Occurrence of "G6PD" Enzyme Deficiency among Children Suffering from Hemolytic Anemia in Gaza - Palestine

Submitted in Partial Fulfillment for the Master Degree of Science in Biological Sciences/Medical Technology

BY
Lina Nimer Aboud

Prof Dr. Mohammad E. Shubair
Professor of Medical Technology
The Islamic University of Gaza

Dr. Mahmoud M. Sirdah
Associate Professor of Blood Pathophysiology
Al – Azhar University - Gaza
DECLARATION

I hereby declare that this submission is my own original work and that, to the best of my knowledge and belief, it contains no materials previously published or written by another person nor materials which to a substantial extent has been accepted for award of any other degree of the university or other institute, except where due acknowledgement has been made in the text.

Signature                                     Name                                                 Date

Lina N. Aboud
Dedication

To my Father’s Pure Soul,

To my Mother,

To my Husband,

To my Sons,

Jawad, Mohammed

and,

To my daughter, Hala,

last but not least,

To the Palestinian people who are

steadfast and patient

on the beloved land of Palestine
Acknowledgement

The DNA Extraction and purification as well as the hematological and biochemical parts of this work were carried out at the Islamic University of Gaza Laboratories, while the molecular characterization of the G6PD gene was carried out at the Associated Regional and University Pathologists, Inc., (ARUP Laboratories), University of Utah School of Medicine Salt Lake City, Utah, USA.

My deepest and profound acknowledgments are to my supervisors Prof Dr. Mohammad E. Shubair and Dr. Mahmoud Sirdah for their continuous support, generous helps, and fruitful and constructive suggestions. I could not have imagined having a better supervisors and mentors for my master thesis.

My special deep and sincere gratitude are to Prof Dr. Josef Prchal (Professor of Medicine, Pathology, and Genetics, University of Utah School of Medicine, USA), Dr. N. Scott Reading (associate Professor of Genetics ARUP Laboratories Utah, USA), for their efforts and helps in the molecular tests performed in the present work and also for their continuous support, helps, and encouragement.

I am especially indebted to the outstanding staff of the Genetics Laboratory at the Islamic University: Mr. Nasser Abu Shaban and Mr. Mohammed Ashour, both were very valuable in many parts of my work.

I am especially grateful to the ARUP Laboratories and University of Utah School of Medicine Salt Lake City, UT, USA for performing the molecular tests of my study free of charge.

I would like to thank the clinical and laboratory staff of Al-Naser pediatric Hospital for patient's recruitment and enrollment to this work.

My thanks also go to Eng. Hammam Al-Rayes who supplied us with the materials and reagents for our work at Gaza on time.

My appreciations are extended to all the patients and their parents for their understanding and cooperation during the study and thereafter.

Finally, not to forget any one, I express my thanks to everyone who supported me through finishing this work.
Abstract

**Background:** The Glucose-6-phosphate dehydrogenase (G6PD) deficiency has been considered as the commonest enzymopathic inherited disorder of red blood cells, which affecting more than 500 million people worldwide. The G6PD gene in human is X-linked so males are more affected than females. The mutation in the G6PD gene may lead to the production of a G6PD enzyme that has diminished functionality and/or stability, leading to wide range of biochemical and clinical presentations principally neonatal jaundice and acute hemolytic anemia triggered by an exogenous agent in most cases. More than 176 mutations, and 500 different variants have been described to date for the G6PD gene, however, the Mediterranean mutation c.563 C>T, whether associated with c.1311 C>T polymorphism or not, is among most common variants that lead to enzyme deficiency and often associated with Favism.

**Objectives:** The main objective of this study is to determine the frequency of the Mediterranean mutation (c.563 C>T) and its association with c.1311 C>T polymorphism among Palestinian G6PD deficient children admitted to Al Nasser pediatric hospital at Gaza due to hemolytic crises.

**Methodology:** In this cross-sectional descriptive study, 80 children (2-8 years old) presenting with hemolytic anemia were included and they represent the period from April 2010 and March 2011. Venous blood samples (2.5 ml) were withdrawn at hospital admission from each hemolytic child and were collected in K$_3$-EDTA tubes and were used for performing complete blood count (CBC), G6PD enzyme assay, DNA extraction and, mutation analysis. Forty school-aged children without G6PD deficiency were selected as the non-G6PD deficient control group for the hematological studies. Additionally, 40 X-chromosomes from apparently healthy, male subjects served as controls tested for the c.1311 C>T polymorphism.

**Results:** The results showed that 65 (60 males & 5 females) out of the 80 children were found to be G6PD deficient. In most G6PD deficient cases (67.7%) hemolytic crisis occurred in early childhood ($\leq$ 40 months), and it was totally (100 %) due to ingestion of fava beans, either green (96.9 %) or dried (3.1 %). Most (98.5%) of G6PD deficient cases went through neonatal jaundice after birth, which last 1-2 weeks. The Mediterranean c.563 C>T and the c.1311 C>T polymorphism were encountered, respectively, in 35.4 % (allele frequency 0.33) and 41.8 % (allele frequency 0.42) of G6PD deficient children. Also the c.1311 C>T polymorphism was identified in 17.5 %.
(allele frequency 0.18) of control blood samples. However, the incidence of c.1311 C>T polymorphism with patients carrying the Mediterranean c.563 C>T mutation was 95.2 percent (allele frequency 0.96) compared to 11.4 percent (allele frequency 0.08) in G6PD deficient individuals without the Mediterranean c.563 C>T mutation. The comparisons of G6PD enzyme activity and erythrogram revealed that the G6PD deficient subjects were admitted to the hospital with significantly lower hematological parameters except for MCHC. In addition, no significant hematological or enzymatic activity differences were reported between the G6PD deficient children carrying the Mediterranean c.563 C>T mutation and those G6PD deficient children carrying non-Mediterranean mutation.

**Conclusions:** It is concluded that the Mediterranean mutation c.563 C>T is common among G6PD deficient Gaza Strip Palestinians and is highly associated with the c.1311 C>T polymorphism. This work could be foundational for further investigations of G6PD molecular studies with emphasis on ancestral origin of these variants. The present study also emphasizes the predominant existence of the G6PD deficiency among Palestinian population, which justifies the necessity of the Palestinian Health policy leaders establishing nationwide programs of newborn screening for G6PD deficiency.

**Key words**

مدى انتشار النقص في إنزيم G6pD بين الأطفال المصابين بفقر الدم الحاللي في غزة، فلسطين

ملخص الدراسة:

مقدمة: يعتبر نقص إنزيم جلوكوز سداسي الفوسفات المختزل G6pD من أكثر اعطالات الإنزيمات الوراثية شيوعًا، حيث أن أكثر من 50 مليون شخص مصاب بهذا المرض على مستوى العالم. مرض نقص إنزيم G6PD مرئي وراثي منتحي مرتبط بالصبغي الجنسي X، حيث يتحكم في تصنيع هذا الإنزيم جين موجود على الصبغي الجنسي. وهذا ما يفسر اصابة الذكور أكثر من الإناث بهذا المرض. ويؤدي حدوث أي خلل في هذا الجين (الطفرة) إلى نقص أو اعتلال في تصنيع هذا الإنزيم، مما يؤدي إلى حدوث تكسر وانحلال كريات الدم الحمراء (الانحلال الدموي) وبالتالي حدوث فقر الدم الذي قد يكون شديداً ومهدداً للحياة. يوجد أكثر من 176 طفرة و400 متغيرًا مختلفًا لهذا المرض وتعتبر طفرة البحر الأبيض المتوسط (c.563C>T) سواء كانت مرتبطة بالعدد الشكلي (c.1311) أو لا هي من بين المتغيرات الأكثر شيوعًا التي تؤدي إلى نقص إنزيم G6PD المرتبط بانيميا الفول.

الهدف من الدراسة:

الهدف الرئيسي من الدراسة هو حساب مدى انتشار طفرة البحر الأبيض المتوسط (c.563C>T) وارتباطها مع الطفرة (c.1311) في الأطفال المصابين بفقر الدم الالتحالي ومرضى بنقص الإنزيم G6PD في مستشفى النصر للأطفال.
الطريق و الأدوات: هذه الدراسة الوصفية شملت 80 طفلا تتراوح اعمارهم من (2-10) سنة و المعالجين في المستشفى نتيجة فقر الدم الانحلالي في الفترة من أبريل 2010 إلى مارس 2011، لقد تم سحب عينات دم وريدي (2.5) مل من كل طفل مصاب بفقر الدم الانحلالي تم دخوله للمستشفى وجمعت في أنابيب K3، وكانت تستخدم لأداء تعداد او صورة دم كاملة EDTA-K3، وتم قياس فعالية و نشاط إنزيم G6PD وتم أيضا استخراج الحمض النووي والميتوحية الطفرات، وقد تم اختيار 40 طفل من المدارس غير مصابين بنقص إنزيم G6PD كمجموعة ضابطة لدراسات الدم بالإضافة إلى ذلك 40 صبغي جنسي من أطفال استخدموا كعينة ضابطة للتحديد الشكلي (c.1311 C>T)

النتائج: أظهرت النتائج ان 65 (60 ذكور و5 إناث) من أصل 80 طفلا من الأطفال المشمولين بالدراسة مصابين بنقص G6PD. في حالات نقص G6PD حوالي (67.7%) أصيب أفراد الدم الانحلالي مركبة في مرحلة الطفولة المبكرة (≤ 40 شهرا)، وكان (100%) بسبب تداول الفول، أما الفول الأخضر بنسبة (96.9%) أو المجفف بنسبة (3.1%). كما أن ما يقارب من (98.5%) من حالات نقص G6PD اصيب بالبرقان الفيسيولوجي بعد ولادة، والتي استمرت 1-2 أسبوع. لقد تم تحديد طفرة البحر الأبيض المتوسط (c.1311C >T) في G6PD و التعدد الشكلي (c.563 C > T) في G6PD، و 35.4% و 41.8% على التوالي في الأطفال المصابين بنقص G6pD. كما تم التعرف على G6pD في 17.5% من عينات الدم الكنترول. كما ان هناك علاقة قوية و جوهرية و ارتباط بين المرضي حامل طفرة المتوسط و ارتباطا مع التعدد الشكلي حيث وجد التعدد الشكلي في 95.2% من حالات المرض التي تحمل الطفرة المتوسطة مقارنة مع 11.4% في الأفراد المصابين بنقص G6PD بدون وجود طفرة البحر الأبيض المتوسط. كشفت المقارنات لقيمة إنزيم CBC والعد الكامل لخلايا الدم (CBC) للأطفال المصابين بنقص G6PD والإنجاز الشكلي (c.1311 C>T) للتحديد الشكلي، و 35.4% و 41.8% على التوالي في الأطفال المصابين بنقص G6pD. كما تم التعرف على G6pD في 17.5% من عينات الدم الكنترول. كما ان هناك علاقة قوية و جوهرية و ارتباط بين المرضي حامل طفرة المتوسط و ارتباطا مع التعدد الشكلي حيث وجد التعدد الشكلي في 95.2% من حالات المرض التي تحمل الطفرة المتوسطة مقارنة مع 11.4% في الأفراد المصابين بنقص G6PD بدون وجود طفرة البحر الأبيض المتوسط. كشفت المقارنات لقيمة إنزيم CBC والإنجاز الشكلي (c.1311 C>T) للتحديد الشكلي.
بالإضافة إلى أنه لا يوجد اختلاف بمعدل
بنقص نشاط الإنزيم ما بين المصابين بظهارة البحر الأبيض المتوسط والغير مصابين بهذه
الظاهرة.

الاستنتاجات: نستنتج من الدراسة أن ظاهرة البحر الأبيض المتوسط (c.563 C>T) هي الأكثر
انتشاراً بمرضى نقص الإنزيم G6PD، وهي مرتبطة لحد كبير مع التعدد الشكلي (c.1311 C>T).
وهي مرتبطة في зрية ممّا لم تؤخذ من الدراسات الجزئية. هذه الدراسة تؤكد أيضاً انتشار نقص الأفيون في إنزيم
G6PD بين سكان فلسطين، الأمر الذي يبرر ضرورة قيام وزارة الصحة الفلسطينية بوضع
برامج لفحص حديثي الولادة لنقص G6PD.

الكلمات المفتاحية:
- ظاهرة البحر الأبيض المتوسط (c.563 C>T)
- التعدد الشكلي G6PD (c.1311 C>T)
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### Abbreviations

<table>
<thead>
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<th>Full Form</th>
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<tr>
<td>2,3-DPG</td>
<td>2,3-diphosphoglycerate</td>
</tr>
<tr>
<td>6-PG</td>
<td>6-Phosphogluconate</td>
</tr>
<tr>
<td>6-PGD</td>
<td>6-Phosphogluconic dehydrogenase</td>
</tr>
<tr>
<td>AHA</td>
<td>Acute hemolytic anemia</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>al</td>
<td>Allele</td>
</tr>
<tr>
<td>AF</td>
<td>Allele frequency</td>
</tr>
<tr>
<td>AR</td>
<td>Autosomal recessive</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CNSHA</td>
<td>Chronic non spherocytic hemolytic anemia</td>
</tr>
<tr>
<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetraacetic Acid</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavine adenine dinucleotide</td>
</tr>
<tr>
<td>G6P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>G6PD</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>G-3-PD</td>
<td>Glyceraldehyde phosphate dehydrogenase</td>
</tr>
<tr>
<td>Hct</td>
<td>Hematocrit</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HMP</td>
<td>Hexose monophosphate pathway</td>
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<tr>
<td>Km</td>
<td>Michaelis constants</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>GSSG</td>
<td>Oxidized glutathione</td>
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<tr>
<td>PCBS</td>
<td>Palestinian central bureau of statistics</td>
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<td>PPP</td>
<td>Pentose phosphate pathway</td>
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<td>Polyacrylamide Gel Electrophoresis</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>UDPG-T</td>
<td>Uridine diphosphate glucuronosyl transferase</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
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Chapter 1
Introduction

1.1 Overview

The Glucose-6-phosphate dehydrogenase (G6PD) deficiency has been considered as the commonest enzymopathic inherited disorder of red blood cells, which affecting more than 500 million people worldwide. The disease has been reported in peoples from nearly all geographical locations; however it occurs most frequently in areas where Plasmodium falciparum malaria had been endemic. G6PD deficiency was discovered for the first time when hemolytic anemia occurred in some persons who consumed anti-malarial drug named Primaquine (1).

The G6PD enzyme is essential for maintaining the integrity of the erythrocytes, preventing the oxidation of hemoglobin and other cellular proteins through providing reducing power in the form of NADPH to all cells including mature red blood cells where the absence of mitochondria restricts the production of NADPH to hexose monophosphate shunt only. The G6PD gene in human is X-linked that maps to the tip of the q-arm of the X chromosome: Xq28, and it consists of 13 exons encoding 515 amino acids with a molecular weight of about 59 kDa, and spans almost 20 Kb in total. Since it is a sex-linked gene, males are more affected than females. Also the heterozygous females for the G6PD gene are genetic mosaics due to X-chromosome inactivation in somatic cells (2, 3).

The G6PD gene is located on the long arm of the X-chromosome (Xq28). Most deficient people do not show any symptoms until or following exposure to oxidative drugs, some infections and ingestion of Fava bean (4).

The mutation in the G6PD gene may lead to the production of a G6PD enzyme that has diminished functionality and/or stability, which demonstrating different levels of enzyme activity and consequently wide range of biochemical and clinical presentations principally neonatal jaundice and acute hemolytic anemia triggered by an exogenous agent in most cases. More than 176 mutations, and 500 different variants have been described to date for the G6PD gene, however most are single base changes, leading to amino acid substitutions (3, 5). The world health organization grouped the G6PD variants into five classes based on their enzyme activity and clinical manifestations, with class I demonstrating the severely deficient cases that associated with chronic non-spherocytic hemolytic anemia (6).
Mediterranean mutation c.563 C>T is among most common variants that lead to enzyme deficiency and often associated with Favism. The best known G6PD-deficient variants that occur at a high frequency are the African variant G6PD A- and the Mediterranean variants. Mediterranean mutations are identified by very low activity in red blood cells (7). Mediterranean variant is the most common variant in Southern Europe, Middle East, and India. The prevalence of Mediterranean mutation c.563 C>T among G6PD deficient patients in the middle east region is varied from one population to another and the range could be from 28-95 %. Favism is always observed in people with G6PD deficiency, Clinical symptoms of favism are pallor, jaundice, hematuria and acute hemolytic anemia occurs 24- 48 h after consumption of fava beans .The main treatment for G6PD deficiency is avoidance of oxidative agents like infection, fava beans and oxidative drugs that induce hemolysis (8). Hemolysis may be so severe that it may even require blood transfusion. Screening of newborns for early diagnosis of G6PD deficiency and proper education can reduce the incidence of clinical symptoms (1).

1.2 Objectives

1.2.1 General objectives

The main objective of this study is to determine the frequency of the Mediterranean mutation (c.563 C>T) among Palestinian G6PD deficient patients in Gaza strip, and to associate it with the c.1311 C>T polymorphism (silent mutation) of the G6PD gene.

1.2.2 Specific objectives

1- To determine the G6PD activity among all children admitted to the pediatric hospital due to hemolytic crises
2- To evaluate all G6PD deficient children admitted to the pediatric hospital
3- To study the demographic and socioeconomical characteristics of the G6PD deficient children.
4- To characterize the Mediterranean c.563 C>T mutation and, c.1311 C>T polymorphism (silent mutation) among all G6PD deficient children admitted to the pediatric hospital
5- To molecularly characterize the c.1311 C>T polymorphism (silent mutation) among a group of apparently healthy subjects used as control group.
6- To find any association between the Mediterranean c.563 C>T mutation and c.1311 C>T polymorphism (silent mutation) as compared to the control group.
1.3 Significance

G6PD deficiency is commonly encountered among hemolytic children at the general pediatric hospital at Gaza strip, Palestine. Although, the Gaza strip is part of the Mediterranean basin where G6PD deficiency has not been reported to significantly exist among the different populations, unfortunately, previous screening nor molecular studies have been performed in the Gaza strip to characterize the G6PD deficiency among the Gaza strip population. The efforts are only limited to provide the G6PD deficient patients with the free clinical cure and management when the crisis occurs. In addition to geographical rationale, green and dried beans (Fava beans) could be considered as the commonest traditional food for the majority of the population in Gaza strip as well as in other Arabic Middle Eastern countries. The 2006 report of the Palestinian central bureau of statistics (PCBS) showed that the average annual household quantities of green, dried and tinned beans is more than 20 Kg (9).

Therefore, we design the present work to determine the frequency of the Mediterranean mutation (c.563 C>T) among Palestinian G6PD deficient children in Gaza strip, and to associate it with the c.1311 C>T polymorphism (silent mutation) of the G6PD gene. This work could be the foundational for further investigations of G6PD gene molecular studies with emphasis on clinical and biochemical aspects of the different variants.
Chapter 2

Literature review

2.1 Hemolytic disease of children

2.1.1 Immune hemolysis:

Immune hemolysis is the most dangerous form of jaundice in the newborn as the bilirubin levels can rise rapidly into the toxic range (10). Significant disease is associated primarily with the D antigen of the Rh group and with incompatibility of ABO factors. Rarely, hemolytic disease may be caused by C or E antigens or by other RBC antigens such as Cw, Cx, Du, K (Kell), M, Duffy, S, P, MNS, Xg, Lutheran, Diego, and Kidd (11).

2.1.1.1 Rh incompatibility

Rh antigens are found only in the RBC membrane. Rh blood group proteins are highly antigenic and are capable of causing severe isoimmunization with a high risk of fetal hydrops and death (12). Hemolytic disease rarely occurs during a first pregnancy because transfusion of Rh-positive fetal blood into an Rh-negative mother tend to occur near the time of delivery, too late for the mother to become sensitized and transmit antibody to her infant before delivery (13). Rh hemolytic disease is now being prevented by the administration of Rho (D) immune globulin (anti-D) (RhoGAM) to unsensitized Rh-negative mothers this usually is done at 28 weeks gestation and again within 72 h after delivery. Other indications of Rho (D) immune globulin or for using larger doses are prophylaxis following abortion, amniocentesis, chorionic villus sampling and transplancental hemorrhage (14).

2.1.1.2 ABO hemolytic disease of the newborn

ABO incompatibility is the most common of hemolytic disease of newborn. Approximately 15% of live births are at risk, but manifestations of disease develop in only 0.3-2.2 % (15). Neonates with group A or B erythrocytes may have increased hyperbilirubinemia hemolysis because of transfer of maternal anti-A or anti-B antibodies into fetal circulation. This can occur in the first born without prior
sensitization of the mother and it is milder and shorter duration than Rh erythroblastosis, this may be due to:

First, an infant with A or B blood groups the antigen is present in all tissues of the body, thus effectively diluting neutralizing the transferred maternal antibodies.

Second, neutralization of maternal antibody A and B antigen prior to entry to fetal circulation.

Third, the relatively weak nature of A or B antibody resulting in less intensive hemolysis (16).

The diagnosis of hyperbilirubinemia secondary to ABO incompatibility is supported by a positive coomb’s test, jaundice in the first 24 hours, presence of spherocytes in the peripheral smear and elevates reticulocytic count (usually exceeding 5 %) (17).

2.1.2 Non immune hemolysis

2.1.2.1 RBCs structural membrane defects

Defects in the erythrocyte membrane and cytoskeletal structure alter the shape and deformability of the cell and result in sequestration of the cell in the narrow splenic sinusoids, so result in hemolysis, hyperbilirubinemia and splenomegaly (18).

2.1.2.1.1 Hereditary spherocytosis

Usually is transmitted as an autosomal dominant, less frequently, as an autosomal recessive disorder. The most common molecular defects are abnormalities of spectrin or ankyrin, cause spherering of the RBCs and associated increase in cation permeability, cation transport, ATP utilization, and glycolysis. The decreased deformability of the spherocytic RBCs impairs cell passage from the splenic cords to the splenic sinuses, and the spherocytic RBCs are destroyed prematurely in the spleen (19).

2.1.2.1.2 Hereditary elliptocytosis

Is transmitted as autosomal dominant, the defect is also in the spectrin, unlike spherocytosis, hemolysis and hyperbilirubinemia are unusual in the neonatal period (20).
2.1.2.2 Hemoglobinopathies

Hemoglobinopathies are inherited defects in globin structure which are common in African, Indian, Asian, and Mediterranean populations. Most of these involve a single amino acid substitution in one of the globin chains. The resulting structural defect can be clinically silent or produce a significant anemia; this hemolysis can be a characteristic of inherited disorders of hemoglobin synthesis, including sickle cell anemia and, thalassemias.

2.1.2.2.1 The thalassemias

Inherited multifactorial anemia characterized by defects in the synthesis of the alpha or beta subunit of the hemoglobin tetramer (α2β2). The deficiency in one globin chain leads to an overall decrease in hemoglobin and the intracellular precipitation of the excess chain, which damages the membrane and leads to clinically evident hemolysis in the severe forms of alpha thalassemias (hemoglobin H disease), and beta thalassemia (intermedia, and major). Beta thalassemia can be diagnosed by hemoglobin electrophoresis, which shows elevated levels of hemoglobin A2 and F, while diagnosis of alpha thalassemia requires genetic studies. Thalassemias are characterized by hypochromia and, microcytosis; target cells frequently are seen on the peripheral smear (21).

2.1.2.2.2 Sickle cell anemia

Is an inherited disorder caused by a point mutation leading to a substitution of valine for glutamic acid in the sixth position of the α chain of hemoglobin. Membrane abnormalities from sickling and oxidative damage caused by hemoglobin S, along with impaired deformability of sickle cells, leads to splenic trapping and removal of cells. Some degree of intravascular hemolysis occurs (22).
2.1.2.3 RBCs enzymatic defects

2.1.2.3.1 Pyruvate Kinase deficiency

The condition is autosomal recessive disorder that has been described in all ethnic groups but is most common in northern European (23). Diagnosis of this condition is by elevated 2, 3-DPG levels and ATP levels, reduced and low levels of pyruvate kinase activity in RBCs (24). One third of those affected present in neonatal period with jaundice and hemolysis that require phototherapy or exchange transfusion. Occasionally the disorder causes hydrops fetalis and neonatal death (25).

2.1.2.3.2 Glucose-6-phosphate dehydrogenase

The ancient Greeks knew Favism, as the advice-attributed to Pythagoras to avoid the Vicia faba or fava beans (26). G6PD deficiency was first discovered in the mid-1950s as accounting for why certain people were sensitive to the hemolytic effect of the anti-malarial drug primaquine. It soon became clear that the vast majority of affected individuals are asymptomatic (27). In 1957, Beutler E. showed by application of “the glutathione stability test” that the susceptibility of primaquine sensitive cells to hemolysis was related to their lower content of glutathione, the major sulfhydryl compound of the erythrocyte (28). Examination of the pathway of glutathione metabolism within the red cells led to the Hexose monophosphate shunt and revealed that the abnormality was related to a deficiency of the red cell enzyme G6PD (29). With the confirmation that the basic defect was a deficiency of G6PD, X-linkage was confirmed by estimation of enzyme activity (30). By the 1960’s, four syndromes, which included massive intravascular hemolysis as an idiosyncratic reaction to multiple drugs and chemicals; hemolysis after ingestion of fava bean (Favism); severe hemolysis as an unusual complication of illnesses; and severe neonatal jaundice causing kernicterus, were all explained by their occurrence predominantly in those who have inherited the G6PD deficient genotype (31). Larrizza in 1960 was the first who described chemicals and food-induced hemolytic anemia using the term enzyme deficiency hemolytic anemia (32). In 1967, a committee of the World Health Organization (WHO) proposed standard procedures to characterize G6PD variants using certain parameters such as: (a) red cell G6PD activity; (b) electrophoretic migration; (c) Michaelis constants (Km) for G6P and NADP; (d) relative rate of utilization or substrate analogues such as 2-deoxy G6P (2d G6P); (e) thermal stability; and (f) pH optimum (33).
2.2 Erythrocyte metabolism

In the process of maturation, red cells lose their nucleus, mitochondria and ribosomes. Loss of its mitochondria deprives the red cell of the most efficient means of energy production, oxidative phosphorylation. The cell is thus entirely dependent upon the relatively inefficient mechanism of anaerobic glycolysis via the Embden Meyerhof pathway for the fulfillment of its considerable energy requirement. Protection of the red cell against the oxidative stresses imposed by its environment and metabolic processes is provided primarily via the Hexose monophosphate pathway (pentose shunt, pentose phosphate pathway, aerobic pathway) (34). Under normal circumstances, about 90% of glucose entering the red cell is metabolized by the anaerobic pathway and 10% by the aerobic pathway. Under conditions of oxidative stress, however, the oxidative pentose pathway may account for up to 90% of glucose consumption (35).

2.2.1 The Embden Meyerhof pathway

Glucose is catabolized anaerobically to pyruvate or lactate. Two moles of high-energy phosphate in the form of adenosine triphosphate (ATP) is utilized in preparing glucose for its further metabolism, up to 4 moles of adenosine diphosphate (ADP) may be phosphorylated to ATP during the metabolism of each mole of glucose, giving a net yield of 2 moles of ATP per mole of glucose metabolized (figuer2.1) (36). In addition to being the sole source of ATP generation in the erythrocyte, the Embden-Meyerhof pathway has two other key functions: first, the generation of 2, 3-diphosphoglycerate [2, 3-DPG] through the DPG, or Rapoport-Luebering, cycle and, second, the generation of NADH at the G-3-PD step (37). Energy liberated from ATP serves in active transport of sodium and potassium, maintenance of low intracellular calcium levels and phosphorylation of membrane proteins and substances of glycolysis itself. The only ATP that is formed in the phosphoglycerate kinase step, rather than that synthesized in the pyruvate kinase step, is available for iron pumping. Each one of these functions aids in maintaining red blood cell ability for normal deformability, thus, allowing the cells to flex and change their shape during passage through the microcirculation (38). NADH is a necessary cofactor for NADH methemoglobin reductase, which maintains heme iron in the reduced state. 2, 3-DPG decreases the oxygen affinity of hemoglobin and increases oxygen delivery to peripheral tissues (39).
2.2.2 The hexose monophosphate pathway

The hexose monophosphate pathway generates reducing potential for the red cell in the form of nicotinamide adenine dinucleotide phosphate (NADPH), which is an essential component of the glutathione cycle (figure 2.1). Under normal circumstances, hexose monophosphate pathway activity consumes about 5-10% of glucose-6-phosphate formed as the first step of glycolysis. However, the rate of activity can be increased by a factor of up to 30 times when required to deal with an increased level of oxidants (40).

The reduction of NADP occurs at the initial and most important step of the HMP, G-6-PD, and again at the 6-PGD step in which 6-PG is converted to ribulose-5-P (37).

Mature erythrocytes synthesize relatively high concentrations of reduced glutathione (GSH). GSH protects erythrocytes from oxidants such as hydrogen peroxide ($H_2O_2$), superoxide anions ($O_2^-$), and hydroxyl radicals (OH), which are produced as byproducts of the oxidation of heme by oxygen. Oxidants are also produced by neutrophils (i.e., during infection) and by erythrocytes after exposure to certain agents. The detoxification of $H_2O_2$ is markedly enhanced by glutathione peroxidase. GSH is converted to oxidized glutathione (GSSG) and to mixed disulfides with protein thiols. GSH levels are restored by glutathione reductase (figure 2.2). In this process, NADPH is oxidized to NADP, which stimulates the HMP, regenerating NADPH (39). A second function of HMP is conversion of hexoses to pentoses. For the most part, the latter are recycled into glycolytic pathway; however, D-ribose-5-phosphate may be used for nucleotide synthesis (38).
Fig 2.1 Reaction scheme of the major metabolic pathways in the human erythrocyte: glycolysis, the 2,3-DPG (Rapoport–Luebering) shunt and the PPP(41).
Fig 2.2 The action of glucose-6-phosphate dehydrogenase (G6PD) in the pentose phosphate pathway (PPP) (4).

2.2.3 Function of G6PD in red blood cells

In the red blood cell, G-6-PD action is the first and rate-limiting step in the pentose phosphate pathway (PPP) that converts NADP into reduced NADPH (37). As an alternative to progressing through the energy-generating pathways of glycolysis, the citric acid cycle and oxidative phosphorylation, glucose can be converted into five-carbon sugars. As a first step in this conversion, G6PD acts on glucose-6-phosphate (G6P) to produce 6-phosphoglucono-δ-lactone, which in turn produces ribose-5-phosphate, 6-phosphogluconate and ribulose-5-phosphate. These reactions can be summarized as follows:

\[
G6P + 2\text{NADP}^+ + H_2O \rightarrow R5P + 2\text{NADPH} + 2H^+ + CO_2
\]

Ribose sugars are required as precursors in the biosynthesis of a number of important molecules, such as ATP, CoA, NAD, FAD, RNA and DNA. In addition, the ribose-5-phosphate formed by the PPP can be completely converted back into glycolytic intermediates. The NADPH produced in the PPP by the action of G6PD and 6-
phosphogluconate dehydrogenase serves as an electron donor in reductive biosynthesis, notably of cholesterol and fatty acids, as well as in the synthesis of nitric oxide (4). G6PD is distributed in all the cells. However, in G6PD deficiency the cells other than RBC do not have significant deficiency (42).

2.2.4 Mechanism of hemolysis

In the absence of G-6-PD, NADPH will not be formed, hydrogen ions will not be available for the reduction of oxidized glutathione, and the latter will not be available to participate in the body’s antioxidant mechanisms (43). In the presence of oxidizing agents, the PPP of the normal red cell is stimulated several fold, the levels of NADPH and GSH remaining unchanged. This has recently been shown to be mediated through an increase in the expression of G6PD. In the G6PD-deficient red cell, however, where the PPP is already operating at a near-maximal rate, the flow of glucose through it cannot be increased, and a fall in the levels of GSH and NADPH is observed (44). Most is known about favism, in which the compounds divicine and isouramil, found in fava beans, have a causal role in the irreversible oxidation of GSH and other protein-bound SH groups. Effects resulting from this include electrolyte imbalance, membrane cross-bonding and erythrocyte phagocytosis. A striking observation in favism is the increase in red cell calcium levels, and in some cases the degradation of erythrocyte calcium ATPase. A combination of both increased passive permeability and a decreased efficiency of the calcium pump may explain the disruption of erythrocyte calcium homeostasis. G6PD-deficient red cells are also more susceptible to calcium-induced vesiculation than normal cells, and this is correlated with the extent of complement-mediated hemolysis (4). Oxidant-induced membrane damage acts as a signal for red cells to be removed from the circulation by erythrophagocytosis. The recognition of these cells by macrophages may result from a modification of membrane carbohydrate. G6PD-deficient cells have been shown to undergo glycoprotein modifications, which may lead to removal from circulation even in non-acute hemolysis (45). A significant increase in the glycosylation of haemoglobin has been reported in G6PD-deficient cells (46).
2.3 Structure of G6PD

The G6PD monomer consists of 515 amino acid subunits with a calculated molecular weight of 59,256 daltons. The active enzyme exists as a dimer and contains tightly bound NADP (figuer2.3). Aggregation of the inactive monomers into catalytically active dimers and higher forms requires the presence of NADP. Thus, NADP appears to be bound to the enzyme both as a structural component and as one of the substrates of the reaction. The glucose-6-phosphate binding site has been identified at amino acid 205 by locating a lysine at this position that is reactive with pyridoxal phosphate in competition with glucose-6-phosphate (1).

![Fig 2.3 A three-dimensional model of the active G6PD dimer. (4).](image)

2.3.1 Genetics and molecular biology of G6PD

G6PD deficiency was first proved to be an X-linked trait through its genetic linkage to the Protan-Deutan colour-blindness phenotype, the only common X-linked polymorphism known at the time. Xq28, the telomeric band of the long arm of the X chromosome where the G6PD gene is located, has since received considerable attention through the mapping of important loci to the region, the creation of a physical and transcription map, and the increasing amount of genomic sequence that is available. The G6PD gene itself was cloned in 1986 (4).

The gene consists of 13 exons and is over 20 Kb in length. The entire gene composed of 20, 114 bp out of which coding sequence consists of 1548 bp and encodes 515 amino acids of G6PD protein (42). Transmission occurs from mother to son but not from father to son. Male hemizygotes (XY) and female homozygotes (XX) are
invariably more severely affected than female heterozygotes (XX). Heterozygous females usually have levels of G6PD intermediate between those of fully affected males and normal subjects. Some heterozygotes have normal red cell G6PD activity: others have a quantitative deficiency as severe as hemizygotes (figuer2.4). The behavior of the X-linked gene in G6PD deficiency bears out Lyon’s hypothesis of X inactivation. The variable amount of total G6PD activity and the double population of cells or mosaicism in heterozygous females are determined by inactivation of the X chromosome bearing either the normal or the deficient gene for G6PD (37).

![Inheritance of G6PD Deficiency](image)

As expected from the fact that most variants of G6PD causing enzyme deficiency have altered biochemical properties, it has been shown that almost all mutations of G6PD affect the coding sequence of the gene (48). The vast majority are single base substitutions leading to amino acid substitutions. 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. In the most common of these combinations (G6PD A→ Val68Met, Asn126Asp), it is suggested that the two mutations act synergistically to produce the deficient phenotype and that the instability of this variant may result from a loss of folding determinants (49). The only deletions seen in the coding sequence are small and in-frame. The absence of large deletions or frame-shift mutations indicates that a total lack of G6PD is incompatible with life (50).
The structure of tetrameric human G6PD has been solved using a natural mutant, G6PD Canton, and a deletion mutant that lacks 25 N-terminal amino acids (51).

2.3.2 Variants of G6PD
2.3.2.1 Biochemical variants

In 1967, a committee of the WHO proposed standard biochemical procedures to characterize G6PD variants such as enzyme activity, Km for G6pD and NADP, heat stability, efficiency of utilization of G6PD and deamino NADP, electrophoretic mobility and pH optima. Biochemical characterization has led to the identification of 442 distinct variants. Of these 442 variants 299 were characterized by methods agreed upon by WHO group. About 100 biochemical variants are found to be polymorphic in various human populations. The variants are grouped to 5 classes by WHO scientific working group based upon the residual enzyme activity and associated clinical symptoms, which are as follows:

Class I: Severe enzyme deficiency with CNSHA.
Class II: Severe enzyme deficiency (< 10% normal) not associated with chronic hemolysis.
Class III: Moderate to mild enzyme deficiency (60-100%), symptomatic hemolysis occurs only with exposure to certain drugs and infectious agents.
Class IV: Mild or no enzyme deficiency (60-100%), the functional activity not modified.
Class V: Increased enzyme activity (> twice normal) (42).

The normal enzyme, G6PD B, is present in 99% of Caucasian Americans and 70% of African Americans. A normal variant, G6PD A+, found in 20% of African Americans, has a faster electrophoretic mobility than G6PD B. G6PD A−, the most common variant associated with hemolysis, is found in about 10% of African Americans and in many African populations. G6PD A− has the same electrophoretic mobility as G6PD A+, but its catalytic ability is decreased. G6PD Mediterranean, the second most common abnormal variant, is common in the Mediterranean area, in India, and in Southeast Asia, with prevalence between 5% and 50 % in certain populations. Its electrophoretic mobility is normal, but its catalytic activity is greatly decreased. G6PD Canton, a common variant in East Asian populations, produces a clinical syndrome similar to G6PD A− (52).
2.3.2.2 Polymorphic variants

Polymorphic variants are those that have achieved a high frequency in some populations. They represent balanced polymorphism. The well known polymorphic variants are G6PD Mediterranean, G6PD African variant (G6PD A–) and oriental variants. Generally each population has its own characteristic mutations although there might be some exceptions occasionally (53). According to a nomenclature recommended by a WHO scientific group such variants are categorized as Class 3 when the residual enzyme activity of the red cells is greater than 10% as in G6PD A- and Class 2 when it is less than 10%, as in G6PD Mediterranean (figuer2.4) (54). Some Africans have an enzyme with the same rapid mobility encountered among deficient individuals, but the activity of the enzyme is normal. This African enzyme is designated G6PD A+ (55).

2.3.2.3 Sporadic Variants

Sporadic variants are also been reported in many populations, which are characterized by chronic non spherocytic hemolytic anemia (CNSHA), even in the absence of stress, and accordingly are designated Class 1 variants. From the point of view of the hematologist, therefore, sporadic variants are of greater importance. They are not limited to a particular population alone. Biochemical characterization is not enough and molecular characterization should be carried out before a variant can truly be classified as unique (42).

2.4 Molecular basis of G6PD variants

2.4.1 African mutations

G6PD A– mutation is an A → G transition at nt 376. Most individuals show the presence of a second mutation G → A at nt 202. The second mutation in some individuals was found at either nt 680 C → T or at nt 968 T → C (42).
2.4.2 Mediterranean mutations

G6PD Mediterranean is a C → T transition at nt 563. It was found to be not genetically homogenous. It is now known that mutations thought to be G6PD Mediterranean are actually quite diverse (42). Here several different variants, e.g. G6PD ‘Sassari’ and ‘Cagliari’, were differentiated on the basis of biochemical characteristics, but all seem to share the same mutation at nt 563. Variants from other parts of the world, thought to be unique, G6PD Dallas, Birmingham, and Panama, proved to be G6PD Mediterranean 563 T (55).

2.4.3 Oriental mutations

One of the common variants found in oriental population is the G6PD Canton mutation at nt 1376. Considerable heterogeneity of G6PD mutation has been documented in various Asian populations (56).

2.4.4 Sporadic Mutations:

Mutations that cause CNSHA are not propagated in the population but rather a rise sporadically as new mutations. Fifty seven mutations causing CNSHA have been reported. Of these, 16 different mutations are clustered in the region surrounding NADP binding site, which were found in a total span of 26 amino acids (57).
2.5 World incidence and distribution of G6PD deficiency

G-6-PD deficiency is a public health problem of enormous proportions. It has been estimated that 200 to 500 million people worldwide, are affected by G-6-PD deficiency, and that 7.5% of the population carry one or two genes for the condition (59). Gene frequencies range from 0.5% in Northern European populations, to over 25% in parts of central and West Africa, the Middle East and South East Asia (58).
Because it is an X-linked condition, the frequency of G-6-PD deficiency is expressed as the proportion of males found to be hemizygous for the condition.

A further 10% comprise female homozygotes, and an additional 10% are heterozygotes who, because of unequal X-chromosome inactivation, are functionally G-6-PD deficient. Because of migration patterns and ease of travel, G-6-PD deficiency can no longer be regarded as a condition limited to the areas marked on the map. G-6-PD deficiency may be encountered nowadays in virtually any corner of the globe (fig.2.6). It must also be taken into account that the frequencies noted in the accompanying map represent that of the entire population of a country or geographic area. These frequencies may be accurate in countries with homogenous populations. However, within any given area subgroups with a high incidence of G-6-PD deficiency may exist (59). For example, in the US, although the overall incidence is noted to be <3.0 %, African Americans are affected to the extent of 11 to 13%, and the gene frequency of South East Asians has been found to be as high as 0.22 (59). The frequency of G-6-PD deficiency in the Ashkenazi (European origin) Jewish males is only 0.5%, and under 3% of those whose families emigrated from North African countries. However, in the subgroup of Jews with origins in Turkey, Syria, Lebanon, Iraq, Iran and Kurdistan, the incidence has recently been found to be 27%, with 10% of the females being homozygotes. Jews from Kurdistan have an incidence of up to 60%—the highest incidence of G-6-PD deficiency known (60). When evaluating a neonate with unexplained jaundice it is therefore important to ascertain the family background and test for G-6-PD deficiency when appropriate (59).
2.6. Malaria and G6PD deficiency

The malaria hypothesis – that the high incidence of G6PD deficiency has arisen because G6PD-deficient alleles confer some resistance against severe malaria caused by infection with *Plasmodium falciparum* – is now generally accepted. Although it has been eradicated from temperate countries, malaria is on the increase in the tropics and kills over a million people, mainly children, each year. The high rate of mortality has meant that alleles conferring some resistance to malaria have a selective advantage (1).

2.7 Clinical manifestation

2.7.1 Acute hemolytic anemia

2.7.1.1 Drug-induced hemolysis

G6PD deficiency was first described as a result of investigations into the hemolytic effects of primaquine. Thus, the first and best-known morbid effect of G6PD deficiency was drug-induced hemolysis. Primaquine is but one of many drugs that shortens RBC life span in G6PD-deficient persons (Table 2.1).
Table (2.1) Drugs to be avoided in glucose-6-phosphate dehydrogenase deficiency

<table>
<thead>
<tr>
<th>Group</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-malarials*</td>
<td>Primaquine</td>
</tr>
<tr>
<td>Sulphonamides</td>
<td>Sulphanilamide, sulphacetamide</td>
</tr>
<tr>
<td>sulphapyridine,</td>
<td>Sulphamethoxazole</td>
</tr>
<tr>
<td>Sulphones</td>
<td>Dapsone thiazolesulfone</td>
</tr>
<tr>
<td>Other sulphur-containing drugs</td>
<td>Glibenclamide</td>
</tr>
<tr>
<td>Nitrofurans</td>
<td>Nitrofurantoin</td>
</tr>
<tr>
<td>Analgesic/antipyretic</td>
<td>Acetanilid acetylsalicylic acid (Aspirin)</td>
</tr>
<tr>
<td>antibacterial agents</td>
<td>Chloramphenicol, ciprofloxacin</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Naphthalene (mothballs), niridazole, methylene blue, toluidine blue, trinitrotoluene (TNT), urate oxidase, phenylhydrazine, phenazopyridine, isobutynitrite, fuzaolidone, acetanilid, spiramycin</td>
</tr>
</tbody>
</table>

*Quinine, chloroquine and quinidine are all acceptable for the treatment of acute malaria. Chloroquine, mefloquine, halofantrine, proguanil and pyrimethamine (but not maloprim, which contains dapsone, and fansidar, which contains a sulphonamide) are acceptable for prophylaxis (4).

2.7.1.2 Favism

A clinical manifestation of G6PD deficiency closely related to drug-induced hemolysis is the hemolytic anemia induced by ingestion of the fava bean, *Vicia faba* (1). Ingestion of fava bean is known to produce hemolysis in G6PD deficient individuals in Arab and Mediterranean countries. Patients with favism are always G6PD deficient but not all G6PD deficient individuals develop hemolysis when they ingest fava beans. Therefore, G6PD deficiency is a necessary but not sufficient cause of favism. Clinically favism presents with a sudden onset of hemolytic anemia, which usually begins after 24 to 48 hr of ingestion of the beans. Pallor, jaundice and hemoglobinuria are the hallmarks of this condition. Abdominal pain, vomiting may also occur. Acute renal failure may
supervene in adults but is rare in children (43). The highest incidence is in boys aged 2-6, but heterozygous females may also be affected. Favism may occur after the ingestion of fresh, dried or frozen beans, but it appears to be more common with fresh beans and therefore has its peak incidence at the time of the harvest. Hemolysis may occur in breast-fed infants whose mothers have eaten fava beans. Whereas it was formerly believed that favism was only seen with the more severely deficient of the polymorphic variants of G6PD (particularly G6PD Mediterranean), typical attacks have been documented in subjects of African origin with the A– variant (4).

2.7.1.2.1 Mechanism of hemolysis

The mechanism by which drugs and fava beans produce hemolytic anemia is not well understood. Such drugs do not lyse RBCs in vitro. Instead, they appear to inflict oxidative injury on the erythrocytes and, therefore, are often designated as oxidative drugs. Because of its relatively high frequency in some areas in the Mediterranean region, the mechanism by which fava beans produce hemolysis has received special attention, with the suggestion that the pathogenesis of favism and drug-induced hemolytic anemia may be essentially the same. Vicine, convicine, ascorbate, and L-DOPA are abundant in fava beans and have been considered candidate toxins. The most likely offenders are vicine and convicine, β-glucosides of pyrimidine compounds that are converted by β-glucosidases to their aglycones, vicine and isouramil, respectively. These compounds form reactive semiquinoid-free radicals and can generate active oxygen species. This results in the formation of ferrylhemoglobin, methemoglobin, and inactivation of various enzymes. The reactions that occur are complex and varied and, therefore, largely unpredictable (1).

2.7.1.3 Infection induced hemolysis

Infection is probably the most common cause of hemolysis in subjects with G6PD deficiency. Numerous bacterial, viral and rickettsial infections have been reported as precipitants, but particularly important are infectious hepatitis, pneumonia and typhoid fever. Viral infections of the upper respiratory and gastrointestinal tracts are reported to cause more severe hemolysis than bacterial infections in G6PD-deficient children. Hemolysis is usually acute and intravascular; renal failure is a well-recognized complication in adults but is rare in children (4).
2.8 Management of G6PD deficiency

Once the diagnosis of G6PD deficiency has been made, prevention is the mainstay of management. The parents should be counseled regarding the pattern of inheritance, avoidance of oxidants, risk of hemolysis during infection and a list of culpable agents provided. Other family members at risk, including parents and siblings, should be screened (43).

2.9 Treatment

Red cell transfusion is likely to be necessary and should not be delayed if the hemoglobin level falls below 9 g/dl, particularly in the face of hemoglobinuria. In some ethnic groups, e.g. African, it is preferable to use phenotype-matched red cells to avoid alloimmunisation due to variation in the frequency of red cell antigens in the donor and recipient population (58).
Chapter 3

Material and Methods

3.1 Study design
This work was performed according to the cross-sectional descriptive study design.

3.2 Target population

3.2.1 Inclusion criteria
All unrelated children aged 2-10 years admitted to Al Naser pediatric hospital due to hemolytic anemia were considered as a target for the present study. Only one child was included for each family.

3.2.2 Exclusion criteria
All other related hemolytic children were excluded from the study. Children with previously known cause of hemolytic anemia were also excluded.

3.3 Study Population
In this descriptive study, 80 children (2-10 years old) presenting with hemolytic anemia at Al Naser pediatric hospital were included and they represent the period from April 2010 and March 2011. Forty school aged healthy children were selected as the non-G6PD deficient control group for the hematological studies. Additionally, 40 X-chromosomes from apparently healthy, male subjects served as controls tested for the c.1311 polymorphism.

3.4 Blood sampling
Venous blood samples (2.5 ml) were withdrawn from each hemolytic child and were collected in K3-EDTA tubes. Blood withdrawals were performed at hospital admission and before any blood transfusion if necessary. The K3-EDTA blood was used for performing complete blood count (CBC), G6PD enzyme assay, DNA extraction and, mutation analysis.

3.5 Ethical consideration
An official approval was obtained from Helsinki committee at the Palestinian ministry of health (annex A). Another official letter of request was obtained from Al Naser Pediatric hospital who approved performing the study on the children presenting
with hemolytic anemia (annex B). The researcher has explained the purpose and objectives of the study to the parent (s) or guardian (s) of all the participants. The inclusion in the study was optional and confidential. After the free acceptance to be enrolled in the study, one of the parents (the father or the mother) was asked to sign the consent form of the study.

3.6 Data collection

The data of the study was collected via questionnaire and also from laboratory investigation of blood sampled for: G6PD enzyme activity, hemogram, the presence of the G6PD Mediterranean c.563 C>T mutation, the silent mutation c.1311 C>T of the G6PD deficient children.

3.6.1 Questionnaire

Part of data was collected by using close-ended questionnaire which was constructed and conducted in Arabic language (Annex C). The questionnaire was designed to include 3 major components with 20 items

1- Socio-demographic and general characteristics of the subjects
2- Health characteristics of the subjects.
3- Health complains of the subjects.

The questionnaire was distributed to the parent who accompanying the hemolytic anemic child on the day of admission to the pediatric hospitals. The researcher has explained the purpose and objectives of the study and declared and committed to the participant about the confidentiality of the study. After the free acceptance, one parent was asked to fill the questionnaire.

3.7 Hemogram of the subjects

Part of the blood in the K3-EDITA tubes was used to perform a hemogram [red blood cell (RBC) count, hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC),] using a Cell Dyne 1800 electronic counter (Sequoia-Turner corporation, California, USA).
3.7.1 G6PD activity assay

G6PD activity was expressed as U/g Hb at 37 °C

3.7.1.1 Principle

In our work, the activity of G6PD enzyme was measured spectrophotometrically at 340 nm using the commercially available G6PDH screening test (Randox Laboratories, Ltd, Antrim, UK) according to manufacturer instructions, and within 12 hours of sample collection. The G6PD activity assay is based on the principle of measurement of rate of absorbance of reduced NADP+ in red cell hemolysate at 340 nm and 37°C. The measurements of absorbance at 340 nm were performed on the Biosystem (Barcelona, Spain) spectrophotometer. The following reagents are provided with the kit:

- reagent 1 (R1) Triethanolamine buffer EDTA
- reagent 2 (R2) NADP
- reagent 3 (R3) Glucose phosphate
- reagent 4 (R4) Digitonin

3.7.1.2 Procedure

Preparation of the blood sample

- A volume of 0.2μl of whole blood was washed with 2ml aliquots of 0.9 NaCl solutions.
- After each wash the sample was centrifuged for 10 min. at around 3000rpm.
- Washing and centrifugation were repeated 3 times.
- The washed centrifuged erythrocytes were suspended in 0.5ml of digitonin solution and then allowed to stand for 15min at 4°C and then centrifuge again.
- The supernatant (hemolysate) was used in the assay within 2 hours.

Assay procedure

Pipette into test tube:

- R1 1000 μl
- R2 30 μl
- Hemolysate 15 μl

Mix, incubate for 5 minutes at 37 °C; and then add:

- R3 15 μl
Mix, read initial absorbance by spectrophotometer and, start timer simultaneously.
Read again after 1, 2, and, 3 minutes

**Calculations**
mU/erythrocytes per ml blood = 33650 X Δ Absorbance at 340 nm
G6PD activity as mU/g Hb= mU/erythrocytes per ml blood X 100 / Hb (g/dl)

**Normal values at 37 °C:**
6.97-20.5 U/g Hb.

### 3.7.2 DNA extraction

Genomic DNA from patient EDTA blood samples was extracted and purified by using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), and adhering to the manufacturer protocol, which can be summarized as follows:

1- Three hundred µl of well mixed blood were added to 1.5 ml Microfuge tube containing 900µl of cell lyses solution (lyses both red blood cells and white blood cells), mixed gently by inversion, and the lysate was incubated at room temperature for 10 minutes. During the incubation period, the tube was periodically mixed (2-3 times) by gentle inversion.

2- The mixture was then centrifuged at 13,000 rpm for 20 seconds, and the supernatant was removed and discarded without disrupting the visible white pellet, then the white pellet was resuspended by vigorous vortexing (10-15 seconds).

3- Three-hundred µl nuclei lyses solution, which lyses the nuclear membrane of white blood cell nuclei, were added to the resuspended pellet with pipetting the solution 5-6 times. The solution by now should become very viscous; the samples with visible clumps should be incubated at 37°C until the clumps are disrupted

4- One-hundred protein precipitation solution, which precipitates nuclear, and cytoplasmic proteins, were added to the nuclear lysate and they were mixed vigorously for 10-20 seconds by the vortex, then centrifuging at 13,000 rpm for 3 minutes at room temperature. A dark brown protein pellet should be visible.

5- The supernatant was then transferred to a clean 1.5 ml microfuge tube containing 300µl isopropanol and was mixed gently by inversion until white thread-like strands of DNA form a visible mass, then centrifuged at 13,000 rpm for 1 minute. The DNA will be visible as a small white pellet.
6- The supernatant was discarded and 300 µl of 70% ethanol were added to the small white pellet and gently inverted several times to wash the DNA pellet, then centrifuged at 13,000 rpm for 1 minute.

7- The ethanol was aspirated carefully, and then the tube was inverted on clean gauze. The pellet was air-dried for 10-15 minutes.

8- The DNA pellet was rehydrated by adding 100µl DNA rehydration solution and incubated at 65°C for one hour. The DNA pellet should be mixed periodically by gently tapping the tube.

9- Finally, the DNA was stored at 2-8°C until molecular analysis is carried out.

10- The extracted DNA was quantified using the NanoDrop™ 2000 instrument (Thermo Fisher Scientific, Lafayette, CO, USA).

11- A genomic DNA concentration of 10 ng/µl was used for all subsequent molecular analyses.

3.7.3 Molecular screening for G6PD Mediterranean c.563 C>T mutation

The DNA segment spanning exons 6-7 of the G6PD gene was amplified and sequenced according to BigDye™ terminator cycle sequencing with an ABI-3130 Genetic Analyzer (ABI Corporation, Carlsbad, CA, USA).

The amplification of Exon 6-7 of the G6PD gene was carried out using 2 µl of genomic DNA in a total PCR reaction of 25 µl.

The PCR reaction contained:
- One X of Failsafe PCR Premix D (Epicentre Biotechnologies, Madison WI, USA)
- Forty hundred nM M13-tailed primers
- forward -'5gttaaaacgacggccagtCTGGGAGGGCGTCTGAATG-3' 
- reverse 5’-caggaaacagctatgaccGCTCTGCCACCCTGTGC-3’
- U Platinum Taq DNA polymerase 1.25 (Life Technologies, NY, USA).
- Molecular grade water.

A touchdown PCR (40 cycles) was carried out in the GeneAmp ®PCR System 9700 (Applied Biosystems, Foster City, USA). The PCR cycling conditions are:
- 2 min at 96 °C
- Ten cycles of 15 s at 96 °C, 15 s at 62 °C decreasing temperature 0.5 °C /cycle, and 1 min at 72 °C.
- Thirty cycles of 15 s at 96 °C, 15 s at 68°C, and 1 min at 72 °C.
3.7.4 Molecular screening for G6PD c. 1311 C>T silent mutation

The silent mutation c.1311 C>T does not create or abolish an existing restriction site, therefore this silent mutation at c.1311 was determined according to the fragment analysis procedure as previously described by Kurdi-Haidar et al., 1990. Where the corresponding DNA segment (207 bp) of exon 11 was amplified using a modified forward and reverse primer:

Forward primer 5’-TGTTCTTCAACCCCGAGGAGT-3’
Reverse primer 3’- CTAGTGGAGTAGGACCTGCAGAA-5’

These modified primers incorporated two mismatched bases (bold underlined) thus creating TGATCA cutting site for BciI restriction enzyme.

The 203 bp amplicons of this PCR reaction are incubated with Bci I restriction enzyme at 54 °C overnight. The PCR and restriction fragment digestion products were analyzed by capillary electrophoresis using the QIAxcel Analyzer System (QIAGEN, Valencia, CA, USA). Normal c.1311 with C are not digested and gave a band corresponding to 203 bp, while the amplicons with the silent mutation c.1311 C>T are digested by the enzyme and produce two fragments 180 bp and 23 bp.

3.8 Statistical analysis

In the present work the researcher used the IBM SPSS Statistics (version 17, IBM Corporation, Somers, NY) for all statistical analyses which included comparisons between the mean values of the hematological parameters as well as G6PD enzyme activity using the independent t-test analysis, allele frequency of c.563C>T and c.1311C>T using the Chi-square test. Statistical significant was established as at p <0.05.
Chapter 4
Results

The present work was performed from April 2010 to March 2011 and included blood samples collected from 80 children aged 2-8 years old who had been referred to Al Nasser Pediatric Hospital in Gaza city due to hemolytic anemia characterized by mild to severe drop in hemoglobin concentration, jaundice, hyperbilirubinemia, and hemoglobinuria. Forty school aged children (29 males and, 11 females) without G6PD deficiency were selected as the non-G6PD deficient control group for the hematological studies. Additionally, 40 X-chromosomes from apparently healthy, male subjects served as controls tested for the c.1311 polymorphism. In this section, the results of the work will be presented and then compared.

4.1 Prevalence of G6PD deficiency among hospitalized hemolytic children

According to the biochemical evaluation of the 80 children admitted to the pediatric hospital due to hemolytic anemia, 65 (60 males & 5 females) of 80 children (81.3%) were found to be G6PD deficient.

4.2 The general characteristics of the study groups

The study population of the present study was composed of 65 subjects identified through biochemical investigations to be G6PD deficient. The general characteristics of those 65 G6PD deficient children are illustrated in table 4.1. Most of the G6PD deficient children are males (92.3%) and females comprised only 7.7% of the G6PD deficient cases. The majority (60%) of the G6PD deficient cases are resident of urban areas, while the remaining 40% (26/65) are resident of rural areas or refugee camps. In most G6PD deficient cases (67.7%) hemolytic crisis occurred in early childhood (≤ 40 months), and it was totally (100%) due to ingestion of fava beans, either green (96.9%) or dried (3.1%). None of the hemolytic cases was found due to drug or bacterial or viral infections.
Table (4.1): General characteristics of the G6PD children (n=65)

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>60</td>
<td>92.3%</td>
</tr>
<tr>
<td>Females</td>
<td>5</td>
<td>7.7%</td>
</tr>
<tr>
<td><strong>Locality</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>City</td>
<td>39</td>
<td>60.0%</td>
</tr>
<tr>
<td>Refugee camp</td>
<td>26</td>
<td>40.0%</td>
</tr>
<tr>
<td><strong>Age of developing hemolytic anemia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 40 months</td>
<td>44</td>
<td>67.7%</td>
</tr>
<tr>
<td>&gt; 40 months</td>
<td>21</td>
<td>32.3%</td>
</tr>
<tr>
<td><strong>Occurrence of hemolytic anemia after</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food</td>
<td>65</td>
<td>100%</td>
</tr>
<tr>
<td>Drug</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Infection</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Food that caused Hemolysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green fava beans</td>
<td>63</td>
<td>96.9%</td>
</tr>
<tr>
<td>Dried fava beans</td>
<td>2</td>
<td>3.1%</td>
</tr>
<tr>
<td><strong>Jaundice after birth (neonatal jaundice)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>64</td>
<td>98.5%</td>
</tr>
<tr>
<td>No</td>
<td>1</td>
<td>1.5%</td>
</tr>
</tbody>
</table>
The clinical history of those G6PD deficient children revealed that most (98.5%) of them went through neonatal jaundice after birth, which last for one week and for two weeks with 66.2% and 32.3% respectively of the G6PD deficient cases.

The results of the 65 G6PD deficient cases revealed family history of G6PD deficiency in 12 (18.5%) cases, 10 (83.3%) of them are brothers/sisters while 2 (16.7%) are cousins. Most parents (98.5%) of the G6PD deficient children showed a previous knowledge about favism and the risks of fava beans consumption on children. The parents were committed to diet constrictions for G6PD deficient hemolytic child as well as their other kids, if any. Also they were committed to the list of avoidable drugs for their kids.
4.3 Age characteristics of G6PD deficient children

The mean age (Table 4.2) of the G6PD deficient children encountered in the present work is $39.5 \pm 3.41$ months, with no significant differences ($p=0.261$) between males ($39.4 \pm 3.29$ months) and females ($41.2 \pm 4.76$ months) regarding the age they developed the hemolytic crisis and consequently were hospitalized due favism.

Table (4.2): Age characteristics of G6PD deficient children

<table>
<thead>
<tr>
<th></th>
<th>Over all n=70</th>
<th>Males n=65</th>
<th>Females n=5</th>
<th>Independent-t-test P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of developing hemolysis in G6PD deficient children</td>
<td>$39.5 \pm 3.41$</td>
<td>$39.4 \pm 3.29$</td>
<td>$41.2 \pm 4.76$</td>
<td>$0.261$</td>
</tr>
</tbody>
</table>

4.4 Consanguinity of parents of the G6PD deficient children according to locality

The consanguinity of the parents of the G6PD deficient children is mentioned in table 4.3, which showed non significant differences in consanguinity prevalence according to locality.

Table (4.3): Consanguinity in parents who reported family history for G6PD according to locality

<table>
<thead>
<tr>
<th>Consanguinity of G6PD family history</th>
<th>Overall</th>
<th>City</th>
<th>Refugee camps</th>
<th>z-score</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brothers</td>
<td>10 (15.4)</td>
<td>6 (15.4)</td>
<td>4 (15.4)</td>
<td>$P= 1.0$</td>
<td></td>
</tr>
<tr>
<td>Cousins</td>
<td>2 (3.1)</td>
<td>0 (0.0)</td>
<td>2 (7.7)</td>
<td>$P= 0.0784$</td>
<td></td>
</tr>
<tr>
<td>Non-Consanguineous</td>
<td>53 (81.5)</td>
<td>33 (84.6)</td>
<td>20 (76.9)</td>
<td>$P=0.435$</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>65 (100.0)</td>
<td>39 (100.0)</td>
<td>26 (100.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.5 Prevalence and allelic frequencies of Med c.563 C>T mutation and c.1311 C>T polymorphism among the G6PD deficient children

The molecular protocols (BigDye™ terminator cycle sequencing for Med c.563 C>T and PCR/RELP for c.1311 C>T) we used in the present work enabled us to characterize the Mediterranean c.563 C>T mutation and the c.1311 C>T polymorphism among the G6PD deficient children (table 4.4). The Mediterranean c.563 C>T mutation was encountered in 23 out of 65 G6PD deficient children representing a mutation frequency of 35.4 %, while the c.1311 C>T polymorphism was encountered in 23 out of 55 G6PD deficient children (50 males and 5 females), representing a frequency of 41.8 %. Due to insufficient sample quantity genetic analysis for c.1311 C>T was not performed in the remaining ten samples. Also the c.1311 C>T polymorphism was identified in 7 out of 40 control blood samples who are known to be none G6PD deficient, representing frequency of 17.5 % (60).

Table 4.4: Allele frequency of Med c.563 C>T and c.1311 C>T polymorphism *

<table>
<thead>
<tr>
<th></th>
<th>Overall G6PD deficient AF (chromosomes tested)</th>
<th>c.1311 C&gt;T AF (chromosomes tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Med c.563 C&gt;T</td>
<td>0.33 (70)</td>
<td>0.96 (23)</td>
</tr>
<tr>
<td>Non Med G6PD mutations</td>
<td>0.67 (70)</td>
<td>0.08 (37)</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>0.18 (40)</td>
</tr>
</tbody>
</table>

In term of allelic frequency which is calculated as the fraction of the affected chromosomes for a specific allele of the total number of chromosomes screened, seventy X-chromosomes (60 male and, 5 female) from G6PD deficient patients were investigated for the Mediterranean c.563 C>T mutation and revealed allelic frequency for Mediterranean c.563 C>T of 0.33 (23 chromosomes/70 chromosomes). The allele frequency of c.1311 C>T among the entire G6PD deficient group was 0.42 (25 chromosomes/60 chromosomes). However, within the G6PD deficient group the incidence of c.1311 C>T polymorphism with patients carrying the Mediterranean c.563 C>T mutation was 95.2 percent (allele frequency 0.96) compared to 11.4 percent (allele frequency 0.08) in G6PD deficient individuals without the Mediterranean c.563 C>T mutation (p-value < 0.001) (60).
The prevalence of c.1311 C>T polymorphism among the control group was 17.5 percent (allele frequency 0.18), which was significantly lower (p-value < 0.001) than the frequency found in the Mediterranean c.563 C>T G6PD deficient group (table 4.4). A comparative analysis of data for Mediterranean c.563 C>T and c.1311 C>T polymorphism prevalence in other regional and worldwide populations is presented in Table 5.1 of the discussion section (60).

4.6 Age characteristics of developing hemolysis in G6PD deficient according to mutation

Results presented in table 4.5 revealed non-significant differences between G6PD deficient children with the Mediterranean c.563 C>T mutation and those with non Mediterranean mutation regarding the age at which the developed hemolysis, 39.09±3.29 and 39.79 ± 3.50 months respectively, p value = 0.434.

Table (4.5): Age characteristics of developing Hemolysis according to mutation

<table>
<thead>
<tr>
<th>age of developing hemolysis</th>
<th>Med mutation</th>
<th>Non Med mutation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>39.09± 3.29</td>
<td>39.79 ± 3.50</td>
<td>0.434</td>
<td></td>
</tr>
</tbody>
</table>

4.7 Distribution of Mediterranean c.563 C>T mutation and c.1311 C>T polymorphism according to locality

Tables 4.6 and 4.7 present respectively the distribution of the Mediterranean c.563 C>T mutation and c.1311 C>T polymorphism according to locality of the G6PD deficient children, which revealed no significant differences of the two variants according to locality.

Table (4.6): Distribution of Mediterranean c.563 C>T mutation according to locality

<table>
<thead>
<tr>
<th>locality</th>
<th>Overall</th>
<th>Med mutation</th>
<th>Non Med mutation</th>
<th>Chi square test P- Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>City</td>
<td>39 (60.0%)</td>
<td>12 (52.2%)</td>
<td>27 (64.3%)</td>
<td>0.245</td>
</tr>
<tr>
<td>Refugee camp</td>
<td>26 (40.0%)</td>
<td>11 (47.8%)</td>
<td>15 (35.7%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>65 (100.0%)</td>
<td>23 (100.0%)</td>
<td>42 (100.0%)</td>
<td></td>
</tr>
</tbody>
</table>
Table (4.7): Distribution of c1311 mutation according to locality

<table>
<thead>
<tr>
<th>locality</th>
<th>Overall</th>
<th>c.1311T</th>
<th>c.1311C</th>
<th>Chi square test</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>City</td>
<td>32 (58.2%)</td>
<td>12 (54.5%)</td>
<td>20 (60.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Refugee camp</td>
<td>23 (41.8%)</td>
<td>10 (45.5%)</td>
<td>13 (39.4%)</td>
<td></td>
<td>0.432</td>
</tr>
<tr>
<td>Total</td>
<td>55 (100.0%)</td>
<td>22 (100.0%)</td>
<td>33 (100.0%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.8 Statistical summary of G6PD enzyme activity and erythrogram based on the presence of c.563 C>T mutation in G6PD as well as non G6PD deficient

The statistical summary and comparisons of G6PD enzyme activity and erythrogram for the G6PD deficient children based on the presence of c.563 mutation in G6PD as well as for 40 non G6PD deficient children used as negative controls are mentioned in table 4.8. These results revealed that the G6PD deficient subjects were admitted to the hospital with significantly lower hematological parameters except for MCHC. In addition, no significant hematological or enzymatic activity differences were reported between the G6PD deficient children carrying the Mediterranean c.563 C>T mutation and those G6PD deficient children carrying non-Mediterranean mutation (60).
Table 4.8 Statistical summary of G6PD enzyme activity and erythrogram based on the presence of c.563 mutation in G6PD as well as non G6PD deficient*

<table>
<thead>
<tr>
<th></th>
<th>Overall n=65</th>
<th>Med c.563 C&gt;T n=23</th>
<th>Non Med c.563 C&gt;T n=42</th>
<th>Non G6PD deficient N=40</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males n=21</td>
<td>Females N=2</td>
<td>Males n=39</td>
<td>Females n=3</td>
</tr>
<tr>
<td>G6PD (U/g Hb)</td>
<td>1.48 ± 1.26</td>
<td>1.12± 1.17</td>
<td>2.26±1.40</td>
<td>1.44±1.15*</td>
</tr>
<tr>
<td>RBC (X 10¹²/L)</td>
<td>3.65 ± 0.98</td>
<td>3.83±0.85*</td>
<td>1.92±0.16</td>
<td>3.60±0.99</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>8.44 ± 2.08</td>
<td>8.78±1.97*</td>
<td>5.35±0.49</td>
<td>8.38±2.07</td>
</tr>
<tr>
<td>Het (%)</td>
<td>25.71 ± 6.30</td>
<td>26.54±6.06*</td>
<td>16.10±1.41</td>
<td>25.57±6.18</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>74.70 ± 10.66</td>
<td>71.30±8.57</td>
<td>83.45±16.33</td>
<td>76.67±10.52</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>24.51 ± 3.68</td>
<td>24.22±3.22</td>
<td>27.70±5.52</td>
<td>24.73±3.69</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>32.96 ± 1.47</td>
<td>33.54±1.80</td>
<td>33.20±0.14</td>
<td>32.69±1.28</td>
</tr>
</tbody>
</table>

* Results were first published in Sirdah et al., 2012 (60)

Med = Mediterranean mutation c.563 C>T

No significant differences were reported between Med and non-Med G6PD deficient groups evaluated by “Independent t-test”

$ No significant differences were reported between males and females among the Non G6PD deficient evaluated by “Independent t-test.

* significant difference between males and females of same group

It is worthwhile to mention that among the G6PD deficient group with Mediterranean c.563 C>T mutation all females were heterozygous for this mutation. Therefore, G6PD deficient females showed a significantly higher enzymatic activity than males 3.27±1.24 vs. 1.33 ± 1.16 U/g Hb respectively, p= 0.001.
Chapter 5

Discussion

G6PD deficiency is commonly encountered among hemolytic children at Al Naser Pediatric Hospital at Gaza Strip. Although, Gaza Strip is part of the Mediterranean basin where G6PD deficiency is highly prevalent, neither previous screening nor molecular studies have been performed to characterize G6PD deficiency among the Gaza Strip population. Medical efforts in Gaza Strip are currently limited to providing G6PD deficient patients with clinical care when a crisis occurs. In addition to geographical rationale, green and dried beans (Fava beans) could be considered as the commonest traditional food for the majority of the population in Gaza strip as well as in other Arabic Middle Eastern countries. (9).

Understanding the prevalence of this disorder in the Palestinian population is important for the prevention, management and treatment of this disease. Through characterizing the nature of G6PD deficiency in the Gaza Strip population it will improve individual and professional decisions in the prevention, treatment and management of the disease. Therefore, we design the present work to determine the frequency of the Mediterranean mutation (c.563 C>T) among Palestinian G6PD deficient patients in Gaza strip, and to associate it with the c.1311 C>T polymorphism (silent mutation) of the G6PD gene.

5.1 Prevalence of G6PD deficiency among hospitalized hemolytic children

Our present work revealed a high proportion (81.3%) of G6PD deficiency among the hemolytic anemic children referred to Al Naser pediatric hospital at Gaza during the one year period of the study. The reported high prevalence of G6PD deficiency among hemolytic children could be attributed to two major factors: the first is a considerable prevalence of G6PD deficiency gene variants among the population, while the second is the huge reduction in the number of new births with β-thalassemic disorders after implementing the premarital test for β-thalassemia as obligatory step in Palestine (61, 62).
5.2 The general characteristics of the study groups

In our cohort who included 65 G6PD deficient children, males represent the majority (92.3%) of those deficient cases admitted to the pediatric hospital due to hemolysis. This high percentage of deficient male children is concomitant with the genetics of the G6PD deficiency gene which is inherited as a sex-linked gene; therefore, males are more affected than females. Also the heterozygous females for the G6PD gene are genetic mosaics due to X-chromosome inactivation in somatic cells (3). It is very noticeable that the hemolytic crisis, in most cases, occurred in early childhood which is about the 3rd year of child life (mean 39.5 ± 3.41 months) and more importantly all hemolytic crises in those G6PD deficient children were due to ingestion of fava beans, either green (96.9%) or dried (3.1%). Surprisingly, none of the hemolytic cases was due to drug or bacterial or viral infections. In Gaza strip, as well as in other Arab countries, green and dried Fava beans which considers as the primary causative agent of hemolytic crisis in G6PD deficiency, are an essential dietary staple for the majority of the population in Gaza Strip. The 2006 report of the Palestinian central bureau of statistics (PCBS) showed that the average annual household quantities of green, dried and tinned beans is more than 20 Kg (9). Meloni and his collage 1983 showed that among 948 G6PD deficient children hospitalized for hemolytic anemia, 923 (97.4%) of these hemolytic crises occurred after ingestion of Fava been either fresh green (94.4%) or dried (3.0%) (62). However Shannon and Buchanan, 1982 showed that severe hemolytic anemia in 11 out of 14 hospitalized black children with G6PD deficiency occurred due to bacterial or viral infections (63). It is worthwhile to mention that almost all (98.5%) our G6PD deficient children of the present work suffered from neonatal jaundice or hyperbilirubinemia directly after birth, lasting for average of one to two weeks. Riskin and his collage 2012 reported a significant association between G6PD deficiency and significant neonatal hyperbilirubinemia with an increased risk for neonatal hyperbilirubinemia with borderline G6PD activity (64). In Egyptian population, Abdel Fattah and his collage 2010 demonstrated that G6PD deficiency is an important cause for neonatal jaundice in Egyptians, which justifies the necessity for introducing of neonatal screening for G6PD deficiency in Egypt (65). While in Indian population 5% of neonatal jaundice cases are due to G6PD deficiency (66).
Additional G6PD deficient cases were report of 12 (18.5%) families of the G6PD deficient children of our study population. In 10 those cases the additional G6PD case was reported among brothers while in 2 cases the additional case was among cousins. Therefore, it could be of important value to screen the family members specially those at childhood for G6PD deficiency and accordingly provide the families with the precautions and measures that required preventing the hemolytic crises in families at risk. A good knowledge, attitude and practices were reported in the families of the deficient children. Almost all (98.5%) of the G6PD deficient parents have a good knowledge about the disease and they are committing to avoidable drugs and also they provide their deficient children with certain diets lacking fava bean and other risk legumes. This could be an early success indicator for national screening and awareness programs for G6PD if adopted for the Palestinian population at Gaza strip. Neonatal screening and health education programs resulted in a substantial decrease in the number of cases of hemolytic crises due to G6PD deficiency (67). Also, Chuu and his colleagues showed the effectiveness of neonatal screening programs for G6PD deficiency to prevent severe neonatal jaundice (68).

5.3 Prevalence and distribution of Med c.563 C>T mutation and c.1311 C>T polymorphism among the G6PD deficient children

The preset work emphasized the predominance (prevalence= 35.4%, AF=0.33) of the Mediterranean c.563 C>T mutation among the Palestinian G6PD deficient at Gaza strip. No significant differences in the prevalence of the Mediterranean c.563 C>T mutation or the c.1311 C>T polymorphism were reported between localities. G6PD Mediterranean mutation has been investigated and reported as the predominant G6PD mutation in different Mediterranean and Middle Eastern countries as well as in India and South Asian countries, however, globally there is a very wide prevalence range, the highest prevalence Sardinians (83.0-100%) and Kurdish Jews (80-97 %). The prevalence of Mediterranean mutation among the Gaza strip population is concomitant with the general trend of this mutation is the region, but, it was lower than its prevalence in the adjacent Arab and Middle Eastern countries like Egypt (62 %), Jordan (65.6 %), Saudi Arabia (51.1-89.1%), Iraq (87.8 %), and Iran (66.2-91.2%) which indicated other candidate mutations to prevail significantly in the Gaza population.
The strong association between the Mediterranean mutation and the c.1311 polymorphism (95.2 % of the cases) is interesting and has been considered as very marked linkage disequilibrium (69). The AF of c.1311 C>T polymorphism among patients carrying the Mediterranean mutation was 0.96 which was significantly higher than its value in patients with non Mediterranean G6PD mutations (0.08) or with the control group (0.18). Concomitant association results between Mediterranean and c.1311 C>T were also repotted in other population of the Middle East and Mediterranean basin (Table 5.1), However, Mediterranean mutation has also been reported predominantly in patients from Indian subcontinent and southern Italy without the association of c.1311 polymorphism. The origins of the c.563 Mediterranean mutation has been postulated to have arisen independently in Europe, Africa and in South Asia (India) based upon its association with the C or T alleles at c.1311 (70, 69, 71) These observations suggest the presence of the Mediterranean mutation within the Palestinian population is more closely associated with European origin than one arising from South Asia of African origin. Different interpretations have been proposed for Mediterranean and c.1311 C>T association pattern. Beutler and Kuhl in 1990; suggested two different origin and Mediterranean mutation may have arisen independently in Europe and Asia, and exceptions in Mediterranean and c.1311 C>T association could result from crossover or population admixture (70). While, Kurdi-Haidar and his colleagues assumed that Mediterranean mutation originally took place in G6PD gene which already had the c.1311 C>T polymorphism, and the absence of 1311 polymorphism in the Italian subjects, might be due to an intragenic event (69). Tishkoff and his colleagues with their haplotyping study expanded the previous proposals into a more historical and population genetics trend/ background/setting. They attributed Mediterranean mutation distribution to a possible spread through the army of Alexander the Great who occupied large territories ranging from Mediterranean, Middle East, North Africa and India during the fourth century B.C. to highlight this association properly (72). Therefore we could recommend a large scale international cooperative study that aims for better understanding of this Mediterranean and c.1311 C>T association based on molecular investigation of G6PD deficient chromosomes from different ethnicities and geographical areas of the world.

The prevalence of c.1311 C>T polymorphism among the control group was 17.5 percent (allele frequency 0.18), which was significantly lower (p-value < 0.001) than
the frequency found in the Mediterranean c.563 C>T G6PD deficient group (table 4.4). A comparative analysis of data for Mediterranean c.563 C>T and c.1311 C>T polymorphism prevalence in other regional and worldwide populations is presented in Table 5.1.

Table (5.1) Prevalence of G6PD c.563C>T and c.1311 C>T polymorphism in different populations of the world*

<table>
<thead>
<tr>
<th>Area</th>
<th>c.563T among G6PD deficient (%)</th>
<th>c.1311T among c.563T (%)</th>
<th>1311T in overall G6PD deficient (%)</th>
<th>In non-G6PD Deficient (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gaza, Palestine</td>
<td>35.4</td>
<td>95.2</td>
<td>41.8</td>
<td>17.5</td>
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<tr>
<td>Bahrain</td>
<td>91.2</td>
<td>95.7</td>
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<td></td>
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<tr>
<td>Croatia</td>
<td>16.7 -21.1</td>
<td>100</td>
<td>15</td>
<td>21</td>
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<tr>
<td>Cyprus</td>
<td>52.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egypt</td>
<td>28.6 -62.0</td>
<td>75-100</td>
<td>52.0</td>
<td>10</td>
</tr>
<tr>
<td>India</td>
<td>60.4</td>
<td>22.8</td>
<td>32.1</td>
<td></td>
</tr>
<tr>
<td>Iran</td>
<td>66.2 - 91.2</td>
<td>90</td>
<td>0.0</td>
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<tr>
<td>Iraq</td>
<td>87.8</td>
<td>97.9</td>
<td>88.0</td>
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<td>72.7</td>
<td>20.7</td>
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<td>Sardinia</td>
<td>83.0- 100</td>
<td>100</td>
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<td></td>
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<tr>
<td>Italy, Sicily &amp; Calabria</td>
<td>52.0</td>
<td>92.3</td>
<td>16.7- 20</td>
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<td>Kurdish Jews</td>
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<td>Jordan</td>
<td>33.3- 65.6</td>
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<td>72.9-74.4</td>
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<td>Oman</td>
<td>75</td>
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<td>11.1-14.3</td>
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<td>United Arab Emirates Al-Ain</td>
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<td>Malaysia</td>
<td>26.7</td>
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* as appeared in Sirdah et al., 2012 (60)
5.4 Statistical summary of G6PD enzyme activity and erythrogram based on the presence of c.563 mutation in G6PD as well as non G6PD deficient

The biochemical and hematological results from the hospitalized G6PD deficient subjects revealed a significantly lower enzyme activity and hematological parameters except for MCHC when compared to the control group. A non significant hematological or enzymatic activity differences were reported between the G6PD deficient children carrying the Mediterranean c.563 C>T mutation and those G6PD deficient children carrying non-Mediterranean mutation. The biochemical and hematological profile of the subjects with Mediterranean c.563 mutation is concomitant to the class III of the Mediterranean mutation provided by the world health organization (6). Among the G6PD deficient group (65 patients) all females (5) were heterozygous for this mutation. Therefore, G6PD deficient females showed a significantly higher enzymatic activity than males $3.27 \pm 1.24$ vs. $1.33 \pm 1.16$ U/g Hb respectively, $p=0.001$, which is due to both the heterozygosity as well as to the mosaicism resulting from X-chromosome inactivation in somatic cells (3).

It is concluded that the Mediterranean mutation c.563 C>T is common among G6PD deficient Gaza Strip Palestinians and is highly associated with the c.1311 C>T polymorphism. This work could be foundational for further investigations of G6PD molecular studies with emphasis on ancestral origin of these variants. The present study also emphasizes the predominant existence of the G6PD deficiency among Palestinian population, which justifies the necessity of the Palestinian Health policy leaders establishing nationwide programs of newborn screening for G6PD deficiency.
CHAPTER 6

Conclusions and Recommendations

Conclusions

- A high proportion (81.3%) of G6PD deficiencies were identified among the hemolytic anemic children referred to Al Naser pediatric hospital at Gaza during the one year period of the study.
- In our cohort, males represent the majority (92.3%) of those deficient cases admitted to the pediatric hospital due to hemolysis.
- In most G6PD deficient cases (67.7%) hemolytic crisis occurred in early childhood (≤ 40 months)
- Ingestion of fava beans, either green (96.9 %) or dried (3.1 %) was the main cause of hemolytic anemia in all G6PD deficient cases.
- Most (98.5%) of G6PD deficient children went through neonatal jaundice after birth, which last for 1-2 weeks
- Family history of G6PD deficiency was reported in 12 (18.5%) cases.
- Most parents (98.5%) of the G6PD deficient children showed a previous knowledge about favism and the risks of fava beans consumption on children.
- The Mediterranean c.563 C>T mutation was encountered among the G6PD deficient children with a mutation frequency of 35.4 %.
- The c.1311 C>T polymorphism was encountered among the G6PD deficient children representing a frequency of 41.8 %.
- The c.1311 C>T polymorphism was identified in control blood samples who are known to be none G6PD deficient, representing frequency of 17.5 %.
• Within the G6PD deficient group the incidence of c.1311 C>T polymorphism with patients carrying the Mediterranean c.563 C>T mutation was 95.2 percent compared to 11.4 percent in G6PD deficient individuals without the Mediterranean c.563 C>T mutation.

• The G6PD deficient subjects were admitted to the hospital with a significantly lower hematological parameters except for MCHC.

• No significant hematological or enzymatic activity differences were reported between the G6PD deficient children carrying the Mediterranean c.563 C>T mutation and those G6PD deficient children carrying non-Mediterranean mutation.

• G6PD deficient females showed a significantly higher enzymatic activity than males.

Recommendations

• Molecular screening for all G6PD deficient patients admitted to pediatric hospitals due to hemolytic crises.

• Establishing and conducting educational awareness programs for G6PD deficiency especially among mothers.

• Specific clinical follow up programs for the deficient children and their families.

• Establishing nationwide programs of newborn screening for G6PD deficiency.
References


31 **Chan TK.** (1983): Glucose-6-phosphate dehydrogenase (G6PD) deficiency. MD Thesis. The University of Hong Kong .


68 Chuu WM, Lin DT, Lin KH, Chen BW, Chen RL, Lin KS. (1996): Can severe neonatal jaundice be prevented by neonatal screening for glucose-6-


ANNEXES

ANNEX A

Palestinian National Authority
Ministry of Health
Helsinki Committee

السلطة الوطنية الفلسطينية
وزارة الصحة
لجنة هيلسكي

التاريخ: 5/3/2012

الاسم: لينا عود
نفيكم عذراً بأن اللجنة قد قررت مقترحكم
حوالي:

Name:

I would like to inform you that the committee has discussed your application about:

“Biochemical and Molecular characterization of Glucose-6-phosphate dehydrogenase deficient children at Gaza, Palestine.”

In its meeting on March 2012
and decided the Following:

To approve the above mentioned research study.

Signature

Member

Member

Chairperson

Conditions:

- Valid for 2 years from the date of approval to start.
- It is necessary to notify the committee in any change in the admitted study protocol.
- The committee appreciate receiving one copy of your final research when it is completed.
ANNEX B

The Islamic University of Gaza

Date: 5/3/2008

To: Dean of Students
From: Director of the Science Program

Subject: Inquiry Regarding the G6PD Deficiency Enzyme

Dear Dean,

I am writing to inquire about the G6PD deficiency enzyme, which is known to cause hemolytic anemia in individuals with the enzyme deficiency. It is particularly important to identify carriers of this enzyme deficiency, as it can lead to complications in children. I would like to conduct a study to examine the prevalence of the G6PD deficiency enzyme among the students of the university.

I am requesting your assistance in organizing a study that involves collecting blood samples from a large number of students to identify those who may be carriers of the enzyme deficiency. The study will be conducted in collaboration with the laboratory department of the university.

I look forward to your positive response to this request.

Sincerely,

[Signature]

[Redacted]
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**ANNEX C**

G-6-PD deficiency enzyme

استبانة حول مرض "انيميا الفول"