Deanship of Graduate Studies

Al-Quds University

Genotyping of Alpha-Globin Gene Mutations among Palestinian Patients with Unexplained Microcytosis

Maysoon Adnan Suliman Hamayel

M.Sc. Thesis

Jerusalem - Palestine

1434 Hijri/ 2013 AD

Genotyping of Alpha-Globin Gene Mutations among Palestinian Patients with Unexplained Microcytosis

Prepared By:

Maysoon Adnan Suliman Hamayel

B.Sc.Medical Technology

Al-Quds University - Jerusalem

Supervisor: Dr. Mahmoud A. Srour.

Co- Supervisor: Mr. Khalid Younis

A thesis submitted in partial fulfillment of requirements for the Degree of Master in Medical Laboratory Sciences / Hematology Track, Faculty of Health Professions - Al-Quds University

1434 Hijri/ 2013 AD

Al-Quds University

Deanship of Graduate Studies

Department of Medical Laboratory Sciences

Thesis Approval

Genotyping of Alpha-Globin Gene Mutations among Palestinian Patients with Unexplained Microcytosis

Prepared by: Maysoon Adnan Suliman Hamayel
Registration Number: 20724022

Supervisor: Dr. Mahmoud A. Srour
Co-Supervisor: Mr. Khalid Younis

Master thesis submitted and accepted, Date: Monday 14.01.2013
The names and signatures of the examining committee members are as follows:

1. Head of committee: Dr. Mahmoud Srour ……..

2. Internal examiner: Dr. Rula Abdalghani ……..

3. External examiner: Prof. Moien Kanaan ……..

4. Committee member: Mr. KhalidYounis ……..

Jerusalem/Palestine

1434 Hijri / 2013 AD

Dedication

To my parents

To my grandparent

To my brother

To my sisters

To all my family

For their love and support
Life with them has never been dull

Maysoon Adnan Suliman Hamayel

Declaration:

I certify that this thesis submitted for the degree of Master of Medical Laboratory Sciences/ Hematology track, is the result of my own research, except where otherwise acknowledged, and that this study has not been submitted for higher degree to any other university or institution.

Signed

Maysoon Adnan Suliman Hamayel

Date: 14/01/2013

Acknowledgement

I want to thank Al-Quds University for giving me the opportunity to be one of their students, especially the Department of Medical Laboratory Sciences represented by their kindness compassionate teachers, who always supported and encouraged me.

I really want to thank my supervisors Dr. Mahmoud Srour and Mr. Khalid Younis who were always with me in this research step by step, supporting and advising me.

I would like to give the great thanks for the people who helped me to accomplish this work, the general manger of Specialized Medical Laboratories Mr. Husam Thaher, the technicians Ms. Barehan Al-hindi, and Ms. Duaa' Faza', who helped me in samples collection and preservation. My thanks extend to the manager of the Medical laboratory Mrs. Huda Nassar at the Palestinian Red Crescent Society Hospital in Al-Bireh, who helped me in the collection of neonate samples.

I would like to thank also, Mr. Ibraheem Salem, and Mr. Issa Shtayeh for their valuable advices and help.

Thank you all my teachers, my friends, and my family.

Abstract

Alpha-thalassemia is an autosomal recessive disorder characterized by microcytic hypochromic anemia. The clinical phenotype ranges from asymptomatic carrier to a lethal hydrops fetalis syndrome. Alpha-thalassemia is a common disorder worldwide, and is especially frequent in the Mediterranean region, Middle East, South East Asia, Africa and Indian subcontinent. The molecular basis of α-thalassemia has been addressed by several studies from the Mediterranean region but not from Palestine. Therefore, this study aimed to investigate the frequency and genotype of α-globin gene mutations ($-\alpha^{3.7}$, $-\alpha^{4.2}$, α2-IVSI-5nt, $--^{MED}$ and $\alpha\alpha\alpha^{anti3.7}$) in a cohort of Palestinian patients with unexplained microcytosis. For this purpose, 73 patients' samples with unexplained microcytosis not due to β-thalassemia or iron deficiency as well as 19 neonates' samples with an MCV <95 fL, were analyzed. Gap-PCR, DNA sequencing and ARMS PCR were used for detection of α-thalassemia mutations common to the Mediterranean region. Of the 73 patient samples analyzed, 50.7 % of samples were carrier for at least one of four mutations, $-\alpha^{3.7}$, α2-IVSI-5nt, $--^{MED}$ and $\alpha\alpha\alpha^{anti3.7}$. The $-\alpha^{3.7}$ and α2-IVSI-5nt mutations were the most frequent mutations among the patients' samples and were found in 48.9 % and 44.7 % of the mutant chromosomes. The $--^{MED}$ and $\alpha\alpha\alpha^{anti3.7}$ mutations were found in 4.3 % and 2.1 % of mutant chromosomes, respectively. In the neonates' samples, the $-\alpha^{3.7}$ and α2-IVSI-5nt mutations were also the most frequent and were found in a frequency close to that observed in patients' samples. The $-\alpha^{4.2}$ mutation was not detected among the study samples, as this mutation is present in low frequency in the Mediterranean region. The high frequency of the α2-IVSI-5nt mutation is novel to the Palestinian α-thalassemia patients and has not been reported in other populations. Analysis of the correlation between red cell parameters and the different α-thalassemia mutations reveled that none of these parameters could predict the presence of α-thalassemia mutations. The red cell indices are general indicators that are altered in different hemoglobin disorders. In conclusion, 50.7 % of the study samples have one or two of the α-thalassemia mutations investigated in this study. Molecular diagnosis of α-thalassemia is strongly recommended in cases with unexplained microcytosis in Palestine. The $-\alpha^{3.7}$ and α2-IVSI-5nt mutations are the most frequent α-thalassemia mutations among the samples analyzed in this study.

Table of Content

List of Tables

List of Figures

List of Appendices

List of Abbreviations

Bp	Base Pair
DMSO	Dimethyl Sulfoxide
DPG	Diphosphoglycerate
FIL	Filipino
Hb	Hemoglobin
HbCS	Hb Constant Spring
HbF	Fetal Hemoglobin
HbS	Sickle Cell Hemoglobin
HPFH	Hereditary Persistence of Fetal Hemoglobin
HS-40	Hypersensitive Site 40 kb
IDA	Iron Deficiency Anemia
Kb	Kilo Base
LCR	Locus Control Region
MCH	Mean Corpuscular Hemoglobin
MCV	Mean Corpuscular Volume
MED	Mediterranean
Nt	Nucleotide
PCR	Polymerase Chain Reaction
(ARMS) PCR	Amplification Refractory Mutation System
(RFLP) PCR	Restriction Fragment length Polymorphism
RBC	Red Blood Cell
RDW	Red Blood Cell Distribution Width
SEA	South East Asia
THIA	Thailand
WHO	World Health Organization
α- Thalassemia	Alpha-Thalassemia
β-Thalassemia	Beta-Thalassemia

Chapter One: Introduction

1. Introduction

Hemoglobinopathies are a heterogeneous group of inherited disorders of hemoglobin (Hb) characterized by reduced synthesis of one or more globin chains (Thalassemias) or synthesis of a structurally abnormal Hb variant (Clark and Thein, 2004). The WHO estimates that about 7 % of the world populations are carriers to Hb disorders. The expected number of new cases of thalassemia will be around 900,000 in the next 20 years. So it is considered one of the most common monogenic diseases and one of the world's major health problems (Vichinsky, 2005; Kohne, 2011).

In the past, thalassemias were mainly found in the Mediterranean area and large parts of Asia and Africa. Today due to international migration, thalassemia is found worldwide albeit in different frequencies (Kohne, 2011).

1.1. Human Hemoglobins

Hemoglobin is a globular protein that consists of two alpha chains, each with 141 amino acids, and two beta chains, each with 146 amino acids. Each α or β globin chain folds into eight α helical segments (A-H) which, in turn, fold to form globular structures. The globular structure is stabilized by salt bridges, hydrogen bonds, and hydrophobic interactions. The heme group is located within a hydrophobic pocket of each globin subunit. (Nelson and Cox, 2004).

There are two types of interactions between α and β chains: $\alpha_1\beta_1$ and $\alpha_1\beta_2$. Each poly peptide chain binds to one heme group. The heme group is composed of an iron atom (in the ferrous state) positioned in the center of the protoporphyrin ring (Figure 1). Each iron atom binds

successfully and reversibly to one oxygen atom transporting it from the lungs to the peripheral tissues (Nelson and Cox, 2004; Ribeiro and Sonati, 2008).

All normal hemoglobins are formed as tetramers consisting of two α–like chains and two β-like chains. Normal adult RBCs contain the following types of hemoglobin: 95-97% of HbA ($\alpha_2\beta_2$), 2-3% of HbA_2 ($\alpha_2\delta_2$) and 1-2% of HbF ($\alpha_2\gamma_2$) (Greer et al., 2004; Sarnaik, 2005).

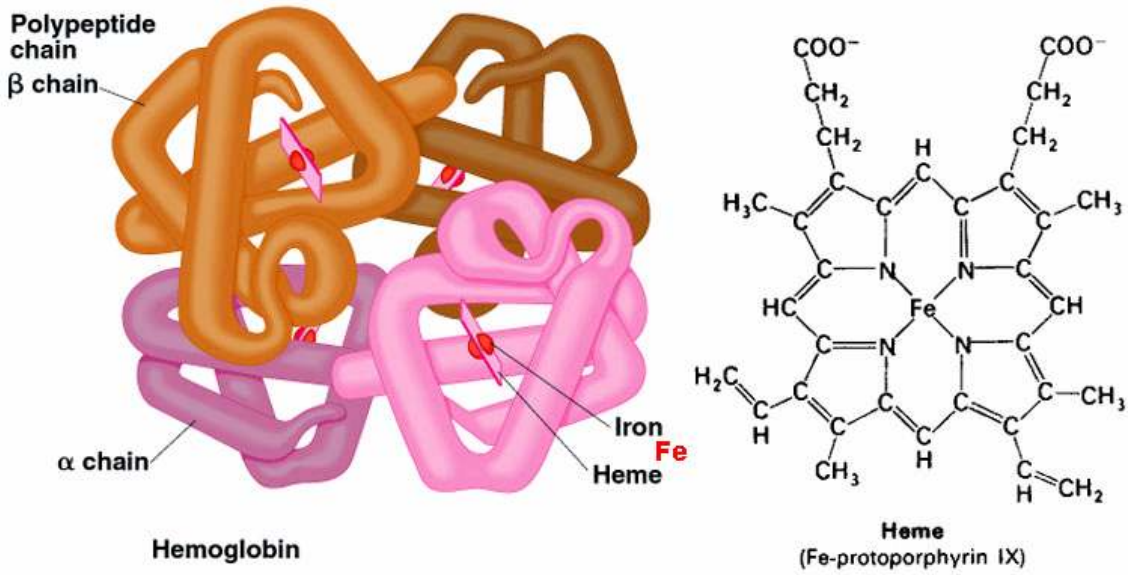

Figure 1: Hemoglobin structure. (Adapted from Mortada, 2009).

1.2. Nomenclature

Hemoglobin was named by using the letters of the alphabet, such as HbA for adult hemoglobin, HbF for fetal hemoglobin and HbS for sickle cell hemoglobin. By the time, this system was unable to provide enough names for new hemoglobins so the need for a new system was increased. In 1960, a new system of nomenclature was developed; the alphabets were used only for normal hemoglobins A and F and abnormal hemoglobin C, E, S and H (Greer et al., 2004).

Abnormal hemoglobins have both a common name which is usually given by the discoverer and a scientific name which shows the variant chain, both the sequential and helical number of the abnormal amino acid and the nature of substitution, thus HbS is designated as $\alpha_2\beta_2^{6Glu\text{-}Val}$ or more precisely as $\alpha_2\beta_2^{6(A3)Glu\text{-}Val}$ (Greer et al.,2004).

1.3. Organization and Structure of Globin Genes

Globin genes occur in clusters, the α- and α-like genes on the short arm of chromosome 16, and the β- and β-like genes on the short arm of chromosome 11(Figure 2). The α-like cluster extends over 40-kilobase range, and the β-like cluster extends over 60-kilobase range. In the α-gene cluster, two α-globin structural genes α_1 and α_2 are placed at 3́ end of the complex. The nucleotide sequences of both genes are the same with slight variation in the sequence of second intron, but the rate of expression of α2 gene is twice that of α_1 gene. Consequently the α_2-gene mutations are more severe than the α_1-gene mutations (Chui, 2005).

The β gene complex consists of six genes arranged in the following 5́ to 3́ order: a single embryonic gene (ε), two fetal genes (γ^G and γ^A), a pseudo-β gene (ψβ), the δ gene, and the β gene. All functional globin genes consist of three exons and two introns (Ribeiro and Sonati, 2008).

Each cluster of genes are controlled by regulatory elements located upstream of the genes. The β cluster is controlled by a group of remote regulatory elements DNAse I–hypersensitive sites collectively known as the locus control region (LCR) (Higgs et al., 2005). Whereas in the α cluster, there is a single known element referred to as HS-40, (so called because it is a DNAse I–hypersensitive site located approximately 40 kb upstream of the 5′ end of the ζ-globin gene) as shown in Figure 2 (Steensma et al., 2005).

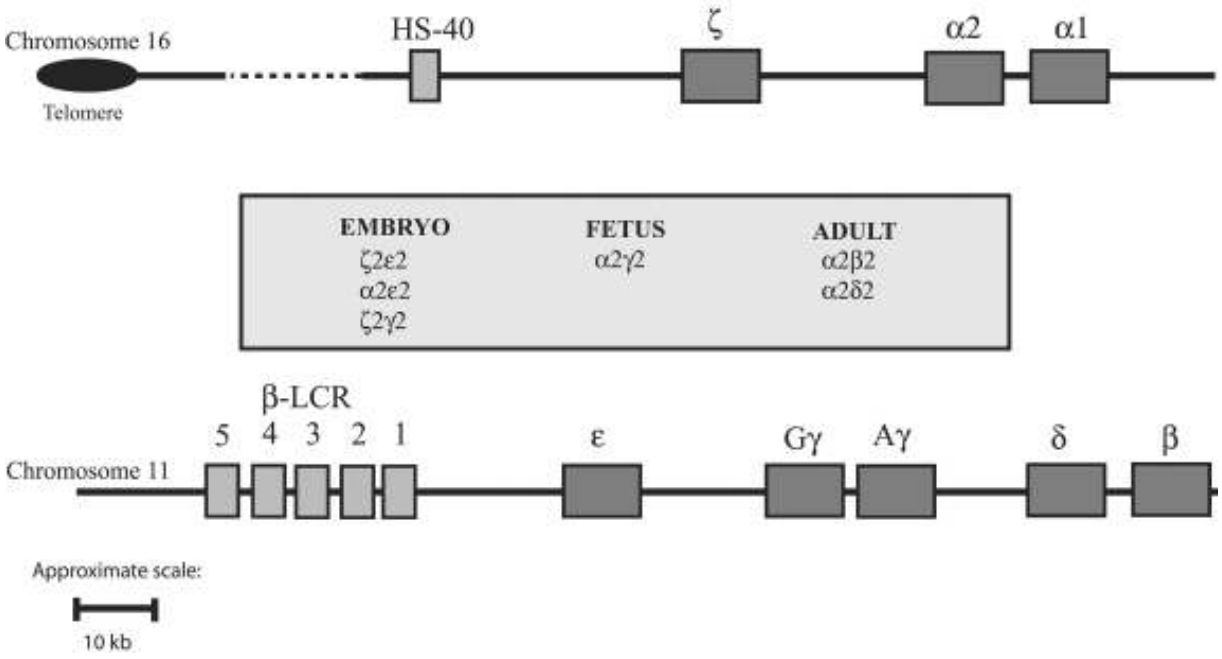

Figure 2: α –Globin gene cluster. In the top, the α-like globin cluster which includes the ζ-, α2-, and α1-globin genes located on chromosome 16 near the telomeric region, and is controlled by HS-40. In the bottom, the β-like globin, located on the short arm of chromosome 11, and is controlled by the locus control region (LCR). (Adapted from Higgs et al., 2005).

1.4. Hemoglobin Switching during Development

Different hemoglobins are produced during development, early in embryogenesis two globin genes switches take place, the embryonic to fetal switch (ε to γ and ζ to α), which are completed at 10 weeks of gestation, and the fetal to adult switch (γ to β) which occurs during the prenatal period (Greer et al., 2004).

During the second and third trimesters, the major hemoglobin is fetal hemoglobin (HbF, α2γ2), with approximately 10% HbA. HbF remains the primary hemoglobin throughout most of gestation. HbF has a higher oxygen affinity compared to adult Hb (HbA α2β2) (Giambona et al., 2009) as a result of low interaction with 2,3-DPG, and these properties make the delivery of oxygen through placenta easier, giving the fetus better access to oxygen from the mother's bloodstream (Mosca et al., 2009).

During the third trimester, the production of γ chains decreases, while the synthesis of β chains increases. At birth, the γ/β ratio is approximately 2:1, the silencing of the γ gene continues after birth until six months of age. After six months, HbA becomes the major hemoglobin with around 1% of HbF, and 2-3 % of HbA2 (Giambona et al., 2009). In normal adults, HbF is heterogeneously distributed among erythrocytes though its synthesis is restricted to a small population of cells, termed F-cells. Approximately 3–7 % of red blood cells are F-cells, containing 20–25% of HbF (Mosca et al., 2009). HbA2 (α2δ2) differs from HbA by only 10 amino acids, but it almost has the same functional properties of HbA. The low level of δ-globin gene expression in comparison to β gene may be due to both transcriptional and posttranscriptional regulation (Giambona et al., 2009).

1.5 Thalassemia Classification

There are several types of Thalassemia that are usually classified based on the type of globin chain affected as α, β, δβ, and γδβ Thalassemia. β-Thalassemia is divided into β^+- and β^0-Thalassemia. In the heterozygous state of β-Thalassemia, abnormalities in red cell morphology are present, and there is an increase in the amount of HbA2, and variable increase in HbF. The homozygous state of β^+ Thalassemia is accompanied by severe anemia, due to reduction in the synthesis of β-globin chain and therefore HbA(Bank, and Ramirez 1978; Weatherall, 1997; Greer et al., 2004). Table 1 shows different genotypes and the related changes in RBC indices, hemoglobin pattern, and the condition of anemia associated with β-Thalassemia.

Other hemoglobin variants may be unstable and cause hemolytic anemia, e.g. Hb Bibba which has low oxygen affinity, Hb Titusville with high oxygen affinity and Hb Chesapeake or methemoglobin presenting as cyanosis. Rarely, homozygosity for unstable α-chain hemoglobin variants such as Hb Taybe can lead to hydrops fetalis (Chui, 2005).

Table 1: States of β-Thalassemia and related disorders (Kohne E, 2011)

Normal Finding	Arrangement of β -globin genes	Hb and RBC indices	Hemoglobin pattern	Symptoms
Heterozygous β-Thalassemia (β-Thalassemia minor)	β+ β0	Hb ♂ 9 to 15 g/dL Hb ♀ 9 to 13 g/dL MCV 55 to 75 fL MCH 19 to 25 pg	HbA2 >3.2% HbF 0.5 to 6%	Mild anemia
Homozygous β-Thalassemia (β-Thalassemia major) Compound heterozygous β-Thalassemia (β-Thalassemia major)	β+/β+ β0/β0 β+/β0	Hb <7 g/dL MCV 50 to 60 fL MCH 14 to 20 pg	HbA2 variable HbF 70 to 90%	Severe illness with long-term transfusion-dependent anemia
Mild homozygous or compound heterozygous β-Thalassemia (β-Thalassemia intermedia)	β+/β+ β+/β0 β0/β0 influential factors	Hb 6 to 10 g/dL MCV 55 to 70 fL MCH 15 to 23 pg	HbA2 variable HbF up to 100%	Moderate disease variable transfusion dependence

1.6. α-Thalassemia

α-Thalassemia is the most common inherited disorder of hemoglobin (Hb) synthesis and probably the most common single-gene disorder in the world (Chong et al., 2000; Harteveld and Higgs, 2010). The α-thalassaemia is inherited as an autosomal recessive disorder characterized by a microcytic hypochromic anemia, and variable clinical picture ranging from asymptomatic to a fatal hemolytic anemia (Harteveled and Higgs, 2010). A normal individual usually has four α-globin genes. Due to deletions and duplications, the number of α-globin genes in each individual can vary from none to as many as eight α-globin genes (Fucharoen and Winichagoon, 2011). Therefore, serious α-globin gene mutations can have an adverse effect upon fetal development, and even cause death in the uterus (Chui and Waye, 1998; Lorey et al*.,* 2001). Multiple gene rearrangements with three to six ζ-like embryonic genes have also been reported (Felice et al*.,* 1986).

1.7. Molecular Basis of α –Thalassemia

Normal individuals have usually four α-globin genes and this genotype is written as αα/αα. The α-thalassaemia is caused commonly by deletion of one (-α) or both (--) α-genes on chromosome 16 (Pirastu et al. 1982; Alcoforado et al. 2012). When a mutation completely removes α-gene from a chromosome this is called α^0-thalassaemia and when the mutation only partially down regulate the expression from the chromosome this is called α^+ thalassaemia (Harteveled and Higgs, 2010). There are at least 40 different deletion mutations affecting the α-globin genes. The size of the deletion is important and affects the clinical phenotype of the disease (Vichinsky, 2009). The most common deletion mutations are shown in Table 2.

Table 2: Classification of the deletion α-Thalassemia (Vichinsky, 2009).

Type of deletion	Phenotype	No. of examples recorded	Examples
Deletion involving one or both α genes Deletion of all or part of one α gene	α^+ Thalassemia	7	$-\alpha^{3.7}$, $-\alpha^{4.2}$
Deletion of all or part of both α genes, without deletion of HS-40	α^0 Thalassemia	20	$--^{SEA}/$, $--^{MED}/$
Deletion of both α genes and of HS-40	α^0 Thalassemia	8	--DUTCH11
Extensive loss of 16p13.3(1-2Mb) including both α genes and HS-40	α^0 Thalassemia	17, with mental retardation and dysmorphism	--BO
Deletion of α1 gene and 18-20 kb downstream of α1 gene	α^0 Thalassemia	1	(α)- ZF*
Deletion of upstream major regulatory element (HS-40) without deletion of genes	α^0 or very severe α+ Thalassemia	12	(αα)RA**

*(α) indicate that the α-gene is present but non-functional.

**(αα) indicate that both α-genes are present but non-functional.

Non-deletion types of α-Thalassemia are much less common than deletion α-Thalassemia. The clinical picture varies from the Hb Bart's hydrops fetalis to the silent carrier state. The most common non-deletion mutations are shown in Table 3. The most common non-deletion variants

are mapped to the α2-gene (Clark and Thein, 2004). The non-deletional α^+ Thalassemias are rare and most of the mutations involve the α2 gene, which has a higher expression rate than the α1-gene with a ratio around 3:1(Fucharoen and Winichagoon, 2011).

Table 3: Classification of non-deletional α-Thalassemia (Vichinsky, 2009).

Type of deletion	Phenotype	No. of examples recognized	Examples
RNA splice site mutation in α_1 or α_2 gene	α^+ Thalassemia	3	α_2 IVS1(-5nt)
RNA polyadenylation signal mutations	$\alpha^+ - \alpha^0$ Thalassemia	4	α_2 AATAAA→AATAAG
Impaired RNA translation consequent on initiation codon or initiation consensus sequence mutation	α^+ Thalassemia, $\alpha^+ - \alpha^0$	5	α_2 ATG→ACG
Impaired RNA translation consequent on a frame shift or nonsense mutation	α^+ or α^0 Thalassemia	5	Codon 30/31 (–4nt) frame shift
Impaired RNA translation consequent on a termination codon mutation leading to an elongated mRNA and α globin chain	α^+ Thalassemia	5	Hemoglobin Constant SpringTAA→CAA ($\alpha^{CS}\alpha$)
Production of highly unstable α chain as a result of point mutation or a small deletion	α^+ Thalassemia	At least 18	Hemoglobin Arginia α^{Agr} α
Lack of a transactivating factor encoded by the ATRX gene	α^+ Thalassemia		ATR-X syndrome

1.8. Clinical and Laboratory Features

1.8.1. α-Thalassemia: Clinical Forms

α-Thalassemia is the most common hemoglobin disorder in the world, with high frequencies in Southeast Asia, the Middle East, including Iran, as well as the Mediterranean populations (Zandian et al., 2008). In α-Thalassemia four clinical pictures are recognized: silent carrier, α-Thalassemia trait, HbH disease, and Hb Bart hydrops fetalis. Figure 3 shows the classification of gene defects and phenotypic expression of these disorders (Harteveld and Higgs, 2010). Table 4 shows the clinical picture for α-Thalassemia.

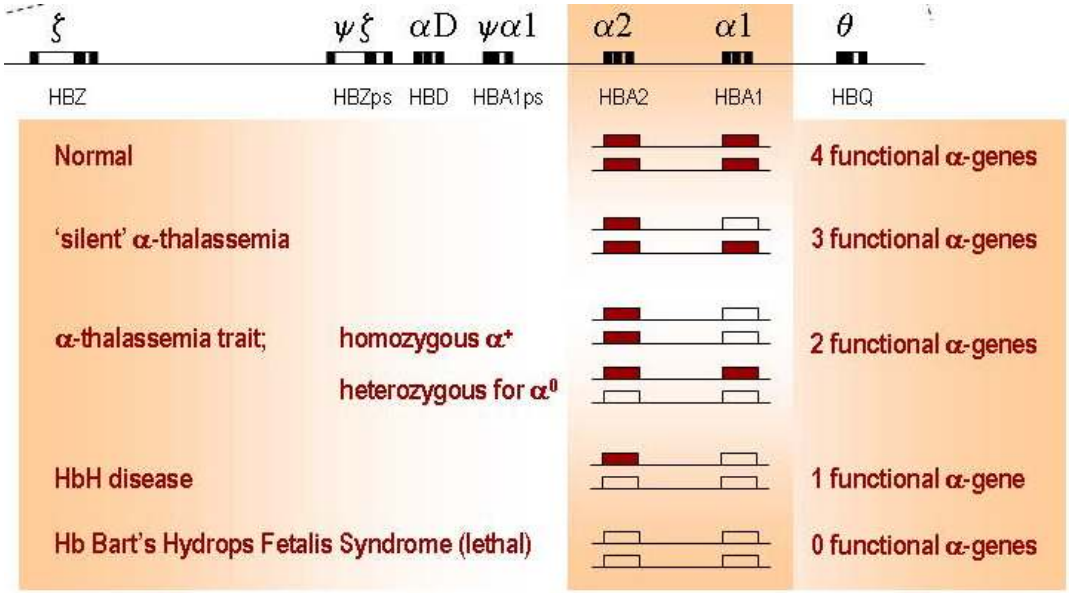

Figure 3: Classification of α-globin gene defects and phenotypic expression (Harteveld and Higgs, 2010).

I.8.1.1 Silent Carrier

This condition results from the presence of a single α-globin gene defect, such as 3.7 and 4.2-kb deletions (designated as $-\alpha^{3.7}/\alpha\alpha$ and $-\alpha^{4.2}/\alpha\alpha$, respectively). The rightward 3.7 kb deletion is caused by reciprocal recombination between Z segments producing a chromosome with only one functional α-gene ($-\alpha^{3.7}/$ or rightward deletion) causing α-Thalassemia and α-triplication allele without a thalassemic effect (Figure 4). Likewise a reciprocal recombination between mispaired X-boxes results in 4.2 kb deletion, called leftward deletion ($-\alpha^{4.2}/$) (Harteveld and Higgs, 2010).

This genotype is diagnosed in the newborn period by a very mild increased percentage (1 to 2%) of Hb Bart, which consists of four γ-globin chains (γ_4). Those patients have completely silent phenotype (normal RBC indices) or present with a moderate Thalassemia like hematological picture, reduced MCV and MCH and very mild anemia with normal HbA2 and HbF, and reduced α to β-globin chain synthesis ratio in the range of 0.8 to 0.9 (Greer et al., 2004).

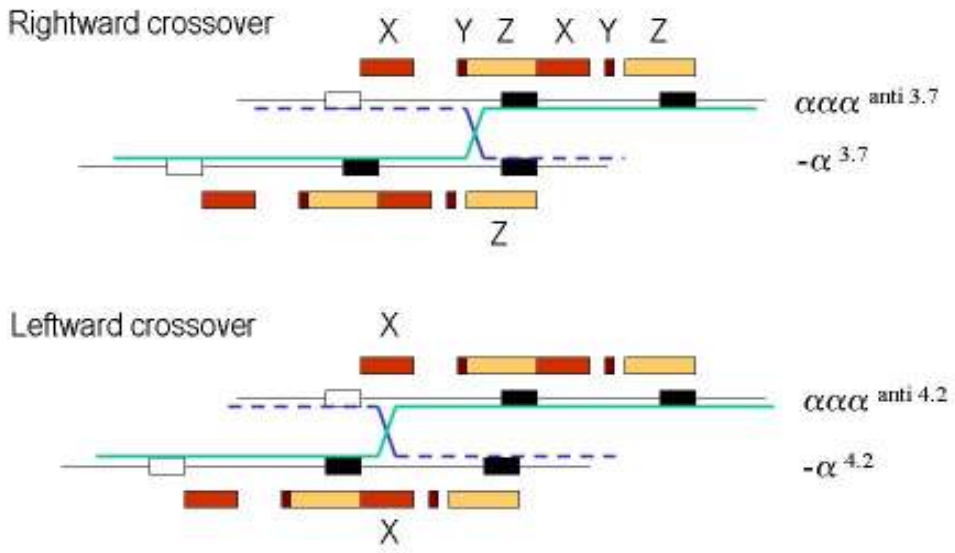

Figure 4: Deletions that cause α^{+}-Thalassemia. A cross-over between the misaligned Z boxes during meiosis gives rise to the $-\alpha^{3.7}$ and $\alpha\alpha\alpha^{anti\ 3.7}$ chromosomes. Cross-over between misaligned X-boxes give rise to $-\alpha^{4.2}$ and $\alpha\alpha\alpha^{anti\ 4.2}$. Adapted from (Harteveld and Higgs, 2010).

1.8.1.2 α-Thalassemia Trait

The α-Thalassemia carrier state results from two α-globin genes deletion, either in cis on the same chromosome (--/αα, heterozygous α^{0}-Thalassemia) or in trans on opposite chromosome (-α/-α, homozygous α^{+} Thalassemia) (Singer, 2009).

These disorders are easily diagnosed in newborns by the finding of high levels of Hb Barts (5- 6 %). In adults the clinical picture vary from mild to moderate microcytic hypochromic anemia (detected on a routine blood count), normal HbA2 and HbF, and a reduced α to β-globin chain synthesis ratio in the range of 0.7 to 0.8 (Greer et al., 2004).

1.8.1.3. HbH disease

This condition results from the deletion of three α-globin genes and the presence of only one active α-globin gene, usually as a result of the compound heterozygous state for α^0 Thalassemia/α^+Thalassemia (--/-α or --/α ^{T}α). Consequentially, the decrease in α-globin chains result in an insufficient production of normal HbA (α2β2) and HbA2 (α2δ2) in the adult patient and of fetal hemoglobin (HbF, α2γ2) in the fetus and the newborn (Baysal, 2011). Defective α-globin production results in excess β- and γ-globin chains, each able to form insoluble tetramers: γ_4 (Hb Bart's) and β_4 (HbH), Which precipitates inside red cells and causes cell membrane damage leading to hemolysis and ineffective erythropoiesis. Also these tetramers have very high oxygen affinity and are therefore useless for effective oxygen delivery (Fucharoen and Viprakasti, 2009; Singer, 2009).

The non-deletional forms of HbH disease (which are considered more severe than deletional forms) result from deletion of two α-globin genes plus inactivation of the third α-globin gene by non-deletional mutation such as Hb Constant Spring, Pakse, or Quong Sze mutations (Chui, 2005). Hb Constant Spring is the most common non-deletional α-globin gene mutation associated with HbH disease. Seventy five percent of HbH cases are caused by deletion mutations (Vichinsky, 2005). The clinical picture of those patients is characterized by moderately to severe hemolytic anemia with microcytosis, hypochromia, low HbA2 and HbF levels and varying quantities of HbH (2–30%) (Baysal, 2011).

Hemoglobin Constant Spring (CS) the most common non deletional α^+-Thalassemia in Southeast Asia, is a variant with elongated α-globin chains. HbCS is caused by a point mutation in the termination codon of α_2-gene that converts it to Glutamine codon (TAA->CAA) and results in an elongated α-chain, with extra 31 amino acid residues. The elongated α-chain is unstable and is produced at a very limited rate (~1 % of normal level) (Fucharoen and Winichagoon, 2011).

Patients with HbH disease usually suffer from hepato- splenomegaly, due to extra-medullary haematopoiesis which may be severe (Sarnaik, 2005). Jaundice may be present in variable

degrees and children may show growth retardation. Other complications include infections, leg ulcers, gall stones, folic acid deficiency and acute hemolytic episodes in response to drugs and infections (Harteveld and Higgs, 2010; Kohne, 2011). HbH disease also presents with a significant increase in RDW and α/β globin chain ratio in the range of 0.5-0.1 (Villegas et al., 1998).

1.8.1.4. Hb Bart's Hydrops Foetalis Syndrome

Hb Bart's hydrops foetalis syndrome is the most severe clinical condition of α-Thalassemia, and results from the deletion of all four α-globin genes, homozygous α^0-Thalassemiaor (--/--) (Kohne, 2011). A fetus homozygous for α^0-Thalassemia produces mainly Hb Barts that has high affinity to oxygen that can't deliver oxygen to tissues and this result in a progressive severe anemia. Many of these fetuses survive to the second or even third trimester of gestation because of the persistence of embryonic ζ-globin chain production (Chui, 2005; Harteveld and Higgs, 2010). In normal fetus, embryonic hemoglobins are responsible for oxygen deliver in the first eight weeks of gestation, those hemoglobins are hemoglobin Gower 1, hemoglobin Gower 2, and hemoglobin Portland, after that a switch to fetal hemoglobin (hemoglobin F, α2/γ2) occurs. In Hb Barts syndrome, this switch does not occur due to absence of α-globin chains. Since α-globin chains are absent, hemoglobin F cannot be synthesized and hemoglobin Bart's becomes the dominant hemoglobin. This anemia underestimates the severity of hypoxia because hemoglobin Bart's has no hem/hem interaction or Bohr effect and binds oxygen irreversibly (Vichinsky, 2009; Harteveld and Higgs, 2010).

The clinical picture of this syndrome presents with very severe anemia (Hb level range, 3 to 8 g/dL), with marked hepatosplenomegaly, generalized edema, signs of cardiac failure, and extensive extramedullary erythropoiesis (Weatherall and Clegg, 2001). In addition, there are maternal complications such as placentomegaly, hypertension and pre-eclampsia, hemorrhage, disseminated intravascular coagulation and others that may be life threatening for the mother (Chui, 2005).

Homozygosis for the South East Asian deletion mutation ($-\alpha^{SEA}/$) is the most common cause of hydrops fetalis in that region. While it is the most common deletion mutation in South East

Asia, it is also found worldwide among many ethnic groups. The -- ^{MED}/ deletion mutation is a common α^0-Thalassemia mutation in Mediterranean region, particularly Greece and Cyprus. In addition to China and Southeast Asia, Hb Bart's hydrops fetalis is now being recognized in Greece, Turkey, Cyprus, India, Sardinia, and other parts of the world (Vichinsky, 2009).

Table 4: The clinical picture of α-Thalassemia (Kohne, 2011)

Phenotype	Genotype	RBCs index	Hb pattern	Symptoms
Normal findings	αα/αα	Hb normal, MCH normal	Normal	No symptoms
Heterozygous α^+-Thalassemia = Silent α-Thalassemia	– α/αα	Hb normal, MCH <27 pg	Normal	No symptoms slight changes to blood count
Homozygous α^+-Thalassemia = α-Thalassemia trait/minor	– α/– α	Hb normal or low, MCH <26 pg	Normal	Mild anemia significant changes to blood count
Heterozygous α^0-Thalassemia = α-Thalassemia trait/minor	– –/αα	Hb normal or low, MCH <24 pg	Normal	Mild anemia significant changes to blood count
Mixed heterozygosity, α^+/α^0-Thalassemia = HbH disease	– –/– α	Hb 8 to 10 g/dL, MCH <22 pg	HbH ≈ 10-20%	Variable chronic hemolytic anemia
Homozygous α^0-Thalassemia = Hb Bart's hydrops fetalis	– –/– –	Hb <6 g/dL, MCH <20 pg	Hb Bart's 80-90%, Hb Portland ≈ 10 -20%, HbH <1%	Life-threatening fetal anemia

1.8.2. Unusual Forms of α-Thalassemia

There are two unusual forms of α-Thalassemia: acquired HbH disease associated with Myelodysplasia Syndromes and α-Thalassemia associated with mental retardation syndrome.

1.8.2.1. Acquired HbH Disease Associated with Myelodysplasia

This condition presents in some patients with Myelodysplasia Syndromes and is characterized with severe hypochromic and microcytic anemia, HbH inclusion bodies in RBCs, anisopoikilocytosis, and decreased α/ β-globin chain synthesis ratio (Greer et al., 2004; Steensmal et al., 2005). It was noticed that acquired α-Thalassemia is not exclusive to Myelodysplasia Syndromes, but it was also reported in other hematological malignancies. The exact cause of acquired α-Thalassemia is not known, but some reports linked this disorder to a mutation in ATRX gene. (Steensmal et al., 2005). The protein encoded by this gene ATRX is widely expressed throughout development, which uses the hydrolysis of ATP as a source of energy. This protein is frequently present in multi-component complexes that remodel chromatin and thereby influence the wide range of the nuclear processes such as DNA replication, DNA repair, recombination, transcription and DNA methylation (Higgs et al., 2005).

1.8.2.2. α-Thalassemia (ATRX) Syndromes

There are two different syndromes in which α-Thalassemia is associated with mental retardation. The first is characterized by relatively mild mental retardation and a variety of facial and skeletal abnormalities. This disorder results from 1-2 mega base pairs deletion resulting from rearrangements of the short arm of chromosome 16. This condition is called α-Thalassemia mental retardation -16 syndromes (Harteveld and Higgs, 2010; Greer et al., 2004). The second is X-Linked mental retardation (XLMR) syndrome, results from an abnormality in the ATRX gene. Clinical diagnosis is based on the presence of mental retardation in combination with α-Thalassemia and a characteristic and recognizable facial appearance, genital abnormalities, and other abnormalities, and defective α-globin synthesis. This disease is rare. About 170 cases have been reported worldwide (Thakur et al., 2011).

1.9. Interaction of α-Thalassemia with other Thalassemia and Hemoglobin variants

The co-inheritance of α-Thalassemia and β-Thalassemia is common in areas where both disorders are frequent, and may improve the anemia, and leads to production of red blood cells with normal indices (Harteveld and Higgs, 2010).

The interaction of α-Thalassemia with other hemglobinopathies such as HbS trait is also common. Individuals with a full complement of α-globin genes have more than 35 % HbS, compared with 28 to 35 % in those with the (-α/αα) genotype, 25 to 30 % in those with the (-α/-α) genotype, and no more than 20 % in those with the rare (-/-α) genotype (Greer et al., 2004).

The inheritance of α-Thalassemia and hereditary spherocytosis was also reported, in a patient suffering from severe hemolytic anemia, and he was transfusion dependent. In that patient, the hemolytic effect of hereditary spherocytosis was not decreased by the effect of hemoglobin H disease (in which there is an increase in osmotic resistance), so a severe hypochromic normocytic anemia was found (Zuysal et al., 1998).

1.10. Prevalence and Geographic Distribution

The overall distribution of α-Thalassemia is similar to that of β-Thalassemia (Alcoforado et al.2012). Approximately, 5 % of the world's population has a globin gene variant, but only 1.7% has α- or β-Thalassemia trait (Muncie and Campbell, 2009). Thalassemia occurs at high frequencies throughout all tropical and subtropical regions of the world (Harteveld and Higgs, 2010). They were originally found mainly in the Mediterranean area and large parts of Asia and Africa (Kohne, 2011) with gene frequencies reaching up to 10 % in some South East Asian countries (Sae-ung et al., 2007).

The Mediterranean region, certain parts of North and West Africa, Middle East, Indian subcontinent, southern Far East, and South East Asia have the highest prevalence of β-Thalassemia and comprise the so-called "Thalassemia belt"(Elgawhary et al., 2008).

The frequency of α-Thalassemia in the Arabian Peninsula is high, particularly in Oman (38.9%), followed by UAE nationals (16.5%) and Yemenis (6.5%) (Baysal, 2011). α-Thalassemia shows an incidence of (4.80 to 5.48%) in Tunisia, (9.0%) in Algeria and (2.2%) in Morocco (Khelil et al., 2010). α-Thalassemia is particularly common in China and Southeast Asia, with up to (40%)of the regional population being carriers (Vichinsky, 2009).

The $-\alpha^{3.7}/$ mutation is the most frequent allele in North Africa. Although it is specific to the Mediterranean regions but it reaches its highest frequencies in Iran (79.1%) and Saudi Arabia (64%) indicating that it could have been introduced in North Africa by Arab conquests (Khelil et al., 2010).

The gene frequencies of α-Thalassemia reach (30-40 %) in Northern Thailand and Laos, (4.5%) in Malaysia and (5 %) in the remote island of the Philippines whereas β-Thalassemia vary between (1 and 9 %) (Fucharoen and Winichagoon, 2011).

HbH disease is predominantly seen in South East Asia, the Middle East and the Mediterranean, similarly the Hb Bart's hydrops foetalis syndrome is predominantly seen in South East Asia (Harteveld and Higgs, 2010). In addition to China and Southeast Asia, Hb Bart's hydrops fetalis is now being recognized in Greece, Turkey, Cyprus, India, Sardinia, and other parts of the world (Vichinsky, 2009).

One of the most frequent α-Thalassemia mutations is the $-\alpha^{SEA}/$ deletion, which deletes both α-globin genes but spares the embryonic globin genes (Vichinsky, 2009). The carrier rate for the common α-globin gene deletion ($-\alpha^{SEA}/$) is reported to be (3–14 %) in various areas in Asia, including (4.6%) in Northern Thailand, (4.1%) in Hong Kong and (4.1%) in Guangdong Province in China (Singer, 2009).

In Palestine, the frequency of α-Thalassemia as well as other hemoglobinopathies is not known. However, Younis et al. (1996) found that the prevalence of β-Thalassemia trait among secondary school students in the West Bank is 3.13 %. To our knowledge, until now there is only one report that examined the molecular genotypes of α-globin gene mutations in a small group of Palestinian Arabs (around 55 patients) living inside Palestine 1948 and all were

referred to the clinic for molecular evaluation of anemia including unexplained microcytosis or suspected of having α-Thalassemia (Oron-Karni et al., 2000). In the latter report the Palestinian Arabs included in this study were unique in having a large proportion of point mutations/small deletion (63/109 mutant chromosomes), three of which were found frequently: α2-IVS1-5nt (29\109 mutant chromosomes), α1 ΔT39 (22/109 mutant chromosomes) and α2 poly (A) nt 6 (11/109 mutant chromosomes). The deletion mutations were less frequent than point mutations/small deletion in this sample (43/109 mutant chromosomes). The authors reported five deletions: $-\alpha^{3.7}/$, $\alpha\alpha\alpha^{anti3.7}$, $\alpha^{4.2}/$, $-\alpha^{MED}/$ and Multi α-genes; and these deletions were found in 30, 6, 1, 5 and 1 chromosomes out of 109 mutant chromosomes analyzed, respectively (Oron-Karni et al., 2000).

Epidemiological surveys suggest that there are approximately 15,000 transfusion-dependent subjects in Europe and that more than 6000 of these subjects are in Italy (Giambona et al., 2009). In Southeast Asia, α-Thalassemia causes HbH disease and Hb Barts hydrops fetalis. Fetuses with the devastating Hb Barts hydrops fetalis due to the complete lack of α globin genes die in uterus or shortly after birth, often during the second or third trimester (Chui, 2005).

The prevalence of α-Thalassemia was analyzed in Eastern Sicily (Italy), the results showed six different genotypes, the most common (36.6 % of the cases) being the heterozygous state for -α3.7 kb deletion ($-\alpha^{3.7}/\alpha\alpha$). The homozygous condition for this defect ($-\alpha^{3.7}/-\alpha^{3.7}$) had a relative frequency of (27.5 %). Ten percent of the individuals were carriers of the $--^{MED}/$ deletion at the heterozygous state $--^{MED}/$ (Bella et al., 2006).

In Tunisia, six α-globin gene molecular defects were found to be responsible for α-Thalassemia, and the most common mutation was $-\alpha^{3.7}$ /, then $--^{MED}$ (Siala et al., 2008). In Iran, the deletion mutations $-\alpha^{3.7}$, $-\alpha^{4.2}$, and $--^{MED}$ were found to be the most common α-globin deletions, and the polyadenylation signal mutation α2-poly (A) (AATAAA>AATGAA) was the most common point mutation (Tamaddoni, 2009).

It seems that each region has its unique spectrum of abnormal Hbs and Thalassemia (α- and β-Thalassemia as well as other types of Thalassemia) mutations (Old, 2003).

The identification of α-gene mutations is essential for identification of patients and carriers as well as in compound heterozygote's resulting from co-inheritance of α-Thalassemia trait and β-Thalassemia trait or Hb variants. The molecular diagnosis is of great importance for understanding of the genotype/phenotype relationship and will enhance the patient's management and counseling (Old, 2003; Clark and Thein, 2004).

Since most of the α-Thalassemia are caused by deletion mutations which show regionally and ethnic distribution (Chong et al., 2000). Screening for α-Thalassemia has been successfully performed with one or two multiplex Gap-PCRs using a combination of primers specific for the most common deletions (Old, 2003). Other point mutations have been detected using RFLP-PCR, ARMS-PCR or DNA sequencing (Oron-Karni et al., 2000; Clark and Thein, 2004).

In Palestine, the frequency and the molecular genotypes of α-Thalassemia are not known. Currently patients diagnosed with microcytosis not due to β-Thalassemia or iron deficiency are not investigated further to establish the correct diagnosis. Thus the inappropriate diagnosis of such patients results inappropriate management of these patients including unnecessary iron therapy.

1.11. Laboratory diagnosis

The most important diagnostic criteria to detect Thalassemia carriers are microcytosis (MCV<80 fL) or hyphochromic red cells (MCH<27 pg) (Borges et al., 2001). The α-Thalassemia trait is suspected when red blood cell indices are reduced, with normal iron stores and HbA_2 levels. The α^0-Thalassemia is usually associated with a reduction in red cell indices (MCV, and MCH) and HbA_2 levels (2.2–2.8%), while α^+-Thalassemia shows a very slight reduction in MCV (74–82 fL) and MCH (24–26 pg) with normal HbA_2 values (2.6–3.1 %) (Giambona et al., 2009; Chui, 2005). It is important to note that altered in red blood cell indices aren't specific for α-Thalassemia, since it is reduced in different type of hemoglobin disorders. No simple biochemical test is able to diagnose the α-Thalassemia trait. Some studies reported high prevalence of α-Thalassemia among individuals with microcytosis and hypochromia not

due to iron deficiency or β-Thalassemia trait (Borges et al., 2001; Bergeron et al., 2005; Alcoforado et al., 2012). Measuring the ratio of α- and β-globin chain synthesis is the most direct approach (at the protein level) to diagnose α-Thalassemia (Harteveld and Higgs, 2010).

The co-inheritance of β-Thalassemia with α-Thalassemia modifies the red blood cell indices, which may be almost normalized without a marked effect on HbA_2 levels. The co-inheritance of β-Thalassemia allele with α^+-Thalassemia is less evident: HbA_2 is unmodified, and red cell indices are slightly higher than with the β-Thalassemia allele alone (Chui, 2005; Giambona et al., 2009).

Individuals with a single α-globin gene deletion or non-deletion mutation can have normal blood counts (Chui, 2005). A deficiency of α-globin chains leads to production of excess γ-globins that form γ_4 tetramers (Hb Bart's) in the fetus, and the amount of Hb Bart's in the cord blood may be used to predict the genotype of α-Thalassemia in the neonates (Fucharoen and Winichagoon, 2011).

HbH inclusion bodies are traditionally used for the detection of α^+-Thalassemia trait, these can be identified by staining the peripheral blood with 1% brilliant cresyl blue (Harteveld and Higgs, 2010). This procedure is laborious, and has limited sensitivity. An enzyme-linked immunosorbent assay (ELISA) to detect embryonic ζ-globin chains in adult erythrocytes can be used as a screening test for carriers of the ($-\alpha^{SEA}$) α-thalassaemia deletion (Chui, 2005).

In pregnant women with fetuses affected with Hb Barts syndrome, fetal erythrocytes containing only embryonic ζ-globin chains and not the adult α-globin chains can be detected very early in pregnancy (Chui, 2005).

HbA_2 Levels are often performed as part of the laboratory investigation to diagnose Thalassemia carriers. If the HbA_2 level is elevated (>3.5%), the individual is considered to be a carrier for β-Thalassemia. If the HbA_2 level is normal or low, the person is suspected to be a carrier for α-Thalassemia (Urbiant et al., 2006). A borderline level of HbA_2 (3.5% ± 0.4) may also exist when β-Thalassemia mutations are co-inherited with other molecular defects (α- and δ-Thalassemia) (Giambona et al., 2009)

Hemoglobin electrophoresis was shown that hemoglobin Bart's screening of fresh umbilical cord blood is an effective method to evaluate α-globin chain imbalance, this strategy could be used to screen populations of high incidence of α-Thalassemia (Rugless et al., 2006). In neonates affected with HbH disease, Hb Bart's constitute about 25% at delivery with a decreased level of HbA, HbA_2 and HbF. In adults affected with HbH disease, HbH eventually replaces Hb Bart's and makes up from 2% to 40% of the total hemoglobin (Chui et al., 2003).

Hb Bart's has been used as a marker for the presence of α-Thalassemia in newborns although its detection underscores the incidence rate. Other methods for the detection of α-Thalassemia include immunological determinations of Hb Bart's and ζ chains (Baysal, 2011). DNA diagnosis from chorionic villi or amniotic fluid fibroblasts can detect Hb Bart's hydrops fetalis as early as 10-16 weeks of gestation (Fucharoen Winichagoon, 2011)

Hemoglobinopathies including Thalassemia were the first genetic diseases to be diagnosed at the molecular level which represents the definitive diagnosis for α-Thalassemia. Gap-PCR is used to amplify regions across the breakpoints of the deletion of the α-gene. This technique provides a quick diagnostic test for α^{+}-Thalassemia and α^{0}-Thalassemia deletional mutations (Tan, et al., 2001). This method is applied to detect the 2 most common α^{+} Thalassemia deletions $-\alpha^{3.7}$and $-\alpha^{4.2}$ and the five common α^{0}-thalassaemia deletions $-\alpha^{20.5}$, $--^{SEA}/$, $--^{MED}/$, $--^{Thai}$ and $--^{Fil}$ (Harteveld and Higgs, 2010).

Point mutations can be detected by different types of PCR such as: Amplification Refractory Mutation System (ARMS PCR) and Restriction Fragment Length Polymorphism (RFLP PCR). However, DNA sequencing is used to confirm sequence variation or to detect new mutations (Clark and Thein, 2004).

1.12. **Treatment of Thalassemia**

α-Thalassemia minor do not require treatment. Treatment for HbH disease depends on the severity of the clinical picture which can vary widely. Transfusions are rarely indicated. Anemia requires regular substitution with folic acid. For Hb Bart's syndrome, transfusions are required in uterus and continuously after birth. (Chui, 2005; Kohne, 2011).

Nearly all fetuses with the Hb Barts hydrops fetalis syndrome are stillbirths or die shortly after birth (Chui, 2005). Intrauterine transfusion therapy appears promising in minimizing the morbidity and mortality of homozygous α-Thalassemia. Recent advances in stem cell transplantation have resulted in some patients being cured (Vichinsky, 2009).

1.13. Objectives of the study

The objectives of this study are:

1. To determine the prevalence of a five α-globin gene mutations (-$\alpha^{3.7}$/, –$\alpha^{4.2}$/ , α2-IVSI-5nt, --^{MED} / and αα$\alpha^{anti3.7}$) among a cohort of Palestinian patients with microcytosis not due to iron deficiency or β-Thalassemia trait.
2. To determine the approximate incidence rate of α-Thalassemia among a cohort of Palestinian patients with microcytosis not due to iron deficiency or β-Thalassemia trait.

Chapter Two: Materials and Methods

2.1. Materials

2.1.1. Reagents and chemicals

All reagents and chemicals used for this study are shown in Table 5. All materials used were of molecular biology grade.

Table 5: Materials used in the study.

Material	**Source/Manufacturer**
Genomic DNA Extraction kit	BIONEER/ K-3032
HotStart PCR Pre Mix	BIONEER/ K-5050
Gel Purification Kit	BIONEER/ K-3034
Tris base	Sigma
Ethidium bromide (10 mg/ml)	HyLabs/ BP451
DNA molecular weight marker 100bp	GeneDirex
DNAase free nuclease water	Hylab
Dimethyl sulfoxide (DMSO)	Sigma
EDTA	Sigma
Agrose	Hylab

2.1.2. PCR primers

All PCR primers were obtained from Metabion Company (Germany). DNA sequences and nucleotide position of PCR primers are shown in Table 6. The primers P51 to P72 were used for

gap-PCR analysis of deletion mutations in α-globin genes (Oron-Karni et al., 2000). While the primers α-CF, α_1-R_1 and α_2-R_2 were used for DNA sequencing of α-globin genes (Clark and Thein, 2004). The primers IVSID52W, A2D5M and A2R904 were used for ARMS PCR analysis of the α2-IVSI -5nt mutation. These primers were described earlier by Lacerna et al. (2007) but A2D5M was slightly modified and the primer IVSID52W was redesigned to increase the specificity of the RCR.

Table 6: Sequence and location of PCR primers.

Primer	Sequence (5'→3')	GenBank Accession no.	Coordinates	Gene
P51	CTGCACAGCTCCTAAGCCAC	J00153	7254-7273 11072-11091	α_2 α_1
P52	CCTCCATTGTTGGCACATTCC	J00153	7531-7551	α_2
P54	CTCAAAGCACTCTAGGGTCCA	J00153	11497-1517	α_1
P55	GTCCACCCCTTCCTTCCTCA	J00153	5687-5706 9247-9266	Y2[1] Y1[1]
P59	CTCTAGGTCACCCTGTCATCA	J00184	26-46	Ψζ1
P60	CTCTGTCGTGTAGACGCCGA	M33022	423-442	θ2
P71	TACCCATGTGGTGCCTCCATG	J00153	3068-3088	$\Psi\alpha_1$
P72	TGTCTGCCACCCTCTTCTGAC	J00153	8894-8915	NH-II[2]
α-CF	GGAGGGTGGAGACGTCCTG	Z84721	33538-33556 37344-37360	α_2 α_1
α1-R1	CGAGAGGTTCTAGCCATGTGTG	Z84721	38453-38474	α_1
α2-R2	CCATTGTTGGCACATTCCGG	Z84721	34602-34621	α_2
IVSID52W	GGAGGCCCTGGAGACGTG AG	Z84721	33847-33877	α_2
A2D5M	GTATGGTGCGGAGGCCCT GGAGACGC	Z84721	33856-33875	α_2
A2R904	GTCTGAGACAGGTAAACA CCTCCAT	Z84721	34642-34618	α_2

[1] Y1 and Y2 refer to homologous regions Y1 and Y2 overlapping α_2 and α_1-globin genes.

[2] NH-II: non-homologous region 2.

Figure 5 shows the physical maps of the human α-globin gene cluster and the location of PCR primers (P51 to P72) used for analysis of deletion mutations.

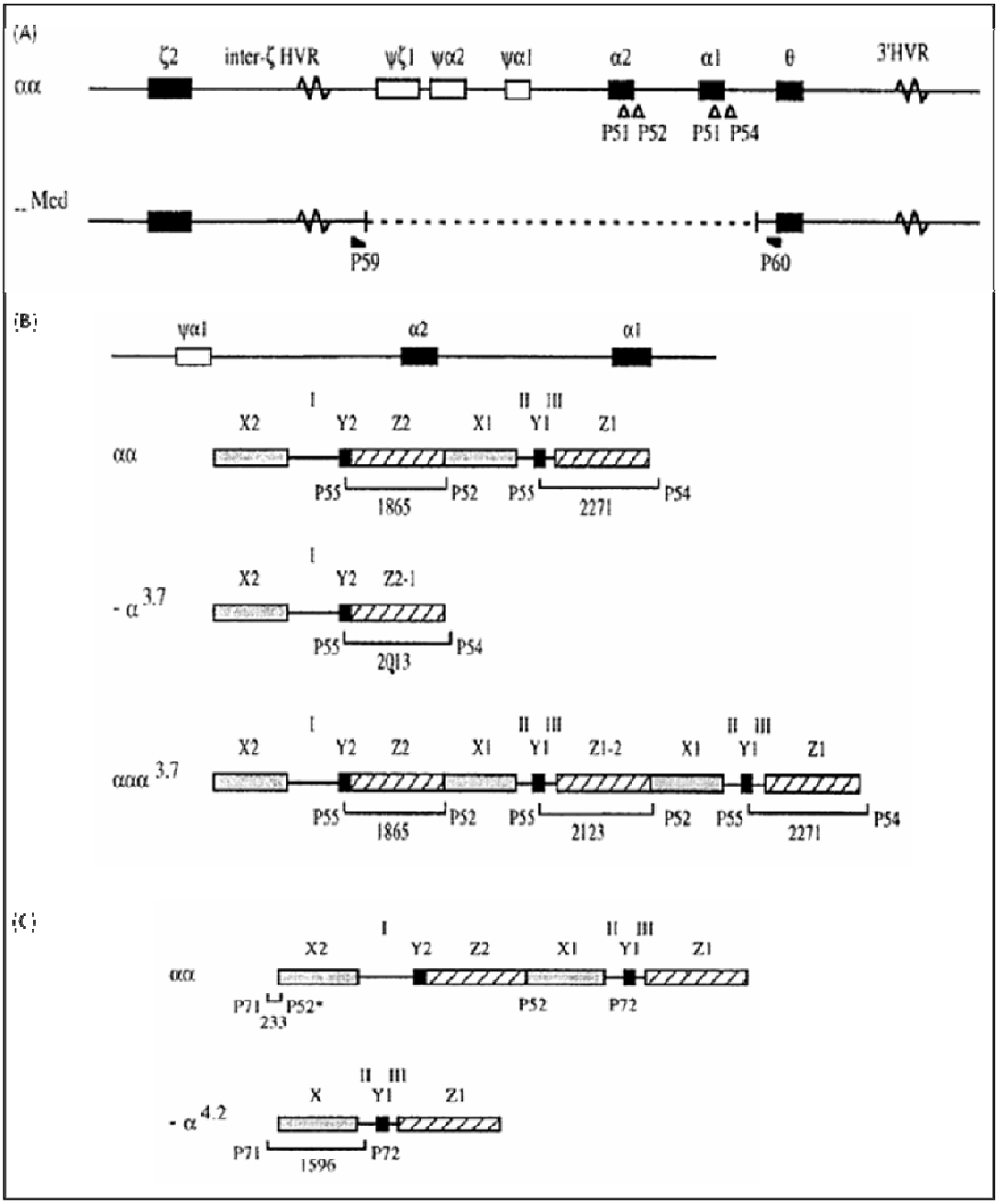

Figure 5: Alpha-globin gene cluster and the location of PCR primers P51 to P72. (A) Physical map of α-globin gene cluster and the --^{Med} deletion. Solid boxes represent functional genes and open boxes represent pseudogenes. The dashed line designates the --^{Med} deletion. (B, C) The locations of the

homologous (X, Y, and Z boxes, denoted as stippled, black, and hatched, respectively) and non-homologous regions (I, II, and III) in the α-globin cluster are shown, as well as the products of unequal crossing over between the Z boxes (--$\alpha^{3.7}$/ and αα$\alpha^{nti3.7}$) and X boxes (--$\alpha^{4.2}$/). The locations of the PCR primers and the sizes of the expected amplicons are shown under each gene map (Oron-Karni et al., 1998).

2.2. Methods

2.2.1. Patients

The study population consisted of two groups: patients and neonates. Patients included in the study were presenting with microcytosis (MCV<80 fL) not due to iron deficiency anemia or β-Thalassemia trait. The β-Thalassemia trait was excluded by the finding of normal HbA_2 level following hemoglobin electrophoresis. For the exclusion of iron deficiency anemia we first assumed that for the investigation of microcytosis, iron studies should be performed as first line tests and if normal, then testing for β-Thalassemia trait should follows. This assumption is based on the clinical protocols adopted by the Primary Health Care clinics in the Ministry of Health. However, the clinical protocols for investigation of microcytosis were not implemented consistently. Therefore, we have applied the Menzer Index (for exclusion of iron deficiency; see section 2.2.3) on all samples with microcytosis and referred for investigation of β-Thalassemia trait. The exclusion of iron deficiency would have been more accurate if the study samples were tested for serum ferritin or serum iron, but such testing was not done in this study and it is a limitation of the present study. Patients fulfilling the above mentioned criteria and referred to the Specialized Medical Laboratories in Ramallah for investigation of β-Thalassemia trait by Hemoglobin electrophoresis were asked to participate in this study.

For the neonatal group, the samples were collected from neonates delivered at the Palestinian Red Crescent Society Hospital at Al-Bireh. During the study period, neonates referred to the hospital laboratory for CBC analysis and were found to have an MCV<95 fL, the neonates' families were asked to donate their blood samples for this study.

2.2.2. Sample collection and processing

EDTA-blood samples were collected from patients referred to the Specialized Medical Laboratories in Ramallah for investigation of β-Thalassemia trait by hemoglobin electrophoresis from September 2010 to January 2012. Samples with MCV<80 fL and normal HbA_2 levels (<3.5 %) were included in this study, provided that iron deficiency anemia is excluded as described in the previous section (2.2.1). Blood samples from neonates were collected during same period from the Palestinian Red Crescent Society Hospital in Al-Bireh. Blood samples were stored at 4˚C after Complete blood count (CBC) analysis and DNA extraction was performed within 1-2 weeks of blood collection.

The Specialized Medical Laboratories in Ramallah performs hemoglobin electrophoresis using HPLC based method (Tosoh Instrument) and is one of the main medical laboratories that performs this test in the West Bank. Specialized Medical laboratories receive samples from various medical laboratories in the West Bank.

The following figure shows the process from sample collection, sample processing, the PCR reactions for deleted mutation, DNA sequencing for representative samples, to ARMS PCR for remaining samples.

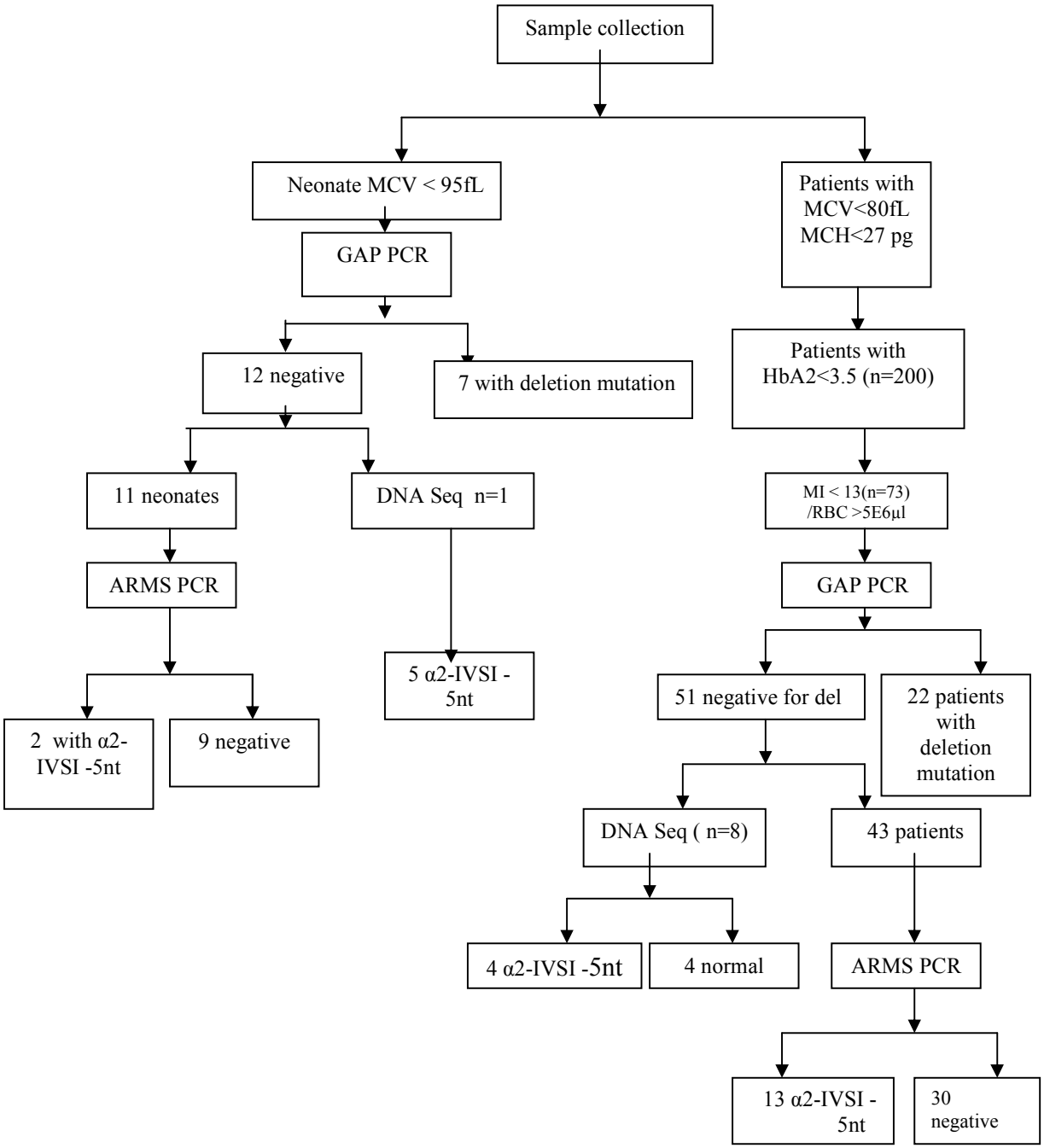

Figure 6: The flow of sample processing and analysis performed in this study. Gap-PCR was performed for detection of the deletion mutations $-\alpha^{3.7}/$, $-\alpha^{4.2}/$ and $--^{MED}/$, and $\alpha\alpha\alpha^{anti3.7}$ triplication. ARMS PCR was performed for detection of the α2-IVSI -5nt mutation. DNA sequencing covered the whole α1- and α2-genes including the promoter region and the 3'UTR.

2.2.3. Mentzer Index

The Mentzer index is used to differentiate iron deficiency anemia from β-Thalassemia trait (Ntaios et al., 2007). The Mentzer index (MCV/red blood cell count) can help distinguish between iron deficiency and β-Thalassemia trait. In iron deficiency, the ratio is usually greater than 13, whereas β-Thalassemia trait yields values less than 13. A ratio of 13 would be considered uncertain (Kohne, 2011). A group of the patients' samples have Mentzer index higher than 13 and have RBC count more five millions per µL. The RBC count is also useful as a diagnostic criterion because β-Thalassemia trait produces a microcytic anemia that is usually associated with an increase in the RBC count. While microcytic anemia, including iron deficiency and anemia of chronic disease, are usually associated with a decrease in the RBC count that is proportional to the degree of decrease in Hb concentration (Clarke and Higgins, 2000). A RBC count less than $5.0x10^6$µL would most likely indicate iron deficiency, but a RBC count above $5x10^6$ µL would most likely indicate β-Thalassemia trait (AlFadhli et al., 2006).

2.2.4. DNA Extraction

Genomic DNA was extracted from peripheral whole blood by the AccuPrep Genomic DNA Extraction Kit (Bioneer) per the manufacturer's instructions. This kit yields an average of 6µg of genomic DNA from 200 µL of whole blood. AccuPrep genomic DNA Extraction Kit employs glass fibers, fixed in a column, which specifically binds DNA in the presence of a chaotropic salt. Proteins and other contaminants are eliminated through a series of short wash and spin steps. Then the genomic DNA is eluted by a low salt solution (10 mM Tris, pH 8.0).

Briefly, 20 µL of protinase K was added to 200 µL of whole blood, then 200 µL of binding buffer was added followed by immediate, short vortexing and incubated at 60°C for 10 minutes. After incubation, 100 µL of isopropanol was added, the lysate was transferred to the reservoir of the binding column tube and spined at 8000 rpm for 1 minute. The binding column was transferred to a new 2 ml tube, washed with 500 µL of washing buffer 1 followed by a second wash with 500 µL of washing buffer 2. The DNA was eluted from the binding column using

200 µL of elution buffer (10 mM Tris, pH 8.0). The genomic DNA was stored at -20 °C until testing.

2. 2.5. Assessment of DNA quality and quantity

For assessment of DNA quality, 5 µL of DNA sample was mixed with 1 µL 6X DNA gel loading dye, loaded on 0.8% agarose gel and electrophoresed at 5-8 volt/cm. A 1 kb DNA ladder was run in each electrophoresis run and samples with positive band only were used for the PCR testing.

For quantification of DNA, the DNA concentration was measured spectrophotometrically at 260 nm. The ratio 260/280 was also calculated and used to further assess the quality of DNA samples.

2.2.6. Polymerase Chain Reaction (PCR)

2.2.6.1. Gap-PCR

First trial: Three multiplex Gap-PCRs were performed as described by Liu et al. (2000). This method consist of three multiplex PCR reactions: (1) the α^{0} reaction which detects $--\alpha^{20.5}$ and $--\alpha^{MED}$, (2) the $-\alpha^{4.2}$ reaction which detects $-\alpha^{4.2}$/ deletion and (3) the $-\alpha^{3.7}$ reaction which detects the $-\alpha^{3.7}$/ deletion and $\alpha\alpha\alpha^{anti3.7}$ rearrangement. Each one of these three Multiplex PCRs consisted of 2-3 pairs of primers (Table 2.7), and one of these primer pairs is designed to amplify a sequence alongside the mutation-specific sequence that served as an internal control for each reaction. For this PCR method, several optimization steps were performed and included: testing of each primer pair separately and in combination, adjustment of the annealing temperature, primer concentration and, addition of PCR enhancers such as DMSO (2-10%), Formamide (1-4%) and Betaine (1.5 M) either separately or in combination. Following these optimization steps, the α^{0} reaction gave the internal control amplicon in some samples and detected the $--\alpha^{MED}$/ mutation in some samples. The $-\alpha^{4.2}$ reaction was not successful and did not yield any of the expected amplicons. The $-\alpha^{3.7}$ reaction gave the expected internal control

amplicon in most samples tested and detected the $-\alpha^{3.7}$/ mutation in some samples. The difficulties experienced in the previous reactions can be explained partly by the high GC content of the α-globin gene cluster as well as to the poor primer design. Therefore these three gap-PCR reactions were replaced by a new set of primers, described in the following paragraph.

Table 7: Sequence of the PCR primers as described by Liu et al. (2000).

Primer	**Sequence (5' to 3')**	**Amplicon size**
α^0 multiplex PCR: α-SEAF + α-R + 20.5(F+R) + MED (F+R).		
α-SEAF	CTCTGTGTTCTCAGTATTGGAGGGAAGGAG	1010 bp (Internal control)
α-R	TGAAGAGCCTGCAGGACCAGGTCAGTGAC CG	
20.5(F)	GGGCAAGCTGGTGGTGTTACACAGCAACT C	875 bp
20.5(R)	CCACGCCCATGCCTGGCACGTTTGCTGAGG	
MED(F)	CGATGAGAACATAGTGAGCAGAATTGCAG G	1187 bp
MED(R)	ACGCCGACGTTGCTGCCCAGCTTCTTCCAC	
-$\alpha^{3.7}$multiplex PCR: 3.7(F) + 3.7(R1) + 3.7(R2)		
- $\alpha^{3.7}$:		
3.7(F)	AAGTCCACCCCTTCCTTCCTCACC	1963 bp
3.7(R2)	TCCATCCCCTCCTCCCGCCCCTGCCTTTTC	
ααα$^{anti3.7}$:		
3.7(F)	AAGTCCACCCCTTCCTTCCTCACC	2440 bp
3.7(R1)	ATGAGAGAAATGTTCTGGCACCTGCACTTG	
αα:		
3.7(F) + 3.7(R1) (see sequence above)		2217 bp
3.7(F) + 3.7(R2) (see sequence above)		2213 bp
-$\alpha^{4.2}$ multiplex PCR: 4.2(F) + 4.2(R1) + 4.2(R2)		
-$\alpha^{4.2}$:		
4.2(F)	TCCTGATCTTTGAATGAAGTCCGAGTAGGC	1725 bp
4.2(R2)	ATCACTGATAAGTCATTTCCTGGGGGTCTG	
αα:		
4.2(F)	TCCTGATCTTTGAATGAAGTCCGAGTAGGC	1510 bp
4.2(R1)	TGGGGGTGGGTGTGAGGAGACAGGAAAGA GAGA	

Second trial: In this trial four multiplex Gap-PCRs (A to D reactions were performed), Table 8 in this table summarizes the expected genotypes, primers and amplicon size as in the original article) were performed as described by Oron-Karni et al. (1998) with some modifications. The results reported in this study and the discussion all refer to this trial. The sequences of PCR primers used in this trial are shown in Table 2.6 (primers P51 to P72). Reaction A is a multiplex reaction which detects the $^{-MED}$/ mutation and contains the following primers: P51 (60 ng), P52 (40 ng), P54 (60 ng), P59 (40 ng), and P60 (40 ng). Reaction B detects the - $\alpha^{3.7}$/ mutation and contains the following primers: P55 (60 ng) and P54 (60 ng). Reaction C detects the -$\alpha^{4.2}$/ mutations and contains the following primers: P71 (40 ng), P72 (60 ng), and P52 (20 ng). Reaction D detects the $\alpha\alpha\alpha^{anti3.7}$ triplication and contains the following primers: P55 (59 ng), P52 (63.08 ng).

Table 8: The α-globin gene genotypes detected using the multiplex gap-PCRs and the size of the corresponding amplicons in bp.

PCR reaction							
	$\_\_^{Med}$ A			-$\alpha^{3.7}$ B	-$\alpha^{4.2}$ C		$\alpha\alpha\alpha^{anti3.7}$ D
Primers → / Alleles ↓	P59-P60	P51-P52	P51-P54	P55-P54	P71-P52	P55-P52	P55-P52
Aα	-	298	446	2271		233	1865
-MED	561	-	-	-		-	-
-$\alpha^{4.2}$	-		446	2271	1596	-	-
-$\alpha^{3.7}$	-		446	2013		233	-
$\alpha\alpha\alpha^{anti3.7}$							1865,2123

All PCR reactions (reactions A to D) were performed with a HotStart ready PCR mix (Bioneer) using ~100 ng of genomic DNA and 5 % DMSO. Each 20-µL reaction contained the manufacturer's buffer (1X), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP and Hot-Satrt Taq DNA Polymerase. Bioneer's Hot-Start Taq DNA polymerase is designed for hot-start PCR to provide higher PCR specificity by use of Pyrophosphatase and Pyrophosphate method.

Amplification was performed with an initial heat activation step of 5 minutes at 94°C followed by 35 cycles, each consisting of 94°C 1 minute, 58°C for 1 minute, and 72°C for 2.5 minutes, and a final extension step of 72°C for 10 minutes. Thermal cycling was performed using a Biometra DNA thermal cycler (Germany).

2.2.6.2. ARMS PCR

(ARMS) PCR was used for detection of the α2-globin gene mutation that termed α2-IVSI-5nt deletion. This mutation involves deletion of the first 5' nucleotides in intron 1 (IVSI) in the α 2-globin gene. The PCR reaction was performed with a Hot-start ready PCR mix (Bioneer) using ~100 ng of genomic DNA and 5 % DMSO. Each 20-µL reaction contained the manufacturer's buffer (1X), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.25 µM of each forward and reverse primers and Hot-start Taq DNA Polymerase. The mutant sequence was amplified using the forward primer A2D5M and the common reverse primer A2R904. The Wild type sequence was amplified using the forward primer IVSID52W and the common reverse primer A2R904. Bioneer's Hot-start Taq DNA polymerase is designed for hot-start PCR to provide higher PCR specificity by use of Pyrophosphatase and Pyrophosphate method.

Amplification was performed with an initial heat activation step of 5 minutes at 94°C followed by 30 cycles, each consisting of denaturation at 94°C for 50 seconds, annealing at 60°C for wild type reaction or 62°C for the mutant reaction for 50 seconds, and extension at 72°C for 1 minute and a final extension step of 72°C for 5 minutes. Thermal cycling was performed using a Biometra DNA thermal cycler (Germany).

2.2.7. Agarose gel electrophoresis

For preparation of agarose gels, appropriate amount of agarose was dissolved in 1X TAE buffer by heating, allowed to cool to 50˚C and then ethidium bromide was added to a final concentration of 0.5 µg/ml. A 2% gel was prepared for reactions A and D and 1.4 % gel for

reactions B and C. The gel solution was poured in an electrophoresis tray with appropriate comb and allowed to polymerize at room temperature.

For analysis of PCR amplicons, 10 µL of each PCR reaction product was mixed with 2 µL of 6 X DNA gels loading dye and electrophoresed at 80 voltage for 45 minutes. A 1 Kb DNA leader was run in each run. Then the gels were visualized on a UV transilluminator.

2. 2.8. DNA Sequencing

Nine DNA samples with microcytosis and hypchromia, with normal HbA2 and with no deletion mutations in the α–globin genes were subjected for complete DNA sequencing of α1- and α2-globin genes. The α1- and α2-globin genes were amplified separately using PCR primers described by Clark and Thein 2004, (Table 2). The α2-globin gene was amplified using the primer pair: α-CF and α2-R2, which yields an amplicon of 1084 bp long. While the α1-globin gene was amplified using the primer pair: α-CF and α1-R1, which yields an amplicon of 1131 bp long.

PCR amplification was performed using Hot-start ready PCR mix (Bioneer) using ~100 ng of genomic DNA and 7.5 % DMSO. Each 20-µl reaction contained the manufacturer's buffer (1X), Hot-Start Taq DNA polymerase, 1.5 mM MgCl2, 0.25 mM of each dNTP and 0.25 µM of each primer.

Amplifications were performed with an initial heat activation step of 5 minutes at 94°C followed by 35 cycles, each consisting of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1.5 minute, followed by a final extension step of 72°C for 5 minute. Thermal cycling was performed in a Biometra DNA thermal cycler (Germany).

The PCR amplicons were separated using 1 % agarose gel and the target gel band was excised. The PCR amplicons were purified from the agarose gel slices using the AccuPrep DNA Purification kit (Bioneer) per the manufacturer's instructions. Briefly, the gel slice was dissolved in 3 volumes of Buffer 1 at 60˚C for 10 minutes. The gel mixture was transferred to

the binding column, washed twice with Buffer 2 and finally eluted with 30-50 µl of 10 mM Tris buffer, pH 8.0.

The purified PCR amplicons were sent to the Heredity Research Laboratory at Bethlehem University for DNA Sequencing. The DNA sequencing was performed by the Dideoxy Chain Termination method and Big DyeV1.1 Terminator reagents (Applied Biosystems) and sequenced on the automated ABI 3130 Genetic Analyzer (Applied Biosystems). Each α-gene was sequenced using the forward and reverse primers.

2.2.9. Analysis of DNA sequences

DNA sequences were viewed using the Chromas Lite software 2.01 (http://www.technelysium.com.au/chromas_lite.html) and the DNA sequences were analyzed using the BLAST program (http://blast.ncbi.nlm.nih.gov/) against the DNA sequence of α-globin gene cluster available under the GenBank accession no. Z84721.1.

2.2.10. Statistical analysis

The mean and standard deviation for total Hb, RBC count, MCH, MCV and RDW were calculated using Excel program/ Microsoft office 2007. To compare the hematological parameters mentioned before, a one-way ANOVA was used (SigmaStat 2.03). Comparisons between groups were made with Tukey test. Statistical significance was assessed as a test with $p < 0.05$.

Chapter Three: Results

3.1 Study Samples

This study was carried out to investigate the prevalence of α-globin gene mutations among Palestinian patients with microcytosis not due to iron deficiency or β-Thalassemia trait. For this purpose, a convenient sample of 200 nonrelated patients was collected in the period September 2010 to January 2012 at the Specialized Medical laboratory in Ramallah. All patients included were presenting with microcystosis, referred for investigation of β-Thalassemia trait by Hb electrophoresis and were found to have HbA2 level below 3.5%. The exclusion of iron deficiency was based on application of the Menzer Index <13, or RBC count >5.0 $x10^6/\mu L$). Seventy -three samples were found to have a Menzer Index <13 or RBC count >$5.0x10^6/\mu L$ and thus unlikely to have iron deficiency. These 73 samples (146 nonrelated chromosomes) were further subjected for investigation of α-globin gene mutations. There was a preponderance of male patients (M/F=45/28).

A second group of 19 nonrelated neonates (38 chromosomes) for whom blood samples were referred to the laboratory for CBC analysis and were found to have an MCV<95 fL at the Palestinian Red Crescent Society Hospital at Al-Bireh during the study period. It has been confirmed that an MCV below 90fL and MCH below 30pg is a strong indicator of the presence of α-Thalassemia minor (Tritipsombut et al., 2008; Akram, et al., 2009).

3.2 Hematological parameters for all study samples

The red cell indices, Hb values and HbA2 level for all samples (73 patients and 19 neonates) that fulfilled the study criteria are shown in Appendix A. All patients' samples showed microcytosis and hypochromia, normal hemoglobin A2 level and Menzer Index <13, or RBC

count >5x10^6 / μL (Appendix A). The neonatal samples also showed microcytosis with MCV <95 fL (Appendix A).

3.3 α-Thalassemia mutations

Identification of the α-Thalassemia mutations among the study samples was performed using gap-PCR, DNA sequencing of representative samples and ARMS PCR. Four different multiplex gap-PCR reactions (Reactions A to D) were performed for detection of four deletion mutations affecting the α-globin genes: -$\alpha^{3.7}$/, -$\alpha^{4.2}$/, --^{MED}/ and αα$\alpha^{anti3.7}$. These mutations represent the most prevalent deletion mutations affecting the α-globin genes in the Mediterranean region. Representative agarose gels for each mutation are shown in Figures 7 to 10.

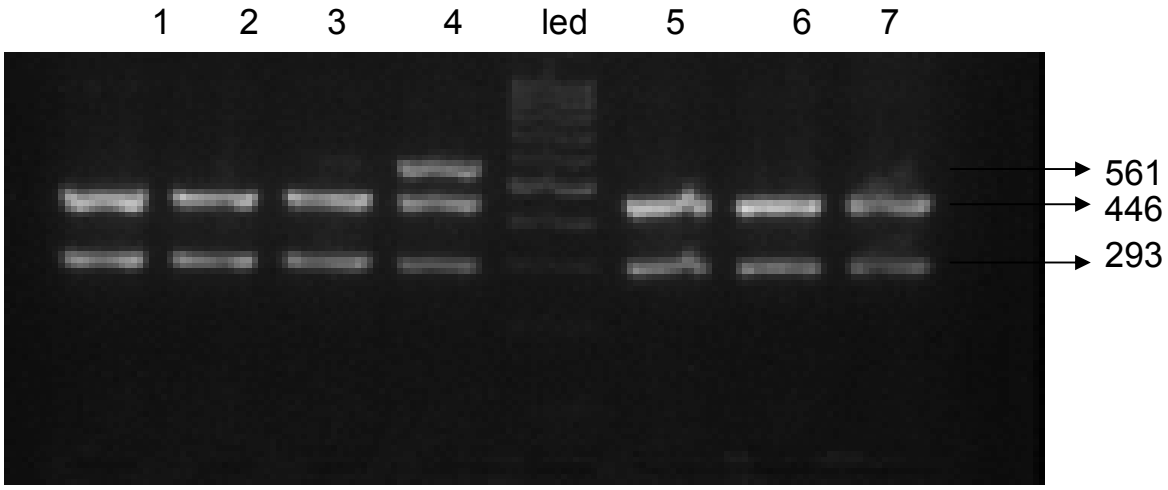

Figure 7: Representative agarose gel for the –^{MED}/ multiplex PCR reaction. Lanes 1-3 & 5-7: negative for --^{MED}/αα mutation, Lane 4: --^{MED}/αα genotype, Lad: 100 bp DNA ladder.

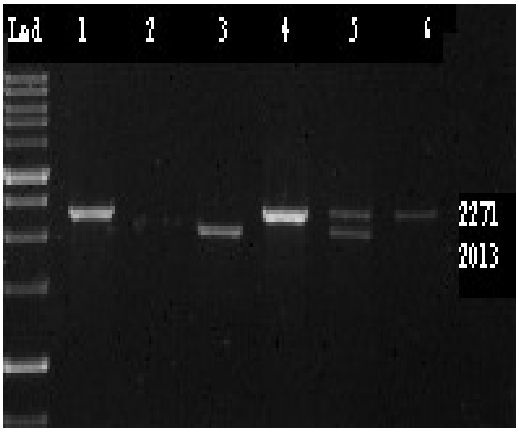

Figure 8: Representative agarose gel for the $-\alpha^{3.7}$ PCR reaction. Lanes 1, 4 & 6: negative for-$\alpha^{3.7}/-\alpha^{3.7}$ mutation, Lane 2 & 3: $-\alpha^{3.7}/-\alpha^{3.7}$ genotype, Lanes 5: $-\alpha^{3.7}/\alpha\alpha$ genotype, Lad: 1 kb DNA ladder.

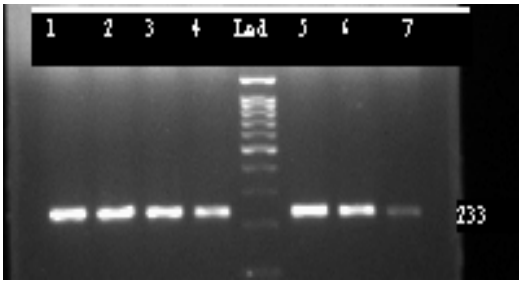

Figure 9: Representative agarose gel for the $-\alpha^{4.2}$ PCR reaction. Lanes 1-7: negative for $-\alpha^{4.2}$ mutation, Lad: 100 bp DNA ladder.

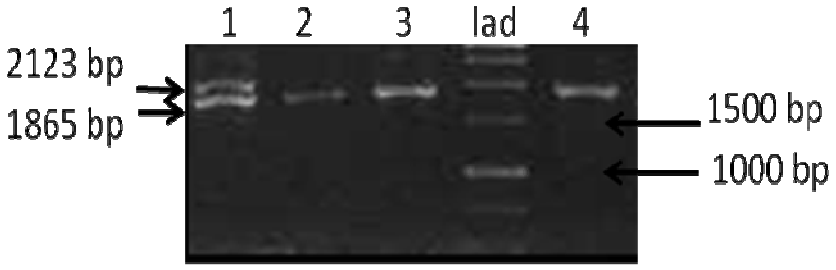

Figure 10: Representative agarose gel for the $\alpha\alpha\alpha^{anti3.7}$ PCR reaction. Lane 1: $\alpha\alpha\alpha^{anti3.7}$ triplication, Lanes 2-4: negative for $\alpha\alpha\alpha^{anti3.7}$ triplication, Lad: 1 kb DNA ladder.

From the samples that were negative for the deletion mutations tested by gap-PCR, eight samples from the patients' group and one sample from the neonate group were selected randomly and subjected for DNA sequencing of α2- and α1-globin genes. DNA sequencing has revealed that four patients' samples and 1 neonate sample were heterozygous for the α2-IVSI-5nt mutation (Figure 11). No other significant genetic variations were observed in DNA sequencing.

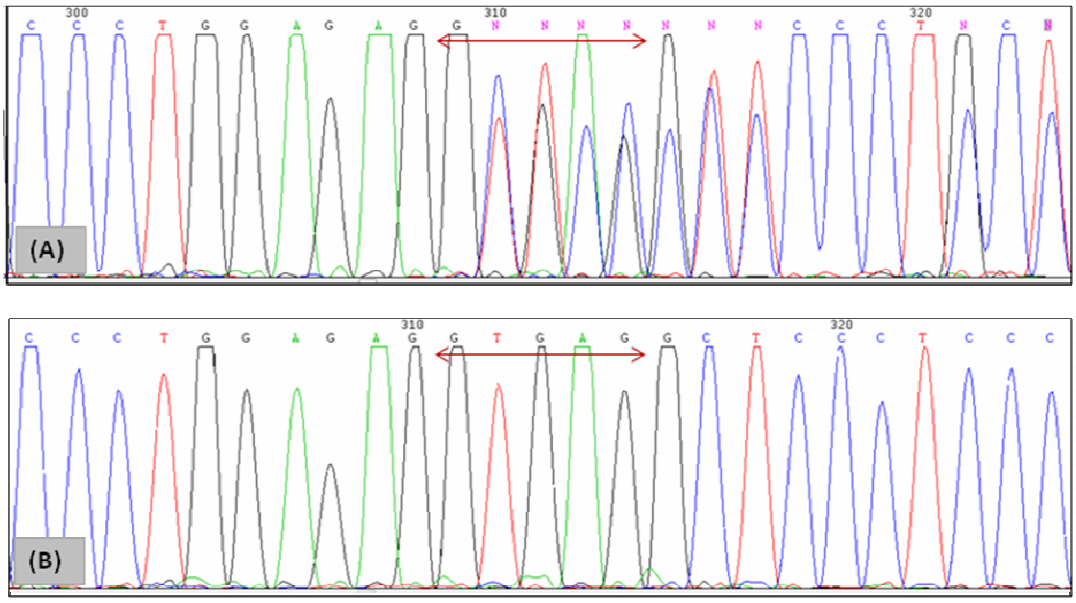

Figure 11: Identification of α2-IVSI-5nt mutation by DNA sequencing. (A) Mutant allele; (B) wild type allele. The double headed arrow indicates the 5 nucleotides deleted in this mutation.

Since the DNA sequencing revealed that more than half of the samples sequenced have the α2-IVSI-5nt mutation, we have screened all study samples for the α2-IVSI-5nt mutation using ARMS PCR (Figure 12).

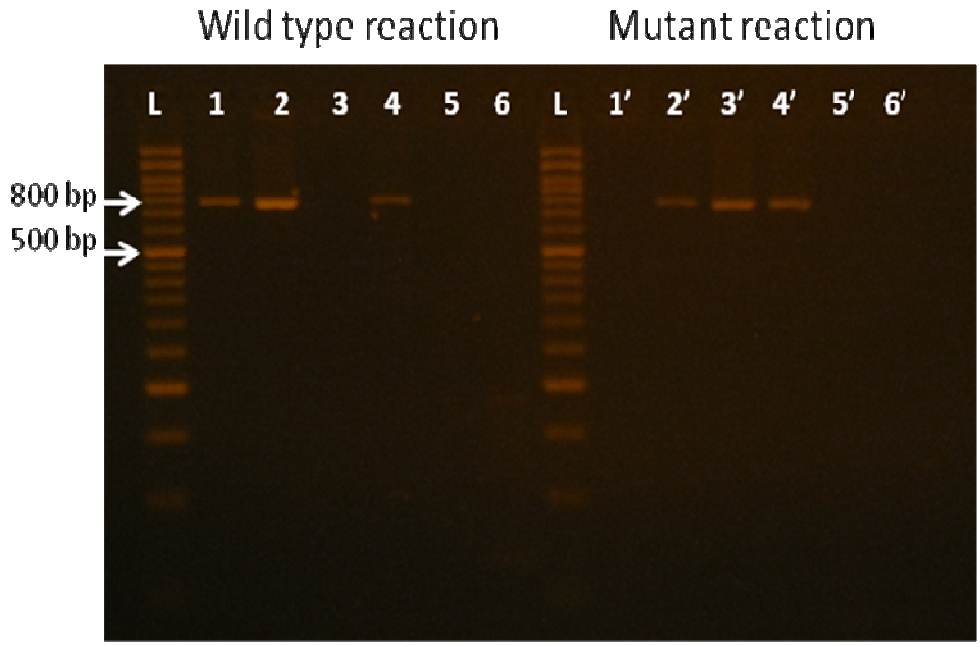

Figure 12: Representative agarose gel for analysis of "α2-IVSI -5nt" mutation by ARMS PCR reaction. The amplicon size is 796 bp in the wild type reaction and 790 bp in the mutant reaction. L: 50 bp DNA ladder; lanes 1 & 1': αα/αα genotype; lanes 2 & 2': α^{-5nt}α/αα genotype; lanes 3 & 3': α^{-5nt}α/α^{-5nt}α genotype; lanes 4 & 4': positive controls; lanes 5 & 5': negative controls; lanes 6 & 6': blanks.

Table 9 summarizes the genotypes of α-globin genes detected in this study. From the 73 patients' samples analyzed, 37 samples (50.7%) were found to have α-glonin gene mutations. Twenty-seven patients were heterozygous for one of the four mutation found (-$\alpha^{3.7}$/; ααα$^{anti3.7}$; --^{MED}/ and α2-IVSI -5nt), eight patients were homozygous for either the -$\alpha^{3.7}$/ or α2-IVSI -5nt and two patients were compound heterozygous for the -$\alpha^{3.7}$/ and α2-IVSI -5nt mutations. The –$\alpha^{4.2}$/ deletion mutation was not detected in the study samples. The most prevalent mutation was the -$\alpha^{3.7}$/ deletion mutation and the second most prevalent one was the α2-IVSI -5nt mutation. As shown in Table 3.10, 48.9 % of the α-Thalassemia mutant chromosomes detected among the patients' group carried the -$\alpha^{3.7}$/ deletion mutation and 44.7 % carried the α2-IVSI -5nt mutation.

Table 9: Genotypes of α-globin genes detected among Palestinian patients and neonates.

Genotype	**Patients**		**Neonates**	
	N	%	*n*	%
(a) Presumably normal genotype	36	49.3	9	47.4
(b) Mutant genotype	37	50.7	10	52.6
$-\alpha^{3.7}/\alpha\alpha$	11	29.7	5	26.3
$-\alpha^{3.7}/-\alpha^{3.7}$	5	13.5	0	0
$\alpha^{-5nt}\alpha/\alpha\alpha$*	13	35.2	4	21.1
$\alpha^{-5nt}\alpha/\alpha^{-5nt}\alpha$	3	8.1	0	0
$-\alpha^{3.7}/\alpha^{-5nt}\alpha$	2	5.4	0	0
$--^{MED}/\alpha\alpha$	2	5.4	1	5.2
$\alpha\alpha\alpha^{anti3.7}$	1	2.7	0	0
Total	73	100	19	100

*The α^{-5nt} refers to the α2-IVSI-5nt mutation.

Table 10: Frequency of α-Thalassemia mutant chromosomes detected among Palestinian patients and neonates.

Mutation	**Patients**		**Neonates**	
	n	%	*n*	%
$-\alpha^{3.7}/\alpha\alpha$	23	48.9	5	50
α2-IVSI-5nt	21	44.7	4	40
$--^{MED}/\alpha\alpha$	2	4.3	1	10
$\alpha\alpha\alpha^{anti3.7}$	1	2.1	0	0
Total	47	100	10	100

For the neonates' group, 10 samples (52.6%) were found to have α-glonin gene mutations and all these 10 samples were heterozygous for one of the three mutation found ($-\alpha^{3.7}/$, $--^{MED}/$ and α2-IVSI -5nt) (Table 9). The $-\alpha^{4.2}/$ deletion mutation was not detected in the neonates group. Among the neonates' group, half (5 out of 10 chromosomes) of the α-Thalassemia mutant

chromosomes detected carried the -$\alpha^{3.7}$/ deletion mutation and 40% of the mutant chromosomes (4 chromosomes) carried the α2-IVSI -5nt mutation (Table 10).

3.4 α-Globin genotypes and red cell parameters

Tables 3.11, 3.12 and 3.13 present the various mean values and SD of the red cell parameters assessed for each genotype group. In the patients' group, the proportions of men in the three groups were as follows: 61% in group 1, 58% in group 2, 67% in group 3. In Table 3.11, the hematological findings of patients' samples are shown for the whole group as well as for males and females separately because the reference range for these parameters is gender dependent.

Table 11: Red cell parameters in the different groups of α-globin genotype detected among the patients' group.

Genotype	Sex	Patient		RBC X10^6 / µL	Hb g/dL	MCV fL	MCH pg/cell	RDW %	HbA2 %
		N	%						
Group 1: Negative for the examined mutations	All Male Female	36 22 14	49.3 30.1 19.2	5.5 ±0.5 5.6 ± 0.6 5.2 ± 0.4	11.5 ± 3.1 12.4 ± 3.3 10.0 ± 2.1	64.5 ± 8.4 66.0± 8.6 62.2 ± 7.7	20.4±3.6* 21.3 ± 3.7 19.0 ± 3.1	17.9± 3.3* 17.0 ± 3.3 19.2± 2.7	2.5 ± 0.4 2.6 ± 0.4 2.4 ± 0.5
Group 2: Single gene defect (-$\alpha^{3.7}$/αα; α^{-5nt}α/αα)	All Males Female	24 14 10	32.9 19.2 13.7	5.5±0.6 5.7±0.6 5.3±0.4	12.2±2.3 12.7±2.6 11.7±1.8	68.0±6.0 68.9±6.2 67.2±5.7	22.4±2.1 23.0±2.2 21.9±1.8	15.9±2.0 15.6±2.4 15.9±1.6	2.7±0.5 2.7±0.4 2.8±0.5
Group 3: double gene defect (-$\alpha^{3.7}$/-$\alpha^{3.7}$; α^{-5nt}α/α^{-5nt}α; -$\alpha^{3.7}$/α^{-5nt}α; - -^{MED}/αα)	All Males Female	12 8 4	16.4 11.0 5.4	5.7±0.5 5.8±0.6 5.3±0.4	11.7±2.1 12.3±2.3 11.7±1.8	62.4±7.1 64.1±8.0 67.2±5.7	20.3±2.5 21.0±2.8 21.9±1.8	16.8±2.9 16.8±2.7 15.9±1.6	2.7±0.4 2.8±0.4 2.4±0.3

Data are presented as mean ± SD. * The MCH and RDW values marked with an asterisk in group 1 are statistically different from respective values in group 2

Table 12: Red cell parameters in the different α-globin genotype detected among the patients' group.

Genotype	Sex	Patient		RBC $X10^6$ / µL	Hb g/dL	MCV fL	MCH pg/cell	RDW %	HbA2 %
		N	%						
$-\alpha^{3.7}/\alpha\alpha$	All	11	15.1	5.6±0.6	12.4±2.6	68.9±6.3	22.5±2.3	15.9±1.8	2.7±0.4
	Males	6	8.2	5.9±0.4	13.3±2.7	72.4±4.1	23.8±1.7	15.4±1.8	2.8±0.2
	Females	5	6.8	5.3±0.5	11.3±2.3	64.8±6.2	21.1±2.1	16.6±1.8	2.7±0.5
$\alpha^{-5nt}\alpha/\alpha\alpha$	All	13	17.8	5.4±0.6	12.3±2.2	67.6±5.8	22.5±2.0	15.6±2.3	2.7±0.5
	Males	8	11.0	5.4±0.7	12.3±2.7	66.2±6.4	22.4±2.5	15.8±2.9	2.0±0.5
	Females	5	6.8	5.4±0.3	12.2±1.3	69.7±4.4	22.7±1.1	15.3±1.2	2.9±0.5
$-\alpha^{3.7}/-\alpha^{3.7}$	All								
	Males	5	6.8	5.7±0.6	12.7±2.8	66.4±8.2	22.1±3.0	16±2.8	2.9±0.5
$\alpha^{-5nt}\alpha/\alpha^{-5nt}\alpha$	All	3	4.1	5.8±0.3	10.7±0.5	56.4±4.5	18.5±0.7	17.7±2.9	2.5±0.2
	Males	2	2.7	6.0±0.3	11.0±0.0	56.7±6.4	18.5±0.9	19.0±2.6	2.6±0.0
	Females	1	1.4	5.51	10.2	55.9	18.6	15.0	2.3
$-\alpha^{3.7}/\alpha^{-5nt}\alpha$	All								
	Females	2	2.7	5.5±0.56	10.3±1.6	61.9±1.7	18.8±0.9	19.0±4.5	2.6±0.5
$--^{MED}/\alpha\alpha$	All	2	2.7	6.09±0.4	12.0±1.6	61.8±7.3	19.9±1.3	15.5±1.6	2.5±0.4
	Males	1	1.4	6.38	13.3	66.9	20.8	16.6	2.8
	Females	1	1.4	5.8	11.0	56.6	18.9	14.4	2.2
$\alpha\alpha\alpha^{anti3.7}$	All	1	1.4	5.68	11.1	61.6	19.5	18.8	2.8
	Males								

Data are presented as mean ± SD. The single sample with α-globin gene triplication ($\alpha\alpha\alpha^{anti3.7}$) is included in this table

Table 13: The Various Mean Values of the Erythrocytic Parameters (± SD) For Eeach Genotype of Neonate Samples.

Genotype Normal range	Patients		RBC 10^6/ µL 4.20-6.30	Hb g/dL 12.0-18.0	MCV fL 80.0-97.0	MCH pg 26.0-32.0	RDW% 11.5-14.5
	No.	%					
$\alpha\alpha/-\alpha^{3.7}$	5	26.3	5.17±1.08	15.8±3.5	90.5±2.3	30±4.3	18.2±6.3
$\alpha/--^{MED}$	1	5.2	4.32	14.2	93.3	32.9	16.5
$\alpha^{-5nt}\alpha/\alpha\alpha$	4	21.0	5.0±0.5	15.6±1	88.5±3.1	31.0±1.9	15.5±1.0
αα/ αα	9	47.4	4.7±0.7	15.3±2.7	93.4±1.1	31.9±1.8	17.7±4.2
Total	**19**	**100**					

Statistical analysis between groups for eythrocytic parameters revealed two significant differences. The MCH is statistically lower for patients in group1 or those patients who were negative for all mutation investigated in this study, than those with a single gene defect (group 2). In all groups, the mean MCH is less than or equal 27 pg. Patients from group 1 have a significantly higher mean RDW compared to patients in group 2. Menzer index is significantly lower in group 3 (10.9±1.4) than group 2 (12.5±0.8). The MCH values in patients with double gene defect (group 3) were slightly lower than group 2, but statistically not significantly probably due to the small number of patients in group 3. The RDW is statistically higher for patients in (group1) than those with a single gene defect (group 2). Interestingly, Hb, MCV, MCH and RDW showed a similar trend among the three groups, where their values were highest in group 2 and values in group 1 and 3 were close together. Although the MCH and RDW values were statistically different when comparing groups 1 and 2, there was no distinct value that can clearly differentiate between these two groups.

The RBC count in group 2 was slightly higher than in group 1 and in turn RBC count in group 3 was slightly higher than in group 2. Single and double gene defects in the α-globin genes are associated with increased erythrocytosis. The HbA2 level in group 2 was slightly higher than in group 1. The mean HbA2 level in group 2 and 3 was very close to each other.

Analysis of the mean values of hematological parameters shown in Table 11 between males and females showed that females have lower values in most of these parameters, but these differences were statistically not significant.

Analysis of the hematological parameters among the different α-globin genotypes detected in this study Table 12 reveals slight differences in mean values but these differences were statistically not significant. Also, no significant differences between males and females were observed when comparing hematological parameters among the different genotypes detected in this study. Samples homozygous for the α2-IVSI-5nt mutation (3 samples) showed a lower mean values for Hb, MCV, MCH and HbA2 and higher RDW values compared to samples with double gene defect ($-\alpha^{3.7}/-\alpha^{3.7}$) or samples with single gene defect. The hematological

parameters observed in samples with compound heterozygous ($-\alpha^{3.7}/\alpha^{-5nt}\alpha$, 2 samples) were slightly different from other genotypes, but the low sample number of this group does not allow a reliable statistical analysis of these differences.

Table 13 compares the hematological parameters among the different genotypes detected in this study. Although slight differences exist among the different genotypes, these differences were not significant, except MCV and RDW in group 1 and Group 2, Menzer index in group 3 and group 2.

Chapter four: Discussion

α -Thalassemia is a group of genetic disorders that result in an imbalance in the synthesis of α and β-globin proteins and is caused by different mutations or deletions (Bergeron et al., 2005). α-Thalassemia is characterized by a microcytic hypochromic anemia, with variable clinical picture ranging from asymptomatic to a fatal hemolytic anemia (Harteveled and Higgs , 2010). α-Thalassaemia is more frequently caused by deletion rather than single point mutations or nucleotide insertions and deletions involving the sequences controlling gene expression (Harteveld and Higgs, 2010). Deletion mutations may involve one or both α-genes on chromosome 16. HbH results from deletion of three α-genes. Hydrops Foetalis Syndrome or Hb Bart's disease results from deletion of four α-genes and it is incompatible with life. So far, more than 35 deletion mutations affecting the α-genes have been reported. Additionally, the number of non-deletional α-globin gene mutations is increasing, and new mutations are still being discovered (Chui, 2005).

The aim of this study was to determine the most prevalent α-globin gene mutations based on targeted mutation among Palestinian patients with microcytosis not due to IDA or β-Thalassemia trait. Additionally, the study aimed to determine the most frequent incidence rate of α-Thalassemia trait among the analyzed patients with microcytosis not due to IDA or β-Thalassemia trait.

A total of 200 patients were studied and all patients had hypochromic microcytic (MCV < 80 fL, MCH <27 pg) anemia. All samples included in the study have a normal Hb electrophoresis profile (HbA2 < 3.5%) and Menzer Index <13 and/or RBC count >$5x10^6$/μL. Menzer index and RBC count are good discrimination function and are used to differentiate IDA from β-Thalassemia trait (Demir et al., 2002; AlFadhli, et al., 2006; Ehsani et al., 2009). Samples with Menzer index <13 or RBC count >$5x10^6$/μl are unlikely to have IDA.

Nineteen neonate samples were included in this study, all have MCV <95 fL. It has been reported that MCV below 90 fL, especially with an MCH below 30pg is a strong indicator of the presence of α-Thalassemia minor in neonates (Tritipsombut et al., 2008; Aktam, et al., 2009).

For detection of α-Thalassemia mutations among the study population, Gap-PCR, ARMS PCR and DNA sequencing were used. Identification of the α-Thalassemia deletion mutations among the study samples was performed using multiplex Gap-PCR. Multiplex Gap-PCR uses primer pairs that are designed to amplify the region flanking a known deletion generating a unique amplicon that will be smaller in the mutant sequence compared with the wild type (Clark and Thein, 2004).

Four different multiplex Gap-PCR reactions were performed for detection of four deletion mutations affecting the α-globin genes: $-\alpha^{3.7}/$, $-\alpha^{4.2}/$, $--^{MED}/$ and $\alpha\alpha\alpha^{anti3.7}$. These mutations represent the most prevalent deletion mutations affecting the α-globin genes in the Mediterranean region (Clark and Thein, 2004). Twenty samples were found having deletion mutations, and one sample with α-globin gene triplication. But in the neonate samples six samples were found having deletion mutations.

To search for point mutations and small insertion/deletion mutations causing α-Thalassemia that could orient the search for additional mutations, the α2- and α1-globin genes were completely sequenced in eight patients' samples and one neonate sample. DNA sequencing has revealed that four patients' samples and one neonate sample were heterozygous for the α2-IVSI-5nt mutation. This mutation results from deletion of five nucleotides at the splice junction between exon 1 and intron 1 of the α2-globin gene (Baysal, 2011). No other significant DNA sequence variants were observed in DNA sequencing results. However, since DNA sequencing was not performed for all study samples, other mutations (other than the mutations investigated in this study) cannot be excluded. The α2-IVSI-5nt mutation was found to be the second most common mutation after the $-\alpha^{3.7}/$ deletion mutation among Arabs/Palestinians referred to an

Israeli Hospital in Jerusalem (Oron-Karni et al., 2000). In the previous report, the α2-IVSI-5nt mutation was reported in 29 chromosomes and the -$\alpha^{3.7}$/ deletion mutation in 30 chromosomes out of the 109 chromosomes studied (Oron-Karni et al., 2000).

Based on the findings of DNA sequencing, which revealed that α2-IVSI-5nt mutation is common in our samples, we have analyzed the study samples for this mutation using ARMS PCR.

From 73 samples that fulfilled the inclusion criteria for this study, 37 samples (50.7%) were found to have α-glonin gene mutations. Twenty-seven patients were heterozygous for one of the four mutations found (-$\alpha^{3.7}$/; αα$\alpha^{anti3.7}$; --^{MED}/ and α2-IVSI-5nt mutations), eight patients were homozygous for either the -$\alpha^{3.7}$/ or α2-IVSI-5nt mutation and two patients were compound heterozygous for the -$\alpha^{3.7}$/ and α2-IVSI-5nt mutations and one sample has a triplication mutation, αα$\alpha^{anti3.7}$. The most frequent mutation among the patients' samples in this study was the -$\alpha^{3.7}$/, where 48.9 % of the mutant chromosomes carried this mutation. The second most frequent mutation was the α2-IVSI-5nt and it was found in 44.7 % of mutant chromosomes. The other two mutations detected in this study, --^{MED}/ and αα$\alpha^{anti3.7}$, were found in 4.3% and 2.1% of mutant chromosomes.

From the neonate samples (n=19), ten samples have α-glonin gene mutation, five samples were heterozygous for the -$\alpha^{3.7}$ mutation, four samples were heterozygous for α2-IVSI-5nt mutation and one sample was heterozygous for the --^{MED}/ mutation.

The predominance of the $\alpha^{3.7}$/ deletion is consistent with the findings of several studies in different populations and regions. The $\alpha^{3.7}$/ deletion is the most common α-globin gene deletion mutation among Jews and Palestinians/Arabs in Palestine 1948. This mutation was found in 63.9% and 27.5 % of mutant chromosomes studied in Jewish and Palestinian/Arab patients (Oron-Karni et al. 2000). For the α2-IVSI-5nt mutation, Oron-Karni et al., (2000) reported that this mutation is found in only about 3% and 26.6% of mutant chromosomes studied in Jewish and Palestinian/Arab patients. Therefore, $\alpha^{3.7}$/ and α2-IVSI-5nt mutations constitute the most frequent mutations among Palestinian/Arab patients in Palestine 1948 (Oron-Karni et al., 2000).

The $\alpha^{3.7}$/ mutation was also reported as the most predominant mutation causing α-Thalassemia in several studies from other Arab countries: Kuwait (Diejomaoh et al., 2000), Tunisia, (Zarai et al., 2002), Iraq (Al-Allawi et al., 2009), Saudi Arabia (Hellani et al., 2009), Oman (Hassan et al., 2010), and United Arab Emirates, (Baysal et al., 2011). Mediterranean countries: Italy (Bella et al., 2006), Iran (Zandian et al., 2008; Hossein et al., 2012), and Turkey (Guvence et al., 2010). Worldwide: Australia (Prior et al., 2004), North America (Bergeron et al., 2005), India (Sankar et al., 2006), China (Xiong et al., 2010), Brazil (Alcoforado et al., 2012), and Afro-Amazonian community (Cardoso et al., 2012). Also, an earlier study from eastern Saudi Arabia revealed that 45% of α-Thalassemia patients are heterozygotes for this mutation (Al-Awamy, 2000). In the United Arab Emirates, it was reported that 45% of neonates are carriers for this mutation (El-Kalla and Baysal, 1998).

In the neonates' samples (n=19), ten samples were heterozygous for one of three α-Thalassemia mutations, $\alpha^{3.7}$/,--^{MED}/ and α2-IVSI-5nt. The $\alpha^{3.7}$/ and α2-IVSI-5nt mutations were the most frequent mutations and constituted 50% and 40% of the mutant chromosomes analyzed, respectively. The findings of the neonates' samples are consistent with the finding of the patients' samples. Baysal (2011) reported that the $\alpha^{3.7}$/ mutation is the most common mutation among neonates in United Arab Emirates.

The –^{MED}/ mutation and ααα$^{anti3.7}$ triplication were found in 4.3% and 2.1 % of mutant chromosomes in the patients' samples, respectively. The –^{MED}/ is the third frequent mutation among our study sample. Oron-Karni et al. (2000) reported that –^{MED}/ mutation and ααα$^{anti3.7}$ triplication were found in 4.6 % and 5.5 %, respectively among the Palestinian/Arab population, which confirms our findings. However, in other studies the –^{MED}/ mutation was the second most frequent α-Thalassemia mutation after the $\alpha^{3.7}$/ mutation, as in Jewish (Oron-Karni et al., 2000) and Kurdish patients (Al-Allawi et al., 2009). In United Arab Emirates (Baysal, 2011) and Iran (Zandian et al., 2008) the –^{MED}/ mutation was found at low frequency, where the α2-PolyA (AATAAA>AATAAG or AATGAA) point mutation was the second frequent α-Thalassemia mutation after the $\alpha^{3.7}$/ mutation.

The $\alpha^{4.2}$/ mutation was not detected among our study samples and this is also consistent with the findings of Oron-Karni et al., (2000) where this mutation was observed in less than 1% of mutant chromosomes among Palestinian/Arab patients. This mutation is also common among α-Thalassemia patients in South East Asia and Pacific Islands (Clark and Thein, 2004) and in North America (Bergeron et al., 2005). Furthermore, this mutation was reported in about 5 % of α-Thalassemia patients in southern Iran (Zandian et al., 2008) and in about 4% in Kurdish patients in Dohuk region of Iraq (Al-Allawi, et al., 2009).

In comparison to other reports, it is interesting to note that our finding that the α2-IVSI-5nt mutation exists in a high frequency among our study sample and in a frequency close to the $\alpha^{3.7}$ mutation. This is novel to the Palestinian population, since till now; similar results have only been reported by Oron-Karni et al. (2000) in Palestinian/Arab patients and have not been reported in other populations.

Statistical analysis of erythrocyte parameters revealed two significant differences concerning the MCH and RDW parameters. The MCH of presumably in (group 1) is statistically lower than that in patients with a single gene deletion (group 2) (Table 3.11). Additionally, the Hb values and MCV in presumably in (group 1) were slightly lower than those in patients with single gene deletion (group 2) (Table 3.11). The sex distribution could not explain for the lower Hb values in group 1 compared to group 2, since the proportion of male patients in both groups is very close, 61.1 % and 58.3 % respectively. However, these differences are probably explained by the fact that group 1 are not a normal control group. In fact group 1 is composed of patients with unexplained microcytosis and probably having anemia of mixed etiology or could have mutations that are not investigated in the present study. Similarly, the higher RDW in group 1 compared to group 2 is explained in this manner. When the patient groups with single and double gene defects are compared, it was observed that the Hb values, MCV and MCH were slightly lower in the group with double gene defect (Table 3.11). While the RBC and RDW values were slightly higher in the group with double gene defect compared to single gene defect. Furthermore, there is an overlap between the red cell parameters of the three groups analyzed, presumably normal, single and double gene defect (Table 3.11). Further analysis of red cell parameters based on the type of mutation, revealed slight differences among different

groups in some cases, but none of these differences were statistically significant (Table 3.12). Our findings are consistent with previous reports that also showed that no red cell parameter could reliably predict the presence of α-Thalassemia genotype (Bergeron et al., 2005; Mehdi & Dahmash, 2011). Molecular diagnosis remains the only reliable method for diagnosis of α-Thalassemia.

Analysis of the red cell parameters in the neonate group further confirms the value of MCH and MCV in the prediction of α-Thalassemia.

In conclusion, All samples included in the study (the patients' samples and neonate' samples) were having unexplained microcytosis. Almost half of the patient's samples and the neonate's samples were found to carry one or two of the five α-Thalassemia mutations investigated in this study. The $\alpha^{3.7}$ and α2-IVSI-5nt mutations are the most frequent α-Thalassemia mutations among the study samples.

None of the red cell indices could reliably predict the presence of α-Thalassemia. DNA analysis of samples with unexplained microcytosis can reliably detect the presence of α-Thalassemia, especially when a wider range of mutations are analyzed, or when samples are analyzed by DNA sequencing**.**

Recommendations

α-Thalassemia should be considered in Palestinian patients with unexplained microcytosis. This observation was confirmed by the finding that about half of the study samples have one or two of the five α-Thalassemia mutations investigated in the present study ($-\alpha^{3.7}$; $-\alpha^{4.2}$, $\alpha\alpha\alpha^{anti3.7}$; $--^{MED}$ and α2-IVSI-5nt mutations). DNA analysis can reliably detect the presence of α-Thalassemia mutations. Testing for the $\alpha^{3.7}$ and α2IVSI 5nt mutations should be considered as the first step in DNA analysis of α-Thalassemia, as these two mutations were found in 48.9 % and 44.7 %, respectively, of mutant chromosomes identified in this study.

Further studies are needed to study the prevalence and genotypes of α-Thalassemia in a larger cohort of Palestinian patients with unexplained microcytosis. Genotyping of α-Thalassemia mutations should be preferably performed using Gap-PCR for detection of large deletion mutations and DNA sequencing for detection of other types of mutations.

References

Akram, A., Khalida, A., Sawsan, S., and Charles, L. (2009). Birth MCV and MCH are quite reliable parameters for the prediction of alpha Thalassemia trait. Clin. Med.: Blood Disorders. 2, 1-4.

Al-Allawi, N.A.S., Badi, A.I.A., Imanian, H., Nikzat, N., Jubrael, J.M.S., and Najmabadi, H. (2009). Molecular characterization of α-Thalassemia in the Dohuk region of Iraq. Hemoglobin. 33(1), 37-44.

Al-Awamy, B.H. (2000). Thalassemia syndromes in Saudi Arabia. meta-analysis of local studies. Saudi. Med. J. 21(1), 8-17.

Alcoforado, G.H.M., Bezerra, C.M., Lemos, T.M.A.M., De Oliveira, D.M., Kimura, E.M., Costa, F.F., Sonati, M.F., and Medeiros, T.M.D. (2012). Prevalence of α-Thalassemia 3.7 kb deletion in the adult population of Rio Grande do Norte, Brazil. Genet. Mol. Biol. 35, 594-598.

AlFadhli, S.M., Al-Awadhi, A.M., and AlKhaldi, D. (2007). Validity assessment of nine discriminant functions used for the differentiation between iron deficiency anemia and Thalassemia minor. J. Trop. Pediatr .53, 93-97.

Bank, A., and Ramirez, F. (1978). The molecular biology of Thalassemia. Crit. Rev. Biochem. 343-368.

Baysal, E. (2011). Alpha-Thalassemia syndromes in the United Arab Emirates. Hemoglobin. 35(5-6), 574-580.

Bella, C., Salpietro, C., La Rosa, M., Cuppari, C., Piraino, B., Cutri, M.R., and Rigoli, L. (2006). Identification of α-Thalassemia mutations in subjects from Eastern Sicily (Italy) with abnormal hematological indices and normal Hb A2. Ann. Hematol. 85(12), 829–831.

Bergeron, J., Weng, X., Robin, L., Olney, H.J., and Soulieres, D. (2005). Prevalence of α globin gene deletions among patients with unexplained microcytosis in a North American population. Hemoglobin. 29(1), 51-60.

Borges, E., Wenning, M.R., Kimura, E.M., Gervasio, S.A., Costa, F.F., and Sonati, M.F. (2001). High prevalence of alpha-Thalassemia among individuals with microcytosis and hypochromia without anemia. Braz. J. Med. Biol. Res. 34(6), 759-762.

Cardoso, G.L., Takanashi, S.Y., and Guerreiro, J.F. (2012). Inherited hemoglobin disorders in an Afro-Amazonian community: Saracura. Genet. Mol. Biol. 35(3), 553-556.

Chong, S.S., Boehm, C.D., Higgs, D.R. and Cutting, G.R. (2000). Single-tube multiplex-PCR screen for common deletional determinants of alpha-Thalassemia. Blood. 95(1), 360-362.

Chui, D.H. (2005). Alpha-thalassaemia and population health in Southeast Asia. Ann. of Hum. Biol. 32(2), 123–130.

Chui, D.H., and Waye, J.S. (1998). Hydrops Fetalis caused by alpha Thalassemia: an emerging health care problem. Blood. 91(7), 2213-2222.

Chui, D.H., Fishermen, S., and Chan, V. (2003). Hemoglobin H disease not necessarily a benign disorder. Blood. 101(3), 791-796.

Clark, E., and Thein, L. (2004). Molecular diagnosis of hematological disorders. Clin. Lab. Haematol. 26, 159-176.

Clarke, G.M., and Higgins, T.N. (2000). Laboratory investigation of hemoglobinopathies: review and update. Clin. Chem., 46 (8 Pt 2), 1284-90.

Darwish, H., El-Khatib, F., and Ayesh, S. (2005). Spectrum of β- globin gene mutation among Thalassemia patients in the West Bank region of Palestine. Hemoglobin. 29, 119–132.

Demir, A., Yarali, N., Fisgin, T., Duru, F., and Kara, A. (2002). Most reliable indices in differentiation between Thalassemia trait and iron deficiency anemia. Pediatr Int. 44(6), 612-616.

Diejomaoh, F.M.E., Haider, M.Z., Dalal, H., Abdulaziz, A., D'Souza, T.M., and Adekile, A.D. (2000). Influence of alpha-Thalassemia trait on the prevalence and severity of anemia in pregnancy among women in Kuwait. Acta. Haematol. 104(2-3), 92-94.

Ehsani, M.A., Shahgoli, E., Rahiminejad, M.S., Seighali, F., and Rashidi, A. (2009). A new index for discrimination between Iron Deficiency Anemia and Beta-Thalassemia minor: Results in 284 patients. Pak. J. Biol. Sci. 12(5), 473-475.

Elgawhary, S., Elbaradie, M.Y., Rashad, M.W., Mossad, M., Abdella, M.A., Ezzat, G., Wali, Y.A., and Elbeshalwy, A. (2008). Prenatal diagnosis of beta-Thalassemia in Egypt: implementing accurate high-tech methods did not reflect much on the outcome. Pediatr. Hematol. Oncol. 25(6), 541-549.

El-Kalla, S., and Baysal, E. (1998). Alpha-Thalassemia in the United Arab Emirates. Acta. Haematol. 100(1), 49-53.

Felice, E.A., Cleek, P.A., Marino, M.E., Mckie, M.E., Mckie, C.V., Chang, K.A., and Husiman, J.H.T. (1986). Different ζ-globin gene deletions among black Americans. Hum. Genet. 173, 221-224.

Fucharoen, S. and Winichagoon, P. (2011). Haemoglobinopathies in Southeast Asia. Indian J. Med. Res. 134, 498-506.

Fucharoen, S., and Viprakasit, V. (2009). Hb H disease: clinical course and disease modifiers. Amer. Society Hematol., 26-34.

Giambona, A., Passarello, C., Renda, D., and Maggio, A. (2009). The significance of the hemoglobin A (2) value in screening for hemoglobinopathies. Clin. Biochem. 42(18), 1786-1796.

Greer, P.J., Foerster, J., Lukens, N.J., Rodgers, G., Paraskvas, F., and Glader, B. (2004) Wintrobes's Clinical Hematology. (Phiadelphia. A Wolters Kluwer Company).

Guvenc, B., Yildiz, S.M., Tekinturhan, F., Dincer, S., Akyuzluer, I., Okten, S., and Erkman, H. (2010). Molecular characterization of alpha-Thalassemia in Adana, Turkey: A single center study. Acta. Haematol. 123(4), 197-200.

Harteveld, C.L., and Higgs, D.R. (2010). Alpha-thalassaemia. Orphanet J. Rare. Dis. 5, 13.

Hassan, S.M., Hamza, N., Al-Lawatiya, F.J., Mohammed, A.J, Harteveld , C.L., Rajab, A., and Giordano, P.C.(2010). Extended molecular spectrum of beta- and alpha-Thalassemia in Oman. Hemoglobin. 34, 127-134.

Hellani, A., Fadel, E., El-Sadadi, S., El-Sweilam, H., El-Dawood, A., and Abu-Amero, K.K. (2009). Molecular spectrum of alpha-Thalassemia mutations in microcytic hypochromic anemia patients from Saudi Arabia. Genet. Test. Mol. Biomarkers. 13(2), 219-221.

Higgs, D.R., Garrick, D., Anguita, E., De Gobbi, M., Hughes, J., Muers, M., Vernimmen, D., Lower, K., Law, M., Argentaro, A., et al. (2005) Understanding alpha-globin gene regulation: Aiming to improve the management of Thalassemia. Ann. N. Y. Acad. Sci. 1054, 92-102.

Hossein, F., Mohsen, R., Mohesn, M., and Their, M. (2012). α-Thalassemia mutations in two provinces of Southern Iran: Fars & Kohkeloye and Bouyer Ahmad. Hemoglobin. 36(2), 139-143.

Khelil, A., Denden, S., Leban, N., Daimi, H., Lakhdhar, R., Leffrac, G., Ben Chibani, D., and Perrin, P. (2010). Hemoglobinopathies in North Africa: a review. Hemoglobin. 34(1), 1-23.

Kohne, E. (2011). Hemoglobinpathies:clinical mainfestations, diagnosis, and treatment. Dtsch. Arzteb. Int. 108, (31-32) 532-540.

Lacerna, G., Musollino, G., Di Noce, F., Prezioso, R., and Carestia, C. (2007). Genotyping for known Mediterranean α-Thalassemia point mutations using a multiplex amplification refractory mutation system. Haematologica. 92(02), 254-255.

Liu, Y.T., Old, J.M., Miles, K., Fisher, C.A., Weatherall, D.J. and Clegg, J.B. (2000). Rapid detection of α-thalassaemia deletions and α-globin gene triplication by multiplex polymerase chain reactions. Br. J. Haematol. 108(2), 295-299.

Lorey, F., Charoenkwan, P., Witkowska, H.E., Lafferty, J., Patterson, M., Eng, B., Waye, J.S., Finklestein, J.Z., and Chui, D.H. (2001). Hb H hydrops foetalis syndrome: a case report and review of literature. Br. J. Haematol. 115(1), 72-80.

Mehdi, S.R., and Al-Dahmash, B.A. (2011). A comparative study of hematological parameter of α and β Thalassemia in a high prevalence zone: Saudi Arabia. Indian. J. Hum. Genet. 17(3), 207-211.

Mortada, 2009. Aailable at: http://pharamcytimes.wordpress.com/category/human-anatomy-physiology/page/2/(hemglobin structure). Accessed on December 11, 2012.

Mosca, A., Paleari, R., Leone, D., and Ivaldi, G. (2009). The relevance of hemoglobin F measurement in the diagnosis of Thalassemia and related hemoglobinopathies. Clin. Biochem. 42(18), 1797-1801.

Muncie, H.L., and Campbell, J.R. (2009) Alpha and beta Thalassemia. Am. Fam. Physician. 80(4), 339-344.

Nelson, D. and Cox, M. (2004). Lehninger Principles of Biochemistry. (W.H.Freeman).

Ntaios, G., Chatzinikolaou, A., Saouli, Z., Girtovitis, F., Tsapanidou, M., Kaiafa, G., Kontoninas, Z., Nikolaidou, A., Savopoulos, C., Pidonia, I. et al. (2007). Discrimination indices as screening tests for beta-thalassemic trait. Ann. Hematol. 86 (7), 487–491.

Old, J.M. (2003). Screening and Genetic diagnosis of Hemoglobin Disorder. Blood Rev. 17(1), 43-53.

Oron-Karni, V., Filon, D., Oppenheim , A., and Rund, D. (1998). Rapid Detection of the common Mediterranean α-Globin Deletion/ Rearrangement using PCR. Am. J. Hematol. 58(4), 306-310.

Oron-Karni, V., Filon, D., Shifrin, Y., Fried, E., Pogrebijsky, G., Oppenheim, A., and Rund, D. (2000). Diversity of α-globin mutation and clinical presentation of α-Thalassemia in Israel. Am. J. Hematol. 65(3), 196-203.

Pirastu, M., Lee, Y.K., Dozy, M.A., Kan, Y., Stamatoyanopolus, G., and Hadjiminas, G. (1982). Alpha Thalassemia in two Mediterranean population. Blood. 60, 509-512.

Prior, J.F., Bittles, A.H., and Erber, W.N. (2004). A community profile of alpha thalassaemia in Western Australia. Community Genet. 7, 211-215.

Ribeiro, D.M., and Sonati, M.F. (2008). Regulation of human alpha –globin gene expression and alpha Thalassemia. Genet. Mol. Res.7 (4), 1045-1053.

Rugless, M.J., Fisher, C.A., Stephens, A.D., Amos, R.J., Mohammed, T., and Old, J. (2006). Hb Bart's in cord blood: an accurate indicator of alpha Thalassemia. Hemoglobin. 30(1), 57-62.

Sae-ung, N., Fucharoen, G., Sanchaisuriya, K., and Fucharoen, S. (2007). α^0 Thalassemia and related disorder in northeast Thailand: a molecular and hematological characterization. Acta. Hematol. 117(2), 78-82.

Sankar, H.V., Arya, V., Tewari, D., Usha, R., Gupta, R.U., Pradhan, M., and Agarwal, S. (2006). Genotyping of alpha-Thalassemia in microcytic hypochromicanemia patients from North India. J. Appl. Genet. 47(4), 391–395.

Sarnaik, S.A. (2005).Thalassemia and related hemoglobinpathies. Indian J. Pediat. 72(4), 319-324.

Siala, H., Ouali, F., Messaoud, T., Bibi, A., and Fattoum, S. (2008). Alpha-Thalassaemia in Tunisia: some epidemiological and molecular data. J. Genet. 87(3), 229-234.

Singer, S.T. (2009). Variable clinical phenotypes of a-Thalassemia syndromes. Scientific World Journal. 9, 615–625.

Steensma, D.P., Gibbons, R.J., and Higgs, D.R. (2005). Acquired alpha-Thalassemia in association with myelodysplastic syndrome and other hematologic malignancies. Blood. 105, 443-452.

Tamaddoni, A., Hadavi, V., Nejan, N.F., Khosh-ani, A., Siami, R., and Aghai-Melbodi, J. (2009). α Thalassemia analysis in Mazandaran Province, North Iran. Hemoglobin. 33, 115-123.

Tan, A.S., Quah, T.C., Low, P.S., and Chong, S.S. (2001). A rapid and reliable 7-deletional multiplex polymerase chain reaction assay for α-Thalassemia. Blood. 98(1), 250-251.

Thakur, S., Ishrie, M., Saxena, R., Danda, S., Linda, R., Viswabandya, A., and Verma, I.C. (2011). ATR-X syndrome in two siblings with a novel mutation (c.6718C>T mutation in exon 31). Indian J. Med. Res. 134, 483-486.

Tritipsombut, J., Sanchaisuriya, K., Fucharoen, S., Fucharoen, G., Siriratmanawong, N., Pinmuang-ngam, C., and Sanchaisuriya, P. (2008). Hemoglobin profiles and hematologic features of thalassemic newborns: application to screening of alpha-Thalassemia 1 and hemoglobin E. Arch. Pathol. Lab. Med. 132(11), 1739-1744.

Urbinati, F., Madigan, C., and Malik, P. (2006). Pathopysiology and therapy of hemoglobinopathies. Part II: thalassaemias. Expert. Rev. Mol. Med. 8(10), 1-26.

Vichinsky, E.P. (2005). Changing patterns of Thalassemia worldwide. Ann. N. Y. Acad. Sci. 1054, 18-24.

Vichinsky, E.P. (2009). Alpha Thalassemia major-new mutations, intrauterine management, and outcomes. Hematology Am. Soc. Hematol .Educ. Program .1, 35-41.

Villegas, A., Porres, A., Sanchez, J., Gonzalez, F.A., Perez-Clausell, C., Martinez, M., Murga, M.J., Cacha, J., Lozano, M., Fernandez-Fuertes, I. et.al. (1998). Red blood cell phenotypes in α-Thalassemia in the Spanish population. Haematologica. 83, 99-103.

Weatherall, D.J. (1997). The thalassaemias. B. M. J. 314(7095), 1675-1678.

Weatherall, D.J., and Clegg, J.B. (2001). Inherited haemoglobin disorders: An increasing global health problem. Bull. World Health Organ. 79, 704-712.

Xiong, F., Sun, M., Zhang, X., Cai, R., Zhou, Y., Lou, J., Zeng, L., Sun, Q., Xiao, Q., Shang, X., et.al. (2010). Molecular epidemiological survey of haemoglobinopathies in the Guangxi Zhuang Autonomous Region of southern China. Clin. Genet. 78(2), 139-148.

Younis, K., Barghuty, F., Abdeen, Z. (1996). Prevalence of β-Thalassemia trait among Palestinian students of higher education. Publication of Health Related Research Center at Al-Quds University-Palestine.

Zandia, K., Nateghi, J., Keikhaic, B., Pedram, M., Najad, N., Hadavi, V., Oberkanin, C., Azarkeivan, A., Law, H., and Najmabadi, H. (2008). α Thalassemia mutation in Khuzestan-Province South east Iran. Hemoglobin. 32(6), 546–552.

Zorai, A., Harteveld, C.L., Bakir, A., Van Delft, P., Falfoul, A., Dellagi, K., Abbes, S., and Giordano, P.C. (2002). Molecular spectrum of alpha-Thalassemia in Tunisia: epidemiology and detection at birth. Hemoglobin. 26(4), 353-362.

Zuysal, Z., Yildirmark, Y., Akar, N., Basak, N., and Cis, S. (1998). Alpha-Thalassemia and hereditary spherocytosis in the same patient: the interaction of two diseases. Pediatr. Hematol. Oncol. 15(3), 271-277.

Appendix A

Table A.1: Hematological parameters for the study samples.

Sample #	RBCs ($x10^6/\mu L$)	Hb (g/dL)	MCV (fL)	MCH (pg/cell)	RDW%	HbA2 (%)	MI
Patients samples							
1	4.9	9.2	63.3	18.9	20.2	2.1	12.9
2	5.0	9.5	59.2	19.1	23.2	2.1	11.8
3	4.6	8.6	58.6	18.8	15.7	3.0	12.7
4	4.5	7.9	58.3	17.7	17.4	2.0	13.0
5	4.6	8.9	59.4	19.2	19.4	3.4	12.9
6	4.9	9.0	57.9	18.4	16.8	2.5	11.8
7	4.9	9.0	58.1	18.3	17.8	1.8	11.8
8	4.9	10.0	60.2	20.3	16.1	3.0	12.2
9	4.9	10.0	61.8	20.3	17.7	3.0	12.6
10	4.6	8.8	56.5	19.0	19.0	1.5	12.3
11	4.9	8.5	57.4	17.3	19.0	2.2	11.7
12	5.0	10.7	62.5	21.6	21.4	2.0	12.5
13	4.9	10.1	63.8	20.6	18.9	2.9	13.0
14	5.0	10.6	68.1	21.3	15.5	2.4	13.6
15	4.7	5.7	47.0	12.1	19.6	1.5	10.0
16	5.2	17.3	76.1	24.8	13.6	2.4	14.6
17	5.3	11.3	64.8	21.2	16.0	3.0	12.2
18	5.1	8.6	58.4	17.0	20.0	2.4	11.5
19	5.4	9.2	57.9	17.2	22.6	2.1	10.7
20	5.6	12.8	72.6	22.7	14.4	3.2	13.0
21	5.9	11.4	60.7	19.4	15.8	2.2	10.3
22	5.1	7.5	56.6	14.7	21.7	2.5	11.1
23	6.0	14.1	72.4	23.7	14.1	2.5	12.1
24	6.0	9.0	72.4	23.7	18.4	2.5	12.1
25	5.2	9.3	56.7	17.8	18.1	2.5	10.9
26	5.8	14.5	73.8	25.2	14.1	2.6	12.7
27	5.6	11.7	62.4	20.9	19.7	2.5	11.1
28	5.3	12.7	68.8	24.2	14.5	2.8	13.0
29	5.5	10.2	56.2	18.6	18.5	3.2	10.2
30	5.1	9.2	63.1	18.2	22.2	2.9	12.4
31	5.7	11.9	65.6	21.0	15.6	3.0	11.5
32	5.8	11.0	56.6	18.9	14.4	2.2	9.8

Table A.1: cont.

Sample #	RBCs (x1012/L)	Hb (g/dL)	MCV (fL)	MCH (pg/cell)	RDW	HbA2 (%)	MI
33	5.2	8.9	59.9	17.2	23.0	2.7	11.5
34	5.0	11.0	62.4	21.0	15.9	2.9	12.5
35	5.1	8.7	57.5	17.1	21.0	2.0	11.3
36	5.4	10.7	58.9	19.7	22.6	2.0	10.9
37	5.6	9.4	59.0	16.8	21.5	2.3	10.5
38	5.4	12.5	70.6	23.3	17.4	2.7	13.1
39	5.3	11.9	68.5	22.4	16.5	2.5	12.9
40	5.2	9.4	58.5	18.2	19.6	2.5	11.2
41	5.6	13.0	68.9	23.0	14.3	3.0	12.3
42	5.8	14.8	75.5	25.8	14.5	2.8	13.0
43	5.2	11.6	66.7	22.5	16.9	3.5	12.8
44	5.3	9.7	57.1	18.4	20.4	1.5	10.8
45	5.8	11.0	61.2	19.1	17.2	2.6	10.6
46	5.8	14.0	75.8	24.3	13.8	2.5	13.1
47	5.9	11.1	61.6	19.5	18.8	2.6	10.4
48	5.8	13.4	73.3	23.2	13.9	3.0	12.6
49	5.5	11.0	58.9	20.0	17.9	3.5	10.7
50	5.7	10.4	58.7	18.3	17.0	2.6	10.3
51	5.6	12.8	75.5	22.8	14.9	2.8	13.5
52	5.3	11.9	65.2	22.8	15.7	3.0	12.3
53	5.5	10.2	55.9	18.6	15.0	2.3	10.2
54	5.6	14.0	76.6	24.8	13.3	3.0	13.6
55	5.5	16.9	79.9	25.8	13.9	2.6	14.5
56	5.7	11.4	74.5	20.0	17.7	3.0	13.1
57	5.7	15.6	77.2	27.1	13.0	2.6	13.5
58	6.2	15.0	74.8	24.2	14.2	2.8	12.1
59	6.3	13.3	64.1	21.1	16.0	2.5	10.2
60	6.2	11.0	52.2	17.8	20.8	2.6	8.4
61	6.6	16.6	74.0	25.0	22.6	3.0	11.2
62	6.1	14.6	74.0	24.1	14.5	3.0	12.1
63	6.3	15.6	73.1	24.8	12.9	2.6	11.6
64	6.4	16.1	74.4	25.3	12.5	2.5	11.6
65	6.2	12.9	64.7	20.7	15.5	2.8	10.4
66	6.2	16.7	77.2	27.0	13.5	3.0	12.5
67	6.4	14.5	67.6	22.6	14.5	2.9	10.6
68	6.5	14.8	66.5	22.7	14.5	3.0	10.2
69	6.0	16.0	77.4	26.6	12.5	2.8	12.9
70	6.5	14.8	68.3	22.9	13.7	2.4	10.5

Table A.1: cont.

Sample #	RBCs (x1012/L)	Hb (g/dL)	MCV (fL)	MCH (pg/cell)	RDW	HbA2 (%)	MI
71	6.4	13.3	66.9	20.8	16.6	2.8	10.4
72	6.2	15.7	75.7	25.2	14.6	2.6	12.2
73	6.2	14.8	72.0	24.0	15.7	2.8	11.6
Neonatal samples							
1	4.0	12.0	90.8	30.0	27.5	ND	-
2	4.9	13.9	86.0	28.5	15.0	ND	-
3	3.3	10.6	93.7	32.4	15.0	ND	-
4	4.2	14.6	93.2	32.7	18.8	ND	-
5	4.3	14.6	93.8	33.4	15.8	ND	-
6	4.6	14.2	87.6	30.9	15.7	ND	-
7	4.3	14.2	93.3	32.9	16.5	ND	-
8	4.8	16.3	93.5	34.0	15.4	ND	-
9	4.9	16.1	93.0	32.9	16.8	ND	-
10	4.85	15.8	94.3	32.6	18.9	ND	-
11	5.9	18.4	88.8	31.0	15.3	ND	-
12	5.7	18.0	87.2	31.7	14.6	ND	-
13	5.7	18.2	87.7	32.0	29.4	ND	-
14	5.3	13.5	91.1	22.4	16.2	ND	-
15	6.3	20.9	93.9	33.4	16.4	ND	-
16	5.7	18.1	91.1	32.0	15.0	ND	-
17	4.8	14.3	94.9	29.7	15.5	ND	-
18	5.26	17.0	93.2	32.3	18.3	ND	-
19	4.21	12.3	93.3	29.2	12.3	ND	-

ND: not determined.

تحديد **نوع الطفرة في الفا- جلوبين جين بين المرضى الفلسطينين الذين يعانون من صغر غير مبرر في حجم كريات الدم الحمراء**

اعداد: ميسون عدنان سليمان حمايل

المشرف الاول: د. محمود سرور

المشرف الثاني: أ.خالد يونس

ألفا-ثلاسميا هو مرض وراثي متنحي الصفة يتميز بفقر دم مصاحب له صغر في حجم كريات الدم الحمراء ونقص في تصبغها. اما أعراضه فتتراوح ما بين حامل للمرض بدون أعراض الى مرض قاتل. وهو مرض شائع في العالم وخصوصا في منطقة حوض البحر الابيض المتوسط والشرق الاوسط وشرق اسيا وافريقيا وشبه القارة الهندية. الاسباب الوراثية لهذا المرض تمت دراستها في عدة مناطق في حوض البحر الابيض المتوسط ولكن ليس في فلسطين. تهدف هذه الدراسة الى تحديد نسبة ونوع الخلل الوراثي في الفا جلوبين جين_/ ^{MED} --, α2-IVSI-5nt, $-\alpha^{4.2}$/ , $-\alpha^{3.7}$/-) and $\alpha\alpha\alpha^{anti3.7}$) في مجموعة من المرضى الذين يعانون من صغر في حجم كريات الدم الحمراء لسبب غير واضح. من اجل ذلك تم جمع 73 عينة من هؤلاء المرضى بعد ان تم استبعاد ان يكون السبب في صغر حجم كريات الدم الحمراء هو بيت- ثلاسميا او فقر دم ناتج عن نقص الحديد. بالاضافة الى هؤلاء المرضى تم تجميع 19 عينة لاطفال حديثي الولادة بحيث يكزن حجم كريات الدم الحمراء لديهم اقل من 95 فيمتوليتر(95 fL). تم استخدام عدة طرق للكشف عن الطفرات المسببة لالفا-ثلاسميا في هؤلاء المرضى وهي Gap-PCR, DNA sequencing, ARMS PCR.

من بين 73 مريض تمت دراستهم, كان هناك 50.7% حاملين لاحدى هذه الطفرات $\alpha^{3.7}$ اوα2-IVSI-5nt او -- ^{MED} او$\alpha\alpha\alpha^{anti\ 3.7}$. أكثر هذه الطفرات شيوعا بين المرضى كان$\alpha^{3.7}$ و α2-IVSI-5nt وبالنسب الاتية 48.9 % و44.7 % بالترتيب. اما ^{MED}-- و$\alpha\alpha\alpha^{anti\ 3.7}$ فقد كانت موجودة بالنسب الاتيه 4.3 % و2.1 % بالترتيب. في عينات الاطفال حديثي الولادة كانت الطفرات التالية ($\alpha^{3.7}$, α2-IVSI-5nt) ايضا هي الاكثر شيوعا. الطفرة المسببة لالفا-ثلاسميا $\alpha^{4.2}$ لم يتم ايجادها في هذه الدراسة بين هؤلاء المرضى.لأن هذه الطفرة تتواجد بالنسبة ضئيلة في منطقة حوض البحر الابيض المتوسط. طفرة α2-IVSI-5nt هي الطفرة الثانية الاكثر شيوعا بين هؤلاء المرضى بعد طفرة $\alpha^{3.7}$. وهذا لم يتم نشره من قبل في أي مجتمع آخر. في تحليل العلاقة بين قياسات كريات الدم الحمراء ونوع الطفرة لم يتم ايجاد علاقة ذات دلالة احصائية بينهما وبالتالي لا تستطيع هذه القياسات الكشف عن وجود الفا-ثلاسميا. وعليه فان التشخيص الجزيئي هو الاكثر دقة واعتمادا للكشف عن الفا-ثلاسميا.

وفي الخلاصة فان نسبة كبيرة (50.7%) من عينات المرضى الذين يعانون من صغر حجم كريات الدم الحمراء لسبب غير واضح كانوا حاملين لاحدى الطفرات المسببة لألفا-ثلاسميا. كما ان التشخيص الجزيئي ضروري جدا لهؤلاء المرضى.