by brief centrifugation to remove drops from inside the lid. GB (silica) columns were placed in 2 ml Collection Tubes (supplied with kit) and the total mixture (including any precipitate) from previous steps was applied to the GB column. The column cap was then tightly capped and centrifuged at 13,000 revolutions per minute (rpm) for 5 min to bind nucleic acid to the silica gel, the flow-through was discarded and GB column placed back in the Collection Tubes.

400 µl of Wash (W1) Buffer was dispensed into the GB column, centrifuged at 13,000 rpm for 30 s, and the flow-through again discarded. This was followed by addition of 600 µl of Wash Buffer and spinning at 13,000 rpm for 30 s. The flow-through was again discarded; GB column placed back in the collection tube and spun at full speed for 3 min to dry the column matrix, thus, completing the washing step.

The dried GB column was transferred into a clean 1.5 ml microfuge tube. 100 µl of 70 $^{o}$C preheated GB Elution Buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0) was dispensed into the center of the column matrix and allowed to stand at room temperature for 5 min until the buffer was absorbed by the matrix. It was then centrifuged at 13,000 rpm for

30 s to elute purified DNA. This DNA elution step was repeated (two times) to increase DNA recovery in a total elution volume of 200 µl. The eluted DNA was then stored at -20 $^{o}$C.

### 3.5.2 Polymerase Chain Reaction

The coding regions of the *G6PD* gene encompassing the A, A- and Mediterranean mutations were analyzed using Polymerase Chain Reaction (PCR) and oligonucleotide primer sets appropriate for the amplification of exons 4, 5, and 6 of the gene as previously described by Bouanga *et al*., (1998) and Daoud *et al*., (2008) (Table 3.0).

**Table 3.0 Set of primers utilized for amplification of encoding regions of G6PD gene underlying three known variants**

| Variant | Exon | Set of primers used (5' – 3') |
|---|---|---|
| A- | 4 | TACAGCTGTGCCCTGCCCT (F)<br>CCGAAGCTGGCCATGCTGG (R) |
| A | 5 | CTGTCTGTGTGTCTGTCTGTC (F)<br>GAGGGCAACGGCAAGCCTT (R) |
| Mediterranean | 6 | GCAGCTGTGATCCTCACTCC (F)<br>CTCCTGAGTACCACCCCCAC (R) |

A 25 µl amplification reaction mixture for the PCR contained: 4 µl of template DNA (150 ng genomic DNA), 2.5 µl of 10X Buffer with 15 mM $MgCl_2$ for Taq polymerase (Genel Ltd, USA), 1 µl of 10 mM each primer (Table 3.0), 0.8 µl of 10 mM each dNTPs (Bioline Ltd., U.K), 0.25 µl of 5 U/ µl Taq DNA polymerase (Kappa Biosystems, U.K) and 15.45 µl of sterile nuclease-free molecular grade water (Park Scientific, U.K). The reaction master mix was prepared in a PCR Workstation™ (Labcaire Ltd., U.K) to ensure sterile conditions.

After 4 min incubation at 95 $^{o}$C, a total of 30 amplification cycles were run for G6PD variants A and A- primer sets under the following PCR conditions: denaturation at 94 $^{o}$C for 10 s, annealing at 62 $^{o}$C for 30 s and elongation at 72 $^{o}$C for 30 s; then a final elongation at 72 $^{o}$C for 10 min. For G6PD Mediterranean variant, the PCR conditions was: initial denaturation at 95 $^{o}$C for 4 min and 35 amplification cycles at 94 $^{o}$C for 10 s denaturation, 62 $^{o}$C for 45 s elongation and annealing at 72 $^{o}$C for 30 s; then a final elongation at 72 $^{o}$C for 7 min. All PCRs were carried out in a GeneAmp® PCR System 9700 (Applied Biosystems, UK). The PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

### 3.5.3 Restriction Fragment Length Polymorphism (RFLP) Analysis

The PCR amplicons were screened sequentially for three G6PD deficient mutations using restriction enzymes appropriate for such mutations, according to the manufacturer's protocol, as follows: the restriction endonuclease *Fok*I to recognize the 376A→G mutation leading to the G6PD A variation, *Nla*III for the 202G→A mutation

characteristic of the G6PD A- and *Mbo*II for the 563T →C typical of the Mediterranean mutation (Table 3.1) (New England Biolabs, U.S.A).

**Table 3.1 Restriction enzymes for digestion of PCR amplicons**

| Enzyme | Sequence | Recognition site |
|---|---|---|
| *Nla*III[a] | CATG | 5′...CATG▼...3′<br>3′...▲GTAC...5′ |
| *Fok*I[b] | GGATG | 5′...GGATG(N)$_9$▼...3′<br>3′...CCTAC(N)$_{13}$▲...5′ |
| *Mbo*II[c] | GAAGA | 5′...GAAGA (N)$_8$▼...3′<br>3′...CTTCT (N)$_7$▲...5′ |

[a]restriction endonuclease from an *E. Coli* strain that carries the *Nla*III gene from *Neisseria lactamica* (NRCC 2118).

[b]restriction endonuclease from an *E. Coli* strain that carries the *Fok*I gene from *Flavobacterium okeanokoites* (IFO 12536).

[c]restriction endonuclease from an *E. Coli* strain that carries the *Mbo*II gene from *Moraxella bovis* (ATCC 10900)

(Source: Manufacturer, New England Biolabs, U.S.A. http://www.neb.com)

Each 25 µl reaction mixture contained 5 µl of PCR amplicons, 5 units of restriction endonuclease, 2.5 µl 10X NEBuffer 4 and 17 µl of sterile nuclease-free distilled water. In the case of *Nla*III digest, 0.25 µl of 100X Bovine Serum Albumin (BSA) was added and the water component reduced to 16.75 µl. After incubation at 37 $^{o}$C for 1 h, the reaction was terminated through heat inactivation by incubating at 65 $^{o}$C for 20 min in water bath. The resulting DNA fragments were separated by electrophoresis on a 3% agarose gel and visualized by ethidium bromide staining.

### 3.6 Agarose Gels

A 2% solution was prepared by melting 4 g of agarose (Agarose Type PGP, Park Scientific. U.K) in 200 ml 1X TBE (Tris-Borate-EDTA) buffer in a microwave oven (230V, 50Hz, 260W, 12.0A;Sharp Corp. Japan) for 3 min. Four drops (1 drop/g agarose) Ethidium Bromide (Sigma, USA) was added to the molten gel and swirled to mix uniformly. The gel was cast to set in a chamber with combs to make the wells. In preparing 3% w/v 6 g of agarose powder was used, added to 200ml 1X TBE. The gel was run at 100 volts in a Maxicell™ EC360M electrophoretic gel system (E-C Apparatus Corporation, St. Petersburg, Florida, U.S.A) and photographed under Ultra-violet (UV) visualization with MicroDoc™ (Cleaver Scientific Ltd., U.K) gel documentation system.

### 3.7 Statistical Analysis

Cross tabulations and Chi-square tests were used to analyze the data using Minitab 15 statistical software (Minitab Inc., USA) and presented on a percentage frequency

distribution table. The means and standard deviations (SD) were determined and reported as mean ± SD. The statistical difference was tested at 5% significance level.

# CHAPTER FOUR

## 4.0 RESULTS

### 4.1 Sex characteristics of the study population

Two hundred participants consisting of 82 (41.0%) males and 118 (59.0%) females, whose age ranged from 18 to 56 years, consented to donate blood for the study. There was a significant difference ($p = 0.001$) between the number of male and female participants. The males were 18 to 56 years old with a mean age and standard deviation of 33.45 ± 10.71 whiles females were 18 to 42 years old with mean age and standard deviation of 28.95 ± 5.55. The mean age of males and females did not differ significantly ($p = 0.25$), (Table 4.1).

**Table 4.1: Age and sex distribution of participants**

| Sex | Number of Participants | Mean age ± S.D. | Age Range |
|---|---|---|---|
| Male | 82 (41.0%) | 33.45 ± 10.71 | 18 – 56 |
| Female | 118 (59.0%) | 28.95 ± 5.55 | 18 – 42 |
| Total | 200 | | |

## 4.2 Biochemical Characterization

### 4.2.1 Characteristics of G6PD deficiency status among participants

Following biochemical screening (methaemoglobin reduction test), it was observed that 162 (81.0%) persons, made up 64 (78.0%) males and 98 (83.1%) females, had normal G6PD enzyme activity (Table 4.2). Whilst 13 (11.0%) females expressed the partial deficient G6PD phenotype, only 7 (5.9%) females and 18 (22.0%) males manifested the fully deficient enzyme activity (Table 4.2). The prevalence of fully deficient enzyme was found to be 22.0% in the male subjects, and 12.5% in the study population, partial deficient G6PD enzyme activity was 11.0% among females and 6.5% in the study population (Table 4.2).

**Table 4.2: G6PD deficiency status distribution in study population**

| Sex | Full deficient | Partial-deficient | Non-deficient | Total |
|---|---|---|---|---|
| Male | 18 (22.0) | 0 (0.0) | 64 (78.0) | 82 |
| Female | 7 (5.9) | 13 (11.0) | 98 (83.1) | 118 |
| Total | 25 (12.5) | 13 (6.5) | 162 (81.0[a]) | 200 |

*Significant difference among the prevalence rate in G6PD non-deficient, G6PD full deficient and G6PD partial deficient individuals is represented as (a) (P value < 0.05).*

### 4.3 Molecular characterization

Biochemical screening using the methaemoglobin reduction test detected 38 (19.0%) persons as deficient for glucose-6-phosphate dehydrogenase. Out of this number investigated, there were 18 males and 20 females (Table 4.2). There was no significant difference ($p = 0.32$) between the number of male and female deficient subjects. These 38 patients and 12 randomly selected non-deficient controls making a total of 50 individuals were subjected to molecular analysis. Polymerase chain reaction (PCR) amplicons of each sample was screened for three mutations namely the 202 G $\rightarrow$A (A-variant), 376 A$\rightarrow$ G (A variant) and the 563 T $\rightarrow$C (Mediterranean variant).

Sequential screening of these samples by restriction fragment length polymorphism (RFLP) indicated that 33 (86.8%) persons out of the 38 deficient subjects were positive for the *G6PD*A mutation. In all, 16 (88.9%) males and 17 (85.0%) females expressed this deficient genotype. For all the non-deficient controls analysed, none tested positive for any of the mutations investigated. Table 4.3 indicates that two females, representing 10.0% were found to have the G6PD A-, however, none of the male subjects tested positive for this variant. All the 50 samples analysed were negative for the Mediterranean mutation, however, 3 (7.9%) individuals who tested positive for the enzymopathy at the biochemical level were found to be negative for the three mutations at the molecular level.

**Table 4.3 Distribution of G6PD mutations among patients in Cape Coast**

| | G6PD variants | | | | |
|---|---|---|---|---|---|
| Sex | G6PD A | G6PD A- | Unknown | G6PD Med | Total |
| Male | 16 (88.9) | 0 (0.0) | 2 (11.1) | 0 (0.0) | 18 (100.0) |
| Female | 17 (85.0) | 2 (9.1) | 1 (5.0) | 0 (0.0) | 20 (100.0) |
| Total | 33 (86.8) | 2 (5.3) | 3 (7.9) | 0 (0.0) | 38 (100.0) |

The prevalence of *G6PD* A and *G6PD* A- variants among the study population was 86.8% and 5.3% respectively. There was a significant difference ($p < 0.0001$) in the prevalence of *G6PD*A and *G6PD* A- mutants of the enzymopathy. The fragment sizes (in base pairs (bp)) as well as the mutations detected following RFLP have been presented in table 4.4.

**Table 4.4 Point mutations detected by restriction enzyme digest**

| G6PD-Variant | Exon | nucleotide change | amino acid change | Enzyme | Fragment size | |
|---|---|---|---|---|---|---|
| | | | | | PCR uncut | Mutant cut |
| A- | 4 | 202 G→ A | 68 Val→Met | *Nla*III | 203bp | 123bp, 69bp, 11bp |
| A | 5 | 376 A→G | Asn→ Asp | *Fok*I | 272bp | 155bp, 117bp |
| Med | 6 | 563 T→ C | 188 Ser→ Phe | *Mbo*II | 275bp | - |

### 4.3.1 Gel electrograms

Electrograms from agarose gel electrophoresis for amplicons and restriction fragments have been presented as figures 4.1 – 4.5. The sizes of PCR amplicons were 203 base pairs (bp), 272 bp and 275 bp for *G6PD* A-, *G6PD* A and *G6PD* Mediterranean variants respectively (Figures 4.1, 4.3, and 4.5). Fragment sizes for *G6PD* A- variant following restriction digestion analysis was 123 bp, 69 bp and 11 bp (Figure 4.2).In the case of *G6PD* A variant, the mutant cut was 155 bp and 117 bp (Figure 4.4); however, there was no restriction fragments detected for the *G6PD* Mediterranean variant.

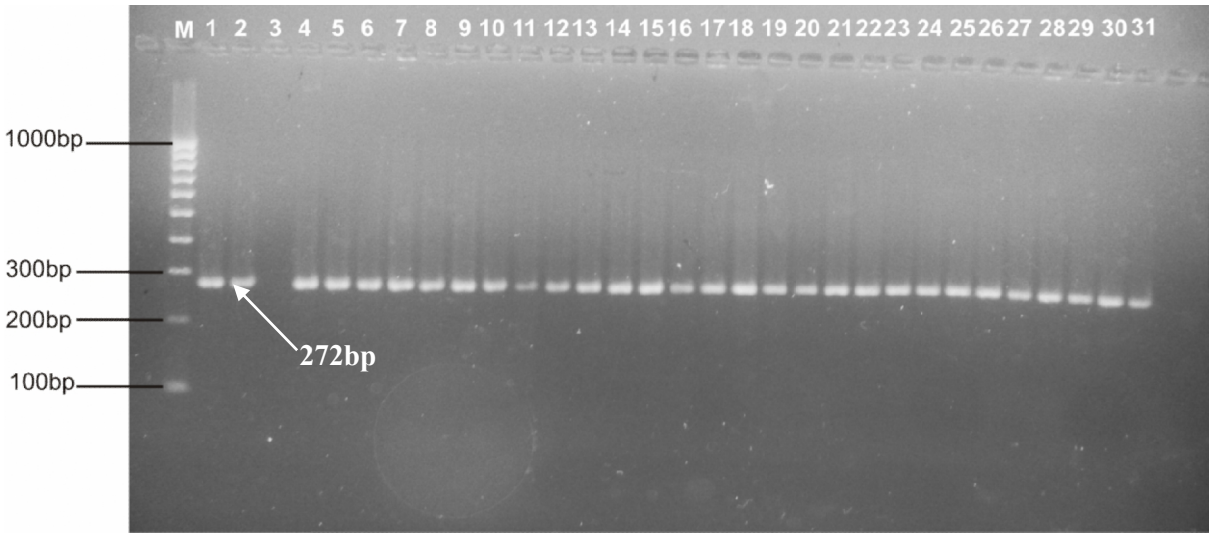


**Figure 4.1**: *G6PD* A uncut PCR amplicons. M = Molecular weight marker, 1-2, 4-31= uncut PCR products, 3- empty (contained control PCR reaction mix)

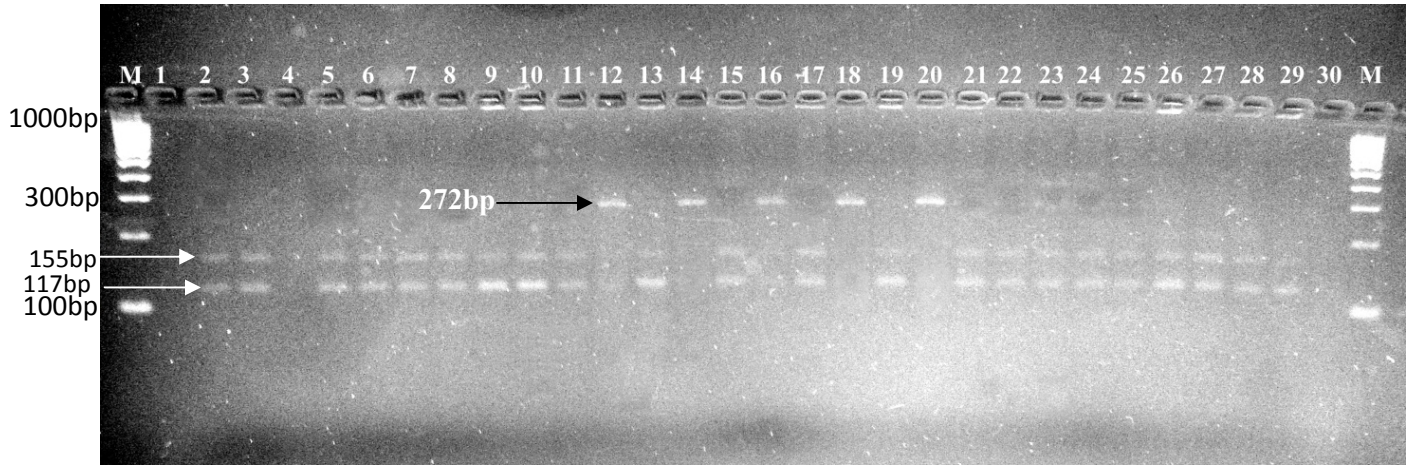


**Figure 4.2**: RFLP of *G6PD* A variant: M - Molecular weight marker; 2-3, 5-11,13,15,17,19,21-29 – PCR- cut products; 12,14,16,18,20 – PCR uncut products; 1,4, 30 – empty. *Fok*I *digests 272bp amplified region of exon 5 of the G6PD gene to 155bp and 117bp.*

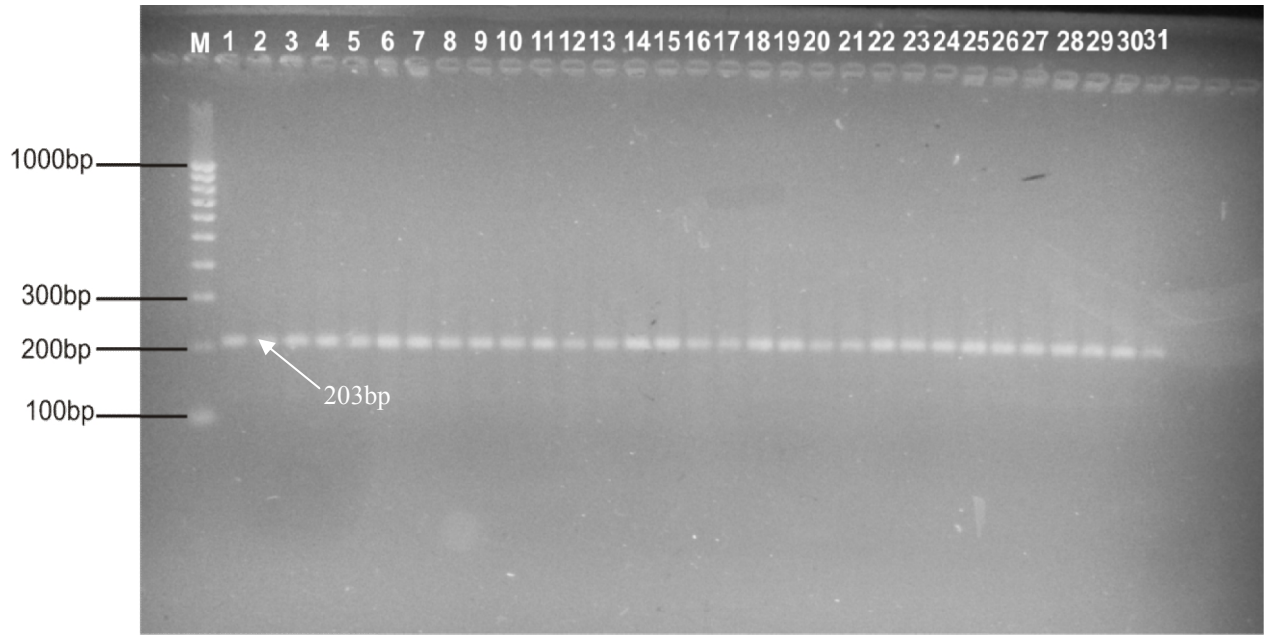


**Figure 4.3**: *G6PD* A- uncut PCR amplicons. M = Molecular weight marker, 1-30 = uncut PCR products.

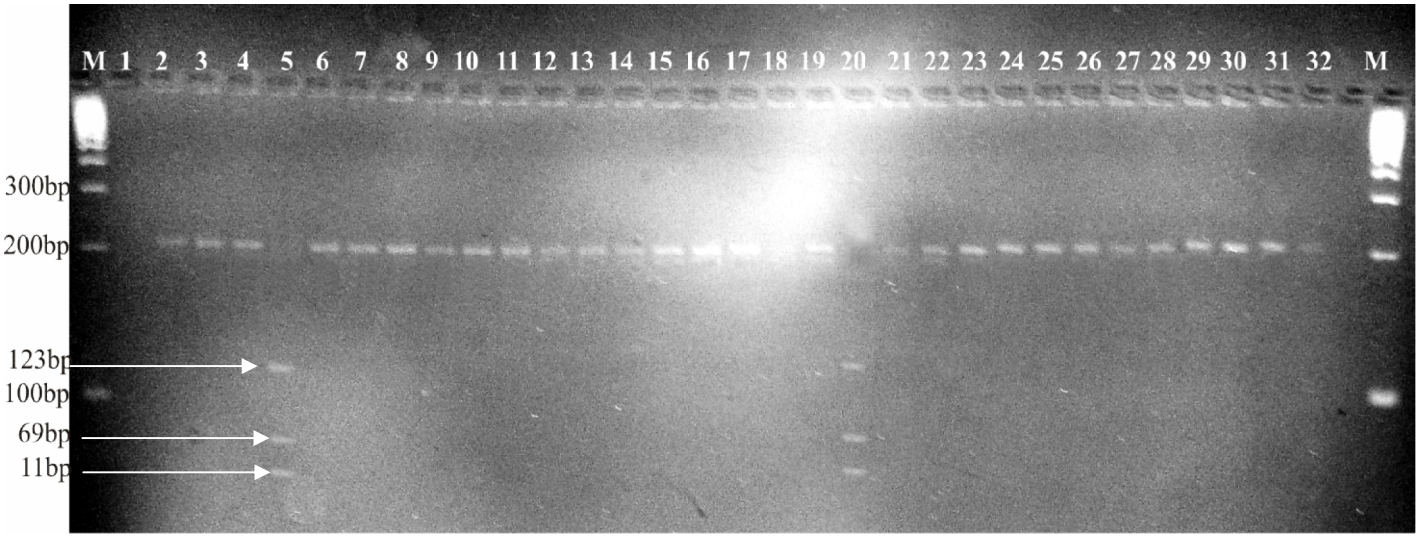


**Figure 4.4**: RFLP of *G6PD* A- variant: M – molecular weight marker; 5 and 21 – PCR cut products; 2- 4, 6-19 and 21 – 31 – PCR uncut products. *Nla*III *cuts the 203bp amplified region of exon 4 of mutant G6PD gene into 123bp, 69bp and 11bp.*

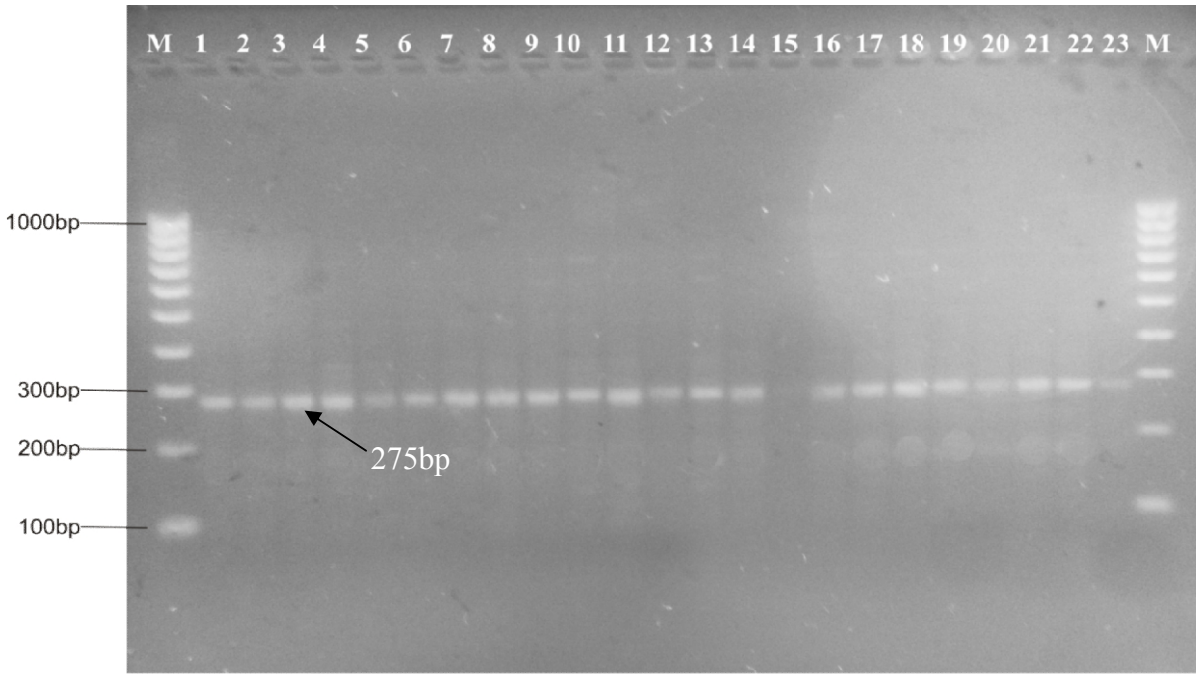


**Figure 4.5**: *G6PD* Mediterranean uncut PCR amplicons. M = Molecular weight marker; 1-14, 16 - 23 = uncut PCR products; 15 = empty (contained control PCR reaction mix).

### 4.3.2 Distribution of G6PD-deficient genotypes

Genotyping of G6PD deficient variants after biochemical screening and molecular characterization indicated that out of the 20 females defective, 17 (85.0%) persons were heterozygous for the *G6PD* A variant, 1 person was heterozygous for the *G6PD* A- deficient gene, and 1 other female showed a homozygous variant of the latter, however, a female was heterozygous for an undetermined variant (Table 4.5). There was a significantly higher ($p < 0.001$) number of females expressing the *G6PD* A variant compared to the *G6PD* A- genotype. On the other hand, 16 (88.9%) males were hemizygous for the *G6PD* A deficient gene whilst 2 other males manifested a hemizygous genotype for an uncharacterized G6PD-deficient allele in the study (Table 4.5).

The prevalence of G6PD-deficient variants among patients in the study population, in the Cape Coast metropolis was 86.8% and 5.3% for *G6PD* A and *G6PD* A- respectively. There was a high significant difference ($p < 0.0001$) in the prevalence of the above genotypes among the participants.

**Table 4.5: Prevalence of G6PD genotypes among deficient subjects**

| G6PD mutation | Patients | Number | Prevalence rate % |
|---|---|---|---|
| *G6PD* A | Female heterozygous | 17 | 44.7[a] |
| | Male hemizygous | 16 | 42.1 |
| *G6PD* A- | Female heterozygous | 1 | 2.6 |
| | Female homozygous | 1 | 2.6 |
| Unknown | Female heterozygous | 1 | 2.6 |
| | Male hemizygous | 2 | 5.3 |

*Significant difference among the prevalence rate in heterozygous and hemizygous genotypes of G6PD* A *variant in deficient individuals is represented as (a) (P value < 0.05).*

# CHAPTER FIVE

## 5.0 DISCUSSION

### 5.1 Biochemical Characterization

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is one of the most common hereditary disorders in humans. According to Ruwende and Hill (1998), the rise in the frequency of the defective gene of the enzymopathy is geographically correlated with malaria endemicity. In Ghana, however, there is scanty available information on the precise extent of G6PD-deficiency state.

A 19.0% prevalence of G6PD deficiency recorded among the study population in this study in conformity with the 15- 26% prevalence rate suggested in Ghana by the World Health Organization (1989).

Results of gender related incidence of the enzymopathy (22.0% and 5.9% full deficiency in males and females respectively, and 11.0% partial deficiency in females) is consistent with the results from three sentinel sites monitoring the G6PD-deficiency prevalence in pregnant women (Anti-Malaria Drug Policy for Ghana, 2009) and the X-linked nature of Glucose-6-phosphate dehydrogenase deficiency as described by Beutler and Vulliamy (2002).

### 5.2 Molecular Characterization

Identification of mutations that cause some pathology is of interest in understanding the nature of the genetic disease and also in obtaining information concerning the

corresponding function of the normal gene in the population. However, the mutations causing G6PD deficiency at the molecular level have not been precisely elucidated.

In this study, *G6PD* A (376 A → G) was found to have a widespread distribution, accounting for 86.8% allelic frequency. Moreover, a high proportion of deficient individuals expressed a heterozygous (44.7%) or hemizygous (42.1%) genotype for this variant. The high prevalence of this variant suggests that it is the precise mutation underlying most cases of the enzymopathy in Cape Coast. Deficient individuals are thus expected to remain asymptomatic in their lifetime as this variant is known to retain at least 85% normal enzyme activity (Ademowo and Falusi, 2002) and is not associated with clinical presentations of the enzymopathy (Arya *et al*., 1995).

The second most prevalent variant detected by restriction enzyme digestion is the *G6PD* A- (202 G →A) with allelic frequency of 7.9% out of the 38 chromosomes tested. This mutant, associated with most clinical conditions (Akanni *et al*., 2010) of G6PD deficiency has been documented as the predominant cause of the disease in sub Saharan Africa (Nkhoma *et al*., 2009), accounting for about 90.0% of the enzymopathy (Martinez de Montemuros *et al*., 1997; Pinto *et al*., 1996). This research in a Ghanaian community however, suggests otherwise. Genotypes of *G6PD* A- variants were not widespread, and may be considered to be benign in adults; but their morbidity, presenting as acute haemolytic anaemia, in young children can be significant. A comprehensive molecular screening for this mutant should therefore, be integrated into routine screening at health facilities, especially in pregnant women and neonates.

The absence of G6PD Mediterranean mutation (563 T → C) among the deficient individuals in the study population is in conformity with its restriction to Mediterranean regions, though it is deemed to be a "rare exported variant", especially through migration and tourism (Daoud *et al*, 2008).

According to Tantular and Kawamoto (2003) and W.H.O (2010), an ideal screening test for G6PD deficiency ought not to give "false-negative" results (i.e., it should not misclassify a G6PD-deficient subject as normal), but it can be allowed to give a few "false-positive" results (i.e., a G6PD-normal subject might be misclassified as being G6PD deficient. For all the 12 randomly selected non-deficient controls analyzed in this study, none was positive for any of the mutations tested at the molecular level. Thus, no deficient individual was misclassified as normal and confirms the methaemoglobin reduction test as being perfectly adequate for diagnostic purposes in patients who are in steady state.

In this study, three G6PD-deficient individuals were found to be negative for all the mutations screened at the molecular level. It is thus possible that they bear mutations not screened for or as yet uncharacterized at the molecular level.

A complete analysis of all the mutations was beyond the scope of this study hence, a more effective approach employed in this research involved screening for common mutations in Africa. This is supported by the fact that 92.1% of deficient patients were genotyped by this approach by analyzing the three variants.

The prevalence of G6PD-deficiency, as well as the underlying causative mutations in females, has been infrequently studied (Reclos *et al*., 2003), therefore knowledge of the

local mutations is beneficial for analysis in females. The study revealed that 85.0% and 9.1% of deficient females had the *G6PD* A and *G6PD* A- variants respectively. Although the enzyme assay (methaemoglobin reduction test) used for the preliminary screening does not form a definitive test in the case of female carriers status (Lin *et al*., 2005), molecular analysis forms an accurate method of assessing the G6PD-genetic status of females (Mehta *et al*., 2000).

In this research, 18 (90.0%) out of 20 deficient females were found to be carriers. The molecular analysis undertaken in this study alone is insufficient in terms of defining the real risk of a female carrying a defective gene; however, it forms the basis for establishing a database of the enzymopathy among Ghanaians, which may be helpful for genetic counseling, anti-malarial drug administration and better management of the disease symptoms.

# CHAPTER SIX

## 6.0 CONCLUSION AND RECOMMENDATION

### 6.1 Conclusion

It is concluded from this study that *G6PD* A variant (376 A ⟶ G) has a widespread distribution than *G6PD* A- variant (202 G ⟶ A) in Cape Coast, however, the Mediterranean variant (563 T ⟶ C) is absent in the metropolis. Furthermore, G6PD-deficient persons may bear mutations uncommon or as yet uncharacterized at the molecular level. However, the present study was underpowered to elucidate such specific variants due to lack of funds.

### 6.2 Recommendation

Future studies should be conducted with more individuals such that events recorded will be substantial enough to predict statistically significant trends in relation to G6PD deficiency and the underlying mutations in Ghana.In addition, biochemically defective but RFLP negative samples must be subjected to single strand conformation polymorphism analysis (SSCP) and subsequent DNA sequencing, to elucidate the specific mutations causing such defects.

# APPENDICES

## Appendix I

### Laboratory buffers and solutions

### Reagents for G6PD assay

A. **Sodium-nitrite-dextrose Solution**: 0.5g Sodium nitrite and 2.0g glucose are bought together in the same flask up to 40ml with distilled water.

B. **Methylene blue solution**: 0.15g trihydrated methylene blue chloride is dissolved in 1000ml (1 litre) of distilled water.

### Primers for PCR

Lyophilized oligonucleotides used in the PCR reactions were reconstituted according to the manufacturer's (The Midland Certified Reagent Company, Midland – Texas, U.S.A) protocol using PCR grade water

### 5X Gel Loading Buffer

A 5X gel loading, which runs approximately 4base pairs on a 1% gel was obtained from Bioline Ltd, U.K.

### DNA Molecular weight size marker

HyperLadder™ IV 100bp DNA molecular weight marker was obtained from the manufacturer (Bioline Ltd. U.K.)

**Restriction Enzymes and Buffers**

The *Fok*I, *Nla*III and *Mbo*II restriction endonucleases and buffers were obtained from New England Biolabs, U.S.A.

**Bands Scoring**

The electrograms bands were scored in MicroDoc™ (Cleaver Scientific Ltd., U.K) gel documentation system with the aid of the product insert supplied with HyperLadder™ IV molecular weight marker.

**Statistical Analysis**

Cross tabulation and Chi-Square analysis, is used to summarize observations by categories and to determine if two discrete variables are associated. For instance, the parameters recorded during the biochemical screening for G6PD-deficiency were gender and G6PD-deficiency status (i.e. Normal, Partial or Full deficiency) (see Appendix II). In analyzing that data in Minitab Statistical Software, each categorical variable was assigned its own column, with each row representing one outcome of the test.

To analyze this data, you choose **Stat > Tables > Cross Tabulation and Chi-Square** in Minitab. Minitab asks you select the variable that will correspond to the table's rows and the table's columns. "Gender" is chosen for rows and "G6PD status" for columns. The options for **Chi-Square analysis** and **Expected cell counts,** are clicked, then press OK, and OK again to run the analysis. Minitab gives an output which summarizes the data with cell counts, percentages within each group as well as Pearson and likelihood ratio chi-square tests with corresponding statistic and p-value.

Analyzed data was presented in percentage frequency distribution tables which display data that specifies the percentage of observations that exist for each data point or grouping of data points.

**Table 4.3 Distribution of G6PD mutations among patients Cape Coast**

| | G6PD variants | | | | |
|---|---|---|---|---|---|
| Sex | G6PD A | G6PD A- | Unknown | G6PD Med | Total |
| Male | 16 (88.9) | 0 (0.0) | 2 (11.1) | 0 (0.0) | 18 (100.0) |
| Female | 17 (85.0) | 2 (9.1) | 1 (5.0) | 0 (0.0) | 20 (100.0) |
| Total | 33 (86.8) | 2 (5.3) | 3 (7.9) | 0 (0.0) | 38 (100.0) |

For instance, in the percentage frequency distribution table above, 16 (88.9%) out of the 18 deficient males had the *G6PD* A variant, whilst 17 (85.0%) of the deficient females had *G6PD* A- variant. In all, 33 (86.8%) out of **38 (100%) deficient individuals** screened had the *G6PD* A variant. There should be no confusion by adding percentages of the *G6PD* A deficient males (88.9%) and females (85.0%) and concluding that the sum is more than 100%. This is so because their computations are done relative to the total number of deficient persons in each specific group (sex). Since 33 persons out of a total of 38 deficient persons had *G6PD* A variant, the percentage is computed relative to

this number [(33÷ 38) X 100%)] and not from the total number recruited for the entire study.

**Appendix II**

**G6PD deficiency status and genotypes**

G6PD DEFICIENCY STATUS AND GENOYPES OF FEMALES (n=20) AND MALES (n=18)

| | | | | GENOTYPES | |
|---|---|---|---|---|---|
| ID | SEX | AGE | G6PD STATUS | A-(202 G) | A (376A G) |
| 4 | F | 28 | PD | | Heterozygous |
| 5 | F | 30 | PD | | Heterozygous |
| 16 | F | 31 | PD | | Heterozygous |
| 24 | F | 23 | PD | | Heterozygous |
| 34 | F | 40 | PD | | Heterozygous |
| 38 | F | 23 | PD | | Heterozygous |
| 79 | F | 29 | PD | | Heterozygous |
| 94 | F | 30 | PD | Heterozygous | |
| 109 | F | 29 | PD | | Heterozygous |
| 113 | F | 38 | PD | | Heterozygous |
| 139 | F | 23 | PD | | Heterozygous |
| 140 | F | 22 | PD | | Heterozygous |
| 141 | F | 30 | PD | | Heterozygous |
| 8 | F | 26 | FD | Homozygous | |
| 23 | F | 34 | FD | | Heterozygous |

| | | | | | |
|---|---|---|---|---|---|
| 27 | F | 36 | FD | | Heterozygous |
| 28 | F | 30 | FD | | Heterozygous |
| 29 | F | 32 | FD | Negative | Negative |
| 36 | F | 33 | FD | | Heterozygous |
| 50 | F | 32 | FD | | Heterozygous |
| 53 | M | 27 | FD | | Hemizygous |
| 56 | M | 25 | FD | | Hemizygous |
| 73 | M | 28 | FD | | Hemizygous |
| 75 | M | 20 | FD | | Hemizygous |
| 76 | M | 32 | FD | | Hemizygous |
| 81 | M | 35 | FD | | Hemizygous |
| 96 | M | 32 | FD | | Hemizygous |
| 107 | M | 27 | FD | | Hemizygous |
| 128 | M | 33 | FD | | Hemizygous |
| 130 | M | 42 | FD | Negative | Negative |
| 162 | M | 25 | FD | | Hemizygous |
| 1 | M | 26 | FD | | Hemizygous |
| 12 | M | 25 | FD | | Hemizygous |
| 15 | M | 25 | FD | | Hemizygous |
| 32 | M | 33 | FD | | Hemizygous |
| 37 | M | 26 | FD | Negative | Negative |
| 84 | M | 30 | FD | | Hemizygous |
| 89 | M | 44 | FD | | Hemizygous |

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