DIAGNOSIS OF IRON DEFICIENCY ANEMIA AND THALASSEMIA USING CONFOCAL AND ATOMIC FORCE MICROSCOPY

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(2017)

Thalasemia
Anemia
Sickle cell disease

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FINAL APPROVAL

It is certificate that we have read the thesis submitted by Ms. Saira Tariq and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M.S Degree in Biotechnology.

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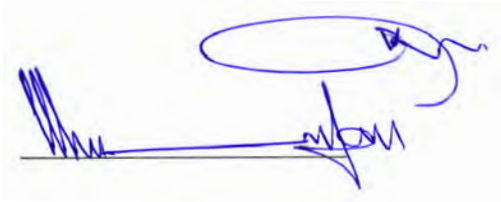

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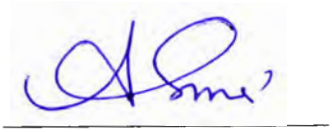

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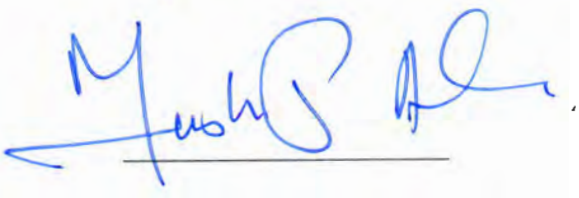

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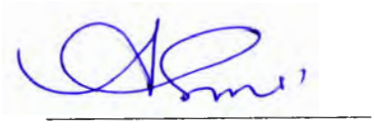

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A thesis submitted to Department of Bioinformatics and Biotechnology,
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DEDICATION

This project is dedicated to Allah Almighty, the One above, who blesses us abundantly every day and His beloved Prophet (PBUH), my parents for their unconditional support at every step, my supervisor for her humble support in tough times and especially to my co-supervisor for being supportive at every step of project and a true mentor for me.

DECLARATION

I hereby declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition.
No part of the thesis has been previously presented for any other degree.

Date 07-02-2017

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CONTENTS

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LIST OF ABBREVIATIONS

RBCs	Red Blood Cells
Hb	Hemoglobin
B	Beta
A	Alpha
CBC	Complete Blood Count
MCH	Mean Corpuscular Hemoglobin
MCV	Mean Corpuscular Vòlume
RDW	Red blood cell Distribution Width
MCHC	Mean Corpuscular Hemoglobin Concentration
Fl	Foot lambert
IDA	Iron Deficiency Anemia
WHO	World Health Organization
CHr	Reticulocyte hemoglobin
AFM	Atomic Force Microscopy
LSCM	Laser Scanning Confocal Microscopy
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
Ml	Microliter

SC disease	Sickle Cell disease
HbA	Adult hemoglobin
HbA2	Hemoglobin A2
HbF	Fetal hemoglobin
HbH	Hemoglobin H
Hb Bart's	Hemoglobin Bart's
ACD	Anemia of chornic disease
g/dL	Gram per deciliter
HIV	Human immunodeficiency Virus
HCV	Hepatitis C virus
g/L	Gram per litre
µg/L	Mu gram per litre
US	United State
ATP	Adenosine Triphosphate
HCP1	Heme Iron transporter
DMT1	Divalent metal ion transporter 1
Mg	Microgram
°C	Degree Centrigarde
G	Gram
%	Percentage
PMTs	Photomultiplier Tube Detectors
D	Dimensional

RGB	Red Green and Blue
Nm	Nanometer
IBM	International Business Machines
RNAi	RNA interference
Cc	Cubic centimeter
EDTA	Ethylene Diamine Tetra Acetic Acid
Rpm	Revolutions per minute
BD	Becton, Diclainson Company
pH	potential Hydrogen
μ	Mu
μm	Mu meter
kHz	Kilohertz
N/m	Newton per meter
L	Length
W	Width
RA	Roughness Analysis
A	Anstrom
Mm	Millimeter
Yrs	Years

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ABSTRACT

Anemia is the most prevalent blood borne disorder, categorize into thalassemia and iron deficiency anemia. In anemia, morphology of erythrocytes is disturbed thus leading to abnormal functioning of erythrocytes. Globally, thalassemia affects 1.3% individuals and is one of the most widespread monogenic disorders in Pakistan. Whereas iron deficiency anemia is other most frequent nutritional deficiency in the world, mostly affecting children and women.

The aim of the current study is to detect the morphological changes of the erythrocytes at the nanometer scale which is also important for the early investigation of anemia. Fifty samples of blood from thalassemia and iron deficiency anemia patient were collected from different hospitals of Rawalpindi and Islamabad. The blood samples were scanned using Atomic Force Microscopy (AFM) and Laser Scanning Confocal Microscopy (LSCM) to check the morphological changes in both types of anemia. According to present study, thalassemia and iron deficiency anemia is most prevalent in females with age group between 5-15 years in thalassemic patients and age group of 16-25 and 36-45years in IDA patients.

Erythrocyte morphology is the significant determinant for diagnosing and distinguishing iron deficiency anemia and thalassemia. The study reported deformed erythrocytes in anemic patients as compared to a healthy control group. Thalassemia erythrocytes showed crenated shape whereas iron deficiency anemia erythrocytes shows elliptocytes shape while healthy erythrocytes show biconcave disk shape by using AFM and LSCM. According to AFM, thalassemia erythrocytes size ranges 20nm whereas iron deficiency anemia erythrocytes range 140nm and healthy erythrocytes ranges 8nm. These techniques seems to be very promising, cheap and less time consuming in determining the structure-function relationship of erythrocyte of thalassemic and iron deficiency anemic patients. The results of LSCM and AFM are quite useful to determine the morphological changes in erythrocytes and to study the disease at molecular level within short period of time. Hence encourages employing these non-invasive techniques for effective diagnosis of anemic patients.

CHAPTER 1

INTRODUCTION

INTRODUCTION

Red blood cells (RBCs, erythrocytes) are a major component of blood (Zeidan and Yelin, 2015). Blood contains4-5×10^9 erythrocytes/ml of blood and it represents 96% of all blood cells (Schaller *et al.*, 2008). The function of RBCs is to transport oxygen to the whole body and they contain hemoglobin, a protein which is able to carry oxygen (Zeidan and Yelin, 2015). It contains protein hemoglobin in a large amount which is responsible for the red color of blood. Each erythrocyte contains approximately 3×10^8hemoglobin molecules (Schaller *et al.*, 2008). The RBC membrane consists of various lipid and protein components. The membrane mass consists of about 52%– proteins, 40% – lipids and 8% – carbohydrates (Zeidan and Yelin, 2015).

Hemoglobin is a heterogeneous protein present in RBCs. One molecule of hemoglobin contains 4 heme groups (Weatherall *et al.*, 1996). Hemoglobin is a hetero-tetramer which consists of two alpha (141 residues present in human hemoglobin) and two beta (146 residues) chains. It is responsible for binding oxygen in the lungs and transports oxygen throughout the body where it is used in aerobic metabolic pathways (Duli´nska *et al.*, 2006).

Anemia is a disease which is characterized by an abnormally decrease amount of hemoglobin or erythrocytes in the blood (Xing *et al.*, 2011). There are two important types of anemia which are iron deficiency and thalassemia. The erythrocytes are smaller in number as compared to a healthy person in anemic patients (Urrechega *et al.*, 2011; Zhang *et al.*, 2012).

Thalassemia is a genetic based group of diseases in which synthesis of hemoglobin (Hb) is impaired, leading to chornic anemia (Wentrup-Byrne *et al.*, 1997). There is an impaired production of alpha and beta chains of hemoglobin (Winichagoon *et al.*, 1993) which significantly increase in erythropoesis and associated absorption of iron from the diet, leading to iron overload (Wentrup-Byrne *et al.*, 1997). It is the most common single gene disorder which is caused by variant or missing genes that can affect the hemoglobin synthesis (Weatherall *et al.*, 2010). With 1.7% of the world population carrying thalassemia genes and thalassemia is one of the most common genetic disorders worldwide. It is common in some parts of the world where it represents a major public health problem (Dulinska *et al.*, 2006). There are different types of thalassemia, i.e. alpha, beta and intermeidate. The most well-known types of thalassemia are

Alpha Thalassemia and Beta Thalassemia whereas in Pakistani population β-thalassemia is most common (Munice and Campbell, 2009).

The disease is global in its distribution and is estimated to have as many as five million carriers in the Mediterranean region alone. In Southeast Asia, the incidence of thalassemia can be particularly high. Every year almost 50,000 pregnancies are at risk of producing an affected fetus, with one fourth of these pregnancies resulting in a thalassemic newborn (Wentrup-Byrne *et al.*, 1997). It is estimated that about 9000 children with beta thalassemia are born every year, although no documentary registry is available in Pakistan. The estimated carrier rate is 5-7%, which accounts as 9.8 million carriers in the total population. The cultural and religious scenario in Pakistan is such that consanguineous marriages are quite common. There is no concept of premarital screening or counseling of individuals with a family history of the disease. Consanguinity was quite high compared to the study done at Lahore showing that 56.7% of the couples were first cousins and 19.8% were relatives. The results showed that 87.5% did not know that the marriages in the same family increased the risk of genetic transmission which is comparable (80%) to a study done at Pakistan Thalassemia Welfare Society Rawalpindi (Arif *et al.*, 2008).

The risks factors are age, gender, blood group, weight, consanguinity and infections. Thalassemia Major is most common in children. Children are normal at the time of birth, but after 3 months of birth they show the symptoms of severe anemia. Patients suffering from thalassemia are at the risk of heart failure, iron overload, splenomegaly, diabetes, hepatitius B C, HIV infection (Borgna-Pignatti *et al.*, 2004; Telfer *et al.*, 2006).

The treatment of this disease depends on the type and severity. Alpha thalassemia patients usually have no symptoms and have no need of any treatment. Intermedia thalassemia patients may need blood transfusions occasionally i.e when they are experiencing stress due to an infection. Major thalassemia is a serious and life threatening illness which is treated with regular blood transfusions, iron chelation therapy, surgery and bone marrow transplant (Galanello and Origa, 2010).

The chemical diagnosis of thalassemia includes complete blood count (CBC), hemoglobin test, mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and red blood cell distribution width (RDW). Many thalassemic patients show mild microcytic anemia in their

complete blood count. The MCV of thalassemia is usually less than 75fl. Supplemental tests include serum ferritin, the peripheral smear, hemoglobin electrophoresis, serum lead level, and bone marrow aspirate. The other tests are serum iron, total iron binding capacity and transferrin saturation which are rarely needed. The Hemoglobin electrophoresis shows the trait is beta thalassemia, alpha thalassemia or iron deficiency anemia (Munice and Campbell, 2009).

Iron deficiency anemia is the most common nutritional deficiencies which can lead to the frequent disability and death all over the world (Urrechaga *et al.*, 2011). Iron deficiency progresses from replete iron stores to a depletion of iron stored in the body. This results in the depletion of the functional iron compartment which can lead to iron deficient anemia (Grant *et al.*, 2007). It is also said that it is insufficient iron intake or menstrual loss in women of childbearing age, or chornic blood loss in the gastrointestinal tract. Iron deficiency develops in sequential changes over a period of negative iron balance. These stages include the iron depletion phase, iron deficient erythropoiesis, and finally IDA (Iron Deficiency Anemia) (Urrechaga *et al.*, 2011).

Iron deficiency is a global health issue. It is difficult to estimate the true global prevalence (Grant *et al.*, 2007). In 2001, more than 80% pregnant women are iron deficient in South Asia (Tapiero *et al.*, 2001). In 2003, UNICEF estimated that 40-50% of children under 5 years old in developing countries are iron deficient. In 2007, the prevalence of iron deficiency in children less than 2 years old living in metropolitan Sydney is 7% and in Auckland is 14%. In comparison, approximately 7% of children in this age group in Europe and the USA are iron deficient. In both Australia and New Zealand the prevalence of iron deficiency in young children varies with ethnicity. In Australia, Aboriginal, Vietnamese and Arabic children are at increased risk. In New Zealand, the Maori, Pacific and children of other non-European ethnic groups are at increased risk (Grant *et al.*, 2007). According to WHO, 39% of children less than 5 years, 48% of children between 5 to 14 years, 42% of women and 52% pregnant women are anemic in developing countries and half of them are iron deficiency anemia (Zimmermann and Hurrell, 2007).

The risk factors of IDA include menstrual blood loss among women, gastrointestinal bleeding, uterine blood loss, poor diet, bleeding after any severe injury, etc. The treatment of IDA involves oral iron therapy via different supplements and parenteral iron therapy i.e iron malabsorption, iron dextran, iron gluconate, iron sucrose (Cook, 2004).

The diagnosis includes screening measurement and definitive measurements of iron status. Hemoglobin tests, transferring saturation test, mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), Zinc protoporphyrin and reticulocyte hemoglobin (CHr) are involved in screening measurements. While definitive measurements are serum ferritin, serum transferring receptor, bone marrow iron and red blood cell distribution width (RDW), hemoglobin electrophoresis and the peripheral smear (Cook, 2004; Muncie and Campbell, 2009). Serum ferritin is the best test to screen iron deficiency anemia. The MCV is less than 80fl in iron deficiency anemia (Muncie and Campbell, 2009).

Iron deficiency anemia and thalassemia can be distinguished by using various chemical lab tests. But these tests are time taking and cannot properly differentiate at a very early time of disease. Now-a-days research is going in the field of biophotonics which provides very simple and easy technology for differentiation of infectious diseases, i.e. laser scanning confocal microscopy and atomic force microscopy (AFM).

Laser Scanning Confocal Microscopy (LSCM) is an invaluable tool for a wide range of investigations in the biological and medical sciences. It is a tool for imaging thin optical sections in living and fixed specimens which ranges in thickness upto 100 micrometers (Claxton *et al.*, 2006). The confocal microscopy is used in the biomedical sciences for imaging fixed or living tissues which can be labeled with one or more fluorescent probes. It is an essential tool for many biomedical imaging applications. The confocal microscopy imaging modes includes single optical sections, multiple wavelength images, three dimensional reconstructions and living cell and tissues sequences (Paddock, 2000).

Atomic force microscopy (AFM) is a powerful technique and allows for the noninvasive examination of specimens under natural conditions and with minimal preparation. It has an ability to obtain topographic information and surface morphology of a specimen in non-aqueous, aqueous or dry condition (Zhang *et al.*, 2012). Atomic force microscopy (AFM) imaging has four applications i.e. measuring persistence lengths, force mapping to measure properties like elasticity of cells, phase mode imaging to detect variations in materials and to obtain topographic information and surface morphology (Hansma *et al.*, 1997). It can be used to study a variety of material in surface science, biochemistry and biology. (Zhang *et al.*, 2012). It is used to investigate chromosomes(Hoshi and Ushiki, 2011), cell membranes (Suresh and Edwardson,

2010), proteins (Engel, 2011), DNA (Di-Bucchianico *et al.*, 2011), RNA structure (Heus *et al.*, 2011), nucleic acid-protein complexes (Miklaszewska *et al.*, 2004), ligand-receptor binding (Odorico *et al.*, 2007), carbohydrates (Lesoil *et al.*, 2010), lipids and living cells(Zhang *et al.*, 2012).

The aim of present study was to check the morphological properties of erythrocytes of iron deficiency anemia, thalassemia and healthy samples by using Atomic Force Microscopy (AFM) and Laser Scanning Confocal Microscopy (LSCM). The results showed that these simple technologies are not time consuming and can easily differentiate the iron deficiency anemia and thalassemia.

The main objectives of current research are:

- Early investigation of anemia through different optical tools.
- Detection of morphological changes to erythrocytes at nanoscale level for differential diagnosis of Iron Deficiency anemia and thalassemia.

CHAPTER 2

LITERAUTRE REVIEW

LITERATURE REVIEW

Blood is a red liquid which is present in the human body. Blood is composed of cells and plasma. The cells include erythrocytes (Red blood cells), leukocytes (White blood cells) and platelets as shown in figure 2.1. The erythrocytes are more than 99 percent of cells. The leukocytes protect the body against infections and cancer. The platelets help in blood clotting. The other part of blood is plasma which is the liquid portion of blood and contains a large number of organic and inorganic substances which are dissolved in water. The hematocrit is the percentage of total blood volume i.e. erythrocytes. The normal hematocrit is 45 percent in men and 42 percent in women (Vander *et al.*, 1990).

2.1 Erythrocytes

Red blood cells (RBCs or erythrocytes) are a major component of blood (Zeidan and Yelin, 2015). Blood have $4\text{-}5\times10^9$ erythrocytes/ml (Schaller *et al.*, 2008). Erythrocytes have biconcave discs shape as shown in figure 2.2 (Guyton and Hall, 2006). The disc is thicker at the edge (Vander *et al.*, 1990). Erythrocytes have a mean diameter of 6-8micrometers and a thickness of 2-2.5micrometers and 1micrometer or less in the center. The average concentration of red blood cells in men is 5,200,000, per cubic millimeter and in women it is 4,700,000. Those people which are living at high altitudes having a greater number of red blood cells (Guyton and Hall, 2006; Schaller *et al.*, 2008).

The major function of RBCs is to transport oxygen to the whole body and contain hemoglobin, a protein which is able to carry oxygen (Zeidan and Yelin, 2015). Hemoglobin is an excellent acid base buffer that's why RBCs are responsible for most of acid base buffering power of whole blood of the body (Guyton and Hall, 2006). Hemoglobin is also responsible for the red color of blood. Each erythrocyte has 3×10^8 hemoglobin molecules approximately (Schaller *et al.*, 2008). The other function of RBCs is that they contain a large quantity of carbonic anhydrase, an enzyme which catalyze the reversible reaction between carbondioxide and water to form carbonic acid (H_2CO_3) and increase the rate of this reaction to several thousand folds (Guyton and Hall, 2006).

The RBC membrane consists of various lipids and protein components. The membrane mass consists of about 52% proteins, 40% lipids and 8% carbohydrates (Zeidan and Yelin, 2015). Erythrocytes lack nuclei and organelles. They also can neither reproduce themselves nor maintain their normal structure (Vander *et al.*, 1990). Erythrocytes are produced in the bone marrow (Schaller *et al.*, 2008). The life span of an erythrocyte is about 100-120days (Vander *et al.*, 1990; Schaller *et al.*, 2008). It means that almost 1% of the body's erythrocytes are destroyed and it must be replaced every day (Vander *et al.*, 1990).

2.2 RBC Morphology

The morphological differentiation of two common microcytic anemia are shown in table 2.1. The elliptocytes and anisocytosis are seen in thalassemia and target cells are seen in iron deficiency anemia. The morphological finding i.e coarse stippling is seen in some cases of thalassemia and is never seen in uncomplicated the iron deficiency. And all other morphologic clues are given in table 2.1 (Ford, 2013).

2.3 HEMOGLOBIN

Hemoglobin is heterogeneous protein which is present in erythrocytes (Weatherall *et al.*, 1996). It gives the red color to blood. It is a protein which contains some non-protein part (Sheller *et al.*, 1996). The function of this protein is to transport oxygen from lungs to all cells in the body (Weatherall *et al.*, 1996; Sheller *et al.*, 1996). The protein portion is called globin and consists of 574 amino acids which are distributed among four polypeptides chain. And the non-protein part is called heme ring which consist of iron (Sheller *et al.*, 1996).

2.3.1 Structure of Hemoglobin

Hemoglobin is a hetero-tetrameric structure which is made up of four protein subunits i.e. two alpha globin protein (141 residues) and two beta globin protein (146 residues) (Duli´nska *et al.*, 2006). The composition of four globin chains describes the hemoglobin type (Munice and Campbell, 2009).

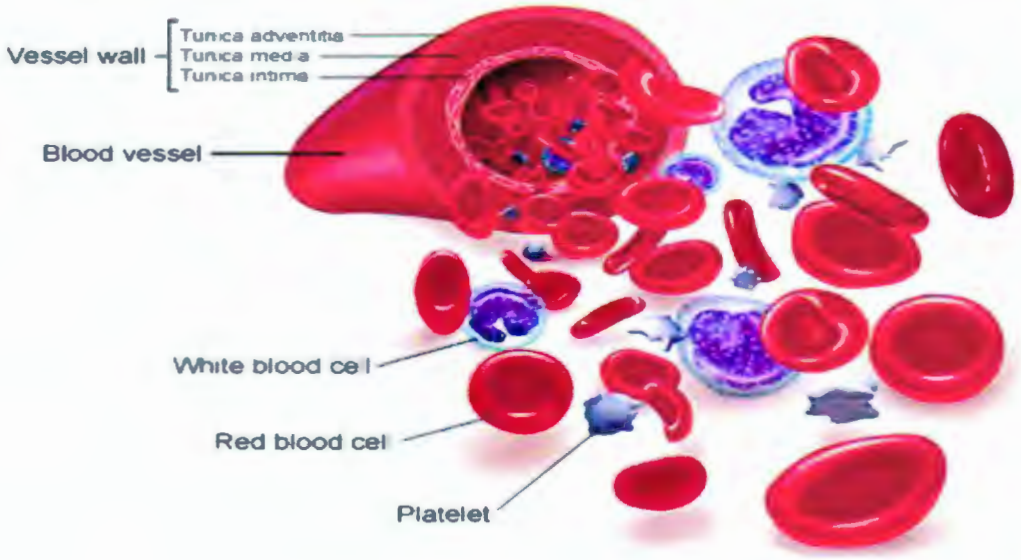

Figure 2.1 Composition of Blood

Figure 2.2 Erythrocytes (RBCs)

Table 2.1 Some Common RBC morphological features related to Iron deficiency Anemia and Thalassemia (Ford, 2013)

RBC Morphology	Morphological Definition	Clinical Associations
Anisochromia	Variation in the amount of central pallor among a population of RBCs.	Iron deficiency, myelodysplasia, hypochromic anemia post transfusion.
Anisocytosis	Variation in size among a population of RBCs.	Common nonspecific finding seen in iron deficiency. Moderate or severe thalassemia, megaloblastic anemia, partially treated anemia of several causes, post transfusion.
Basophilic stippling: coarse	RBCs have variably sized (up to large) basophilic 'granular' discolorations across its entire cytoplasm, on a Wright-stained film.	Thalassemia, Lead poisoning, myelodysplasia, Pyrimidine 5' nucleotidase deficiency, post chemotherapy.
Dimorphism	Two distinct populations of RBC are present, for example, microcytic and normocytic, or hypochromic and normochromic.	Myelodysplasia, post transfusion, partially treated iron deficiency.
Elliptocyte	RBC is oval shaped	Iron deficiency, megaloblastic anemia, hereditary elliptocytosis, post chemotherapy.
Hypochromia	The Zone of central pollar is >1\3	Iron deficiency, thalassemia, and

	the diameter of RBC	anemia of chronic disease.
Target cell	The RBC has a central red area within the zone of central pallor.	Thalassemia, liver disease, hyposplenism,Hgb C dsease or SC disease, hereditary Xerocytosis. May be seen in iron deficiency anemia.

2.3.2 TYPES OF HEMOGLOBIN

There are two types of hemoglobin

1. Normal hemoglobin
2. Abnormal hemoglobin(Weatherall *et al.*, 2005;Muncie and Campbell, 2009)

2.3.2.1 NORMAL HEMOGLOBIN

Normal hemoglobin is present in the body of healthy person (Weatherall *et al.*, 2005). It includes Adult hemoglobin (HbA), Hemoglobin A2 (HbA2) and Fetal hemoglobin (HbF) (Weatherall *et al.*, 2005; Muncie and Campbell, 2009). Fetal hemoglobin (HbF) contains two alpha chains and two gamma chain. Adult hemoglobin (HbA) contains two alpha chains and two beta chains. And hemoglobin A2 (HbA2) contains two alpha chains and two delta chains (Munice and Campbell, 2009). In thalassemia HbA level is low and HbF level is high. HbF is present in fetuses and newborn babies. HbA2 is found in small amounts in adults i.e. 1-3% while HbA and HbF are present in higher amount (Weather *et al.*, 2005). In iron deficiency anemia, HbA2 level becomes lower than normal range (Munice and Campbell, 2009).

2.3.2.2 ABNORMAL HEMOGLOBIN

Abnormal hemoglobin includes hemoglobin H (HbH) and hemoglobin Bart's (Hb Bart's). Hemoglobin H (HbH) contains four beta chains and hemoglobin Bart's (Hb Bart's) contains four gamma chains. Hb Bart's present in alpha thalassemia infants which causes non immune hydropsfetalis in utero, which is always fatal. HbH present in anemic infant that causes hemolysis and severe anemia (Munice and Campbell, 2009).

2.4 Anemia

Anemia is a blood disease in which the amount of hemoglobin or erythrocytes are abnormally decrease in the blood. It is also called microcyctic anemia. This disease also disrupts the structure of erythrocytes (Xing *et al.*, 2011). In anemia, patient erythrocytes are smaller in numbers as compared to healthy persons (Urrechega *et al.*, 2011; Zhang *et al.*, 2012). There are five main problems which are caused by microcytic anemia, i.e, Thalassemia, Iron deficiency

Figure 2.3 Structure of normal and abnormal hemoglobin (Munice and Campbell, 2009)

anemia, Anemia of chornic disease, lead poisoning and congential sideroblastic anemia. Three causes of anemia are common in all over the world i.e Thalassemia, Iron deficiency anemia and anemia of chornic disease (ACD) (Ford, 2013).

2.5 THALASSEMIA

The word thalassemia is derived from two Greek words: **thalassa** means "Sea" and **haima** means "Blood" (Munice and Campbell, 2009; Galanello and Origa, 2010). Thalassemia is a group of inherited autosomal recessive hematologic disorder (Munice and Campbell, 2009). Thalassemia is one of the most common inherited disorders which cause by variant or missing gene that affects the hemoglobin synthesis (Weatherall *et al.*, 2010).

2.5.1 Classification of thalassemia

It is a genetic based group of diseases in which synthesis of hemoglobin is irregular which can lead to chornic anemia (Wentrup-Byrne *et al.*, 1997). It can increase in erythropoiesis and absorption of iron from the diet which can lead to iron overload (Wentrup-Byrne *et al.*, 1997). Anemia in the human thalasemia is caused by a combination of ineffective erythropoiesis (intramedullary hemolysis) and hemolysis of adults RBCs in the peripheral blood (Kuypers *et al.*, 1998). There are two types of thalassemia, i.e. Alpha thalassemia and Beta thalassemia, but in Pakistani population Beta thalassemia is most common (Munice and Campbell, 2009).

2.5.2 Alpha Thalassemia

Alpha thalassemia is heterogeneous autosomal recessive hereditary anemia which is characterized by reduced or absent alpha globin chain synthesis (Danjou *et al.*, 2011). Alpha globin chain production is organized by two genes on each chromosome 16. It is caused by the deletion of one or more genes at chromosome 16. The deletion in one of four genes results in a alpha thalassemia silent carrier, and is asymptomatic with normal hematologic findings. The deletion in two of four genes causes the alpha thalassemia trait with microcytosis and no anemia. The three gene deletion causes the production of hemoglobin H which results in microcytic anemia, hemolysis and splenomegaly. The deletion of four genes results in production of

hemoglobin Bart's (Hb Bart's) which cause the alpha thalassemia major which causes fatal hydropsfetalis (Munice and Campbell, 2009).

2.5.3 Beta Thalassemia

Beta thalassemia is a major form of thalassemia and it is a heterogeneous autosomal recessive hereditary anemia. It is characterized by reduced or absent beta globin chain synthesis resulting in reduced hemoglobin in erythrocytes, decreased red blood cell production and dysregulation of iron which results in iron overload (Weatherall *et al.*, 1998; Danjou *et al.*, 2011; Kuyper *et al.*, 1998). Beta globin synthesis is controlled by one gene on each chromosome 11. Beta thalassemia occurs from more than 200-point mutations and sometimes deletion of two genes. The beta thalassemia trait is caused one gene defect, and it is asymptomatics and the results give microcytosis and mild anemia. If two genes reduced or absent then the person has beta thalassemia major, also known as Cooley anemia. Beta thalassemia major symptoms start at the age of six month (Munice and Campbell, 2009).

2.5.3.1 TYPES OF BETA THALASSEMIA

There are three main forms of beta thalassemia.

- Thalassemia Minor (β-thalassemia trait, heterozygous thalassemia)
- Thalassemia Intermedia
- Thalassemia Major (Cooley's anemia or Mediterranean)

(Galanello and Origa, 2010).

2.5.3.1.1 Thalassemia Minor

It is the carrier state with the heterozygous condition which is usually asymptomatic and characterized through various hematological features. The person lives a normal life without any disease complication because only one of β globin alleles bears a mutation with an increase in the fraction of Haemoglobin A2 (>3.5%) and a decrease in the fraction of Haemoglobin A (<97.5%) (Lahiry *et al.*, 2008).

2.5.3.1.2 Thalassemia Intermediate

Thalassemia intermediate is a condition intermediate between the major and minor forms. Affected individuals can often manage a normal life but may need occasional transfusions. In this type there is a reduced synthesis of β-globin chain. Intermediate form has a wide variety of genotype. The genotype may be homozygous as well as heterozygous. The heterozygous thalassemia is often asymptomatic (Cao and Galanello, 2010). The Symptoms usually develop during the age of 2-6 years and they do not need the regular blood transfusion for survival. The consequences of thalassemia intermediate are characteristic deformities of the bone and face, osteoporosis with pathologic fractures of long bones and the formation of erythropoietic masses that primarily affect the spleen, liver, lymph nodes, chest and spine. Extra medullary erythropoiesis may cause neurological disorders and may also develop gallstones due to ineffective erythropoiesis (Galanello, 2001).

2.5.3.1.3 Thalassemia Major

Beta thalassemia major (also known as Cooley's anemia or Mediterranean anemia) is a severe type of thalassemia. It requires frequent blood transfusions. It is a homozygous or compound heterozygous state with severe hematological features. When fetal RBC's cannot be replaced with normal cells which contains HbA ($\alpha_2\beta_2$) then the β globin synthesis becomes apparent. The major switch from HbF to HbA was during the first year of life. Infants affected with thalassemia major may develop the symptoms like paleness, feeding problems, diarrhea, irritability, enlargement of the abdomen. They have severe anemia associated with low RBCs, low MCV and MCH count. The Hb pattern varies according to the type of β-thalassemia. In beta thalassemia HBA is absent, HbF is 95%-98% and HbA2 is 2%-3%. It is diagnosed at the age of 6-24 months after birth (Thalassemia International Federation 2008). If regular transfusion which are given to the patient that maintain the minimum hemoglobin concentration of 9-10.5g/dl, the children shows the normal growth up to 10-12 years. Complication due to transfusions may occur like iron overload which further include mental retardation, the delay of sexual maturation, etc. Later on it can also damage the organs of the body like liver, heart, kidney etc. which can cause dilated myocardiopathy, diabetes, HIV, HCV and osteoporosis (Borgna-pignatti *et al.*, 2004).

2.5.4 PREVALANCE OF THALASSEMIA

Thalassemia is a major public health problem and 1.7% of the world population carrying thalassemic genes (Zhang *et al.*, 2012). It is identified as the most widespread inherited blood disorder in the world by the World Health Organization (WHO). It is found in more than 60 countries with a carrier population of up to 150 million (Cao and Galanello, 2002). β-Thalassemia has been faced periodically in almost every racial group but β-thalassemia is predominant in the Middle East, Mediterranean countries, Central Asia, Southern China, India and the Far East. In SouthEast Asia, the prevalence of thalassemia can be particularly high (Wentrup-Byrne *et al.*, 1997). Pakistan has the topmost number of children with transfusion dependent thalassemia in the world due to the high rate of cousin marriages, high birth rate, and large population size (Alwan and Modell, 1997). Every year about 9000 children with beta thalassemia are born, predicted, although no documentary registry is available in Pakistan. The expected carrier rate is 5-7%, which accounts as 9.8 million carriers in the total population. The cultural and religious scenario in Pakistan is such that consanguineous marriages are quite common (Arif *et al.*, 2008). B-thalassemia was dominant in Punjabis (60.7%) followed by Saraikis (25.5%). Cast wise, it was most frequent in Rajputs followed by Jatts, Arian, Sheikhs and Pathans (Hafeez *et al.*, 2007). Very high (>81%) consanguinity and low literacy rate are risk factors for the high incidence of β-thalassemia in South Punjab (Baig *et al.*, 2005).

2.5.5 Risk Factors

The common risk factors of thalassemia are cousin marriages, infections, migration of people from one area to the other area of the world. As children are more susceptible for thalassemia major. At the time of birth they are normal but with the passage of time they show the symptoms of severe anemia. Consanguinity or cousin marriages are most important risk factors or both parents with heterozygous condition or carrier state have 100% chances of having a thalassemic child (Munice and Campbell, 2009; Cao and Galanello, 2010).

2.5.5.1 Consanguinity

Consanguinity plays an important role in developing thalassemia. This disease passes through generations after generations in autosomal recessive manner hence there are more chance of getting beta thalassemia major in consanguine marriages.

2.5.5.2 Family history of thalassemia

Thalassemia is a hereditary disorder which is passed from parents to children through mutated haemoglobin genes. So if in a family cases of beta thalassemia are present, then there are more chances that the children born in that family are thalassemic.

2.5.5.3 Comorbidities

Patients suffering from thalassemia are at the risk of heart failure, iron overload, splenomegaly, diabetes etc.

2.5.5.4 Population Migration

Due the migration of individuals from one part of the world to the other, beta thalassemia spread in the world. This is the major risk factor as it is more common in the Mediterranean region so people migrated to Europe and introduced this disease there (Cao and Galanello, 2010).

2.5.6 Treatments of Thalassemia

Alpha thalassemia patient does not have any symptoms. So there is no need of any type of treatment. Beta thalassemia is a serious inherited disease and has many symptoms which have different types of treatment. The treatment of this disease includes bone marrow transplant, blood transfusion, chelation, etc (Munice and Campbell, 2009).

2.5.6.1 Blood transfusion

The blood transfusion will be necessary depending upon the type and severity of the clinical condition. Beta thalassemia patient requires periodic and long life blood transfusion for maintaining the hemoglobin level higher than 9.5g per dL (95g per L) and sustain normal

growth. The need of blood transfusion may start at six month of age (Munice and Campbell, 2009).Transfusions are mostly given after 2-3 weeks (Cao and Galanello, 2010).

2.5.6.2 Chelation

The patients which are depending on the blood transfusion have developed iron overload. They have no physiologic process to remove excess iron from multiple transfusions. They require treatment with an iron chelator that starts between five and eight years of age (Muncie and Campbell, 2009). The chelation therapy will start when the concentration of serum ferritin exceeds 300μg/L (Cao and Galanello, 2010). Deferoxamine (Desferal) has been the treatment of choice. It is nontoxic and expensive. An alternative treatment of this is oral deferasirox (Exjade) which had been approved by the U.S. Food and drug Administration. The effects of deferasirox were transient and gastrointestinal in nature (Munice and Campbell, 2009).

2.5.6.3 Bone Marrow Transplantation

Bone marrow transplantation is an only curative therapy of beta thalassemia in childhood. Hematopoietic stem cell transplantation usually results in an excellent product in low risk persons which are defined as those with no hepatomegaly, no portal fibrosis by liver biopsy, and regular chelation therapy.

2.5.6.4 Preconception genetic counseling

Preconception genetic counseling is strongly recommended for all persons with thalassemia. Two parents, each with a beta thalassemia trait, have a one in four chance of conceiving a child with beta thalassemia major and a three in four chance the child will have thalassemia trait or be normal. Persons with alpha thalassemia trait have a more complex pattern of inheritance. Whether both defective genes are on the same or different chromosomes will alter the outcome.Chorionic villus sampling using polymerase chain reaction technology to detect point mutations or deletions can identify infants affected with beta thalassemia. Persons with alpha thalassemia trait should consider prenatal diagnosis because Hb Bart's increases the risk of toxemia and postpartum bleeding. Preimplantation genetic diagnosis is becoming available in conjunction with in vitro fertilization (Munice and Campbell, 2009).

2.6 IRON DEFICIENCY ANEMIA

Iron deficiency anemia is one of the most common nutritional deficiencies which can lead to the frequent disability and death (Zhang *et al.*, 2012). The insufficient iron intake, menstrual loss in women of the child bearing age, or chronic blood loss in the gastro-intestinal tract which may results iron deficiency anemia. It may change in iron which is present in the human body. These stages include the three phases i.e. iron depletion phase, iron erythropoiesis and iron deficiency anemia (Urrechaga *et al.*, 2011). In iron deficiency anemia the level of iron is low than normal and also has a very low amount of hemoglobin i.e. less than 120g/l (Al-Quaiz, 2001). The morphological properties of erythrocytes are altered in the patients of iron deficiency anemia. The shapes of erythrocytes are deformed and irregular (Zhang *et al.*, 2012).

Iron is a component of proteins which is essential for crucial cellular processes. The roles of iron containing proteins are in oxygen transport, ATP production, DNA synthesis and other physiological processes (Tapiero *et al.*, 2001).

2.6.1 Physiology

The primary site of regulation of iron absorption is in the small intestine (Tapiero *et al.*, 2001; Zimmermann and Hurrell, 2007). The diets of human contain both heme and non heme iron. Each form of iron has specific transporters. The heme iron transporter (HCP1) is upregulated by hypoxia and iron deficiency and might also transport folate as shown in figure 2.4. The non heme iron transporter comes from the intestinal lumen into the enterocytes and mediated by the divalent metal ion transporter 1 (DMT1). DMT1 only transport ferrous iron (Zimmermann and Hurrell, 2007). The enterocyte is a highly specialized, polarized and absorptive cell which is found in the intestinal villus. It controls the dietary iron into the body. In iron deficiency, the iron which is absorbed from non heme iron can increase than heme iron. Once iron comes inside the enterocyte then it transports across the apical membrane, intracellular translocation across the cytosol, then effluence of iron across the basolateral membrane and then comes into circulation (blood). The movement of iron involves transport proteins when it enters into the enterocyte. The transport proteins are ferroportin 1, iron oxidase and hephaestin (Tapiero *et al.*, 2001; Zimmermann and Hurrell, 2007).

2.6.1.1 Ferroportin 1

It moderates the export of iron from other cells. Iron deficiency and hypoxia stimulate duodenal expression of DMT1 and ferroportin which then increase iron absorption.

2.6.1.2 Hepcidin

It is a regulatory hormone which is secreted by the liver. It inhibits the absorption and release of iron from the macrophages and other cells. Hepcidin bind to ferroportin 1 which then causes the internalization and degradation. The internalization and degradation processes can decrease the iron transfer into the blood. In iron deficiency, hepdicin release is decreased from the liver and then increasing iron absorption to the maximum (Zimmermann and Hurrell, 2007).

2.6.2 Prevalence of Iron deficiency anemia

It is difficult to estimate the true global prevalence of iron deficiency, and it is a global health issue. In 2001, according to different studies iron deficiency anemia affects above 2 billion people around the world. In the U.S., ~7.8 milion women and 700,000 children are iron deficient and ~3.3 million women and 240,000 children are facing iron deficiency anemia. In 2001, it is predicated that 50% of pregnant women and upto 80% in SouthAsia have iron deficiency anemia (Tapiero *et al.*, 2001). UNICEF predicted in 2003 that 40-50% of children under 5 years old in developing countries are iron deficient (Grant *et al.*, 2007). In 2007, according to WHO 39% of children less than 5 years, 48% children between 5 to 14 years, 42% of women and 52% of pregnant women are anemic in developing countries (Zimmermann and Hurrell, 2007). In 2016, India is also at increased risk of iron deficiency anemia. About 56% of adolescent girls are affected by iron deficiency anemia in 2016 (Ahankari *et al.*, 2016). In Pakistan, it is predicted that 80% of pregnant women were anemic and 20% were non-anemic in Lahore (Khan *et al.*, 2007). In 2008, the prevalence of anemia among ever-married women aged 15 to 44 is reported to be 26% in urban areas and 47% in rural areas. The prevalence of anemia among pregnant women living in Karachi ranging from 29% to 50% in 2008 (Ansari *et al.*, 2008).In 2012, the prevalence of iron deficiency anemia in children under five years of age is between 40–70% (Habib *et al.*, 2016). In 2015, 75% pregnant women were anemic and 25% nonanemic in Faisalabad (Anjum *et al.*, 2015).

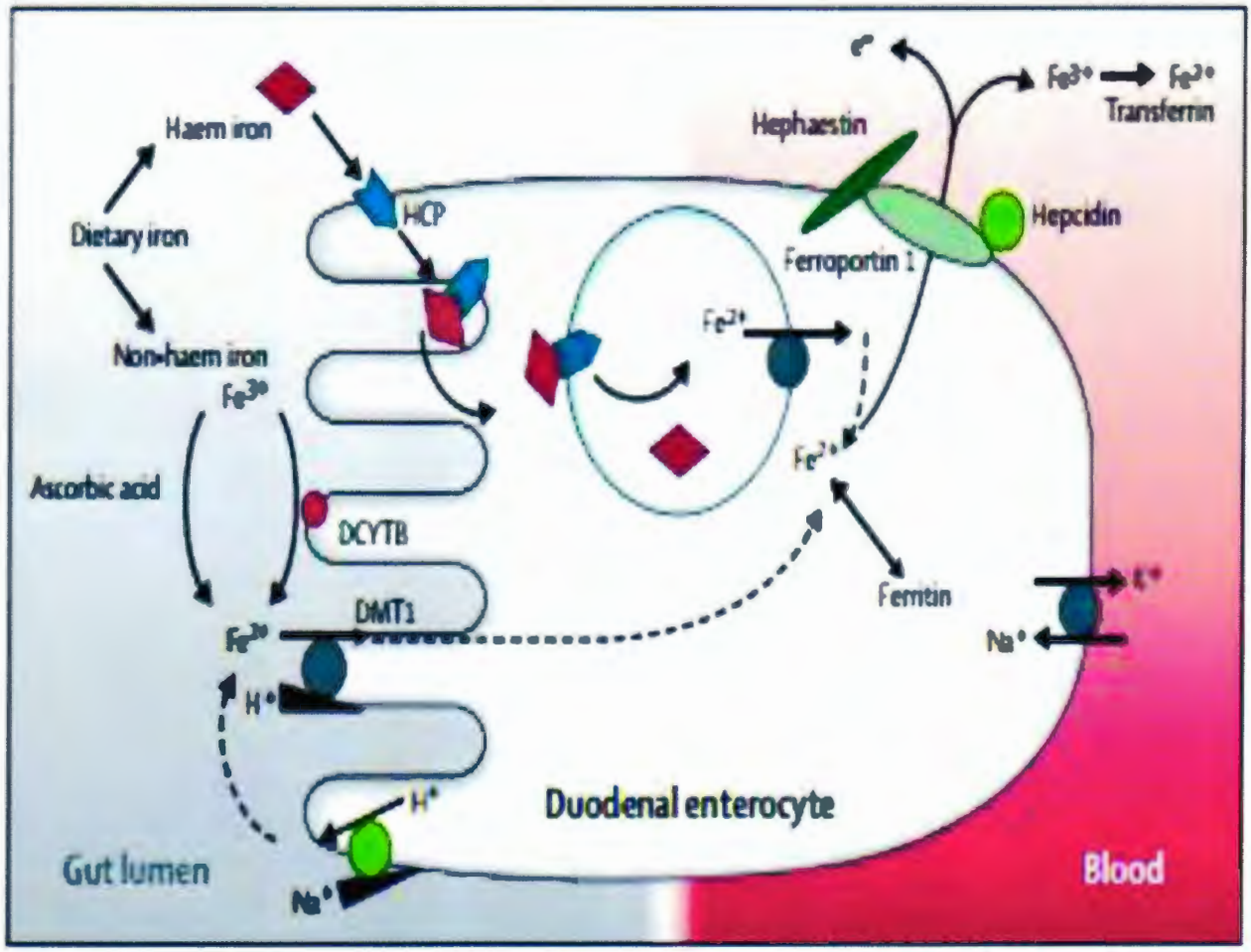

Figure 2.4 Regulation of Intestinal iron uptake (Zimmermann and Hurrell, 2007)

2.6.3 Risk Factors of Iron Deficiency Anemia

The frequency of risk factors of iron deficiency anemia is high in women and children than men. The menstrual blood losses may increase the risk of iron deficiency anemia. Normally, 1ml of blood loss and 0.5 mg iron loss from the body. During heavy menstrual blood loss i.e. >80 ml of blood loss from the body which can increase the risk of iron deficiency anemia. Another risk factor of iron deficiency anemia is pregnancy. During pregnancy, the iron requirement is about 1g. It means the requirement of iron increases the three fold because of expansion of maternal red cell mass i.e. from 350ml to 450ml and growth of the fetal-placental unit (Zimmermann and Hurrell, 2007; Percy *et al.*, 2016). The remaining risk factors are poor diet, bleeding after severe injury, gastrointestinal bleeding, and increased loss of blood and decreased intake of iron (Cook, 2004; Percy *et al.*, 2016).

2.6.4 Symptoms of iron deficiency anemia

The common symptoms of iron deficiency anemia include hair loss, weakness, fatigue, dizziness, headache, cold intolerance, restless legs, irritability, poor concentration and poor work performance (Percy *et al.*, 2016).

2.7 Difference between Iron Deficiency Anemia and Thalassemia

Thalassemia	**Iron Deficiency anemia**
A hereditary disorder	Non hereditary disorder
Iron is present in the bone marrow	Iron is absent in the bone marrow
Iron overload	Iron deficiency
Does not cure by iron therapy and iron supplements	Cure by iron therapy and iron supplements
Erythrocytes counts are high	Erythrocytes counts are low

2.8 Laboratory Diagnosis of Iron Deficiency Anemia and Thalassemia

There are many laboratory methods for identifying iron deficiency anemia and thalassemia. The laboratory measurements include hemoglobin measurement, transferring saturation test, mean corpuscular hemoglobin (MCH), serum ferritin, and serum transferring receptor, hemoglobin electrophoresis and red blood cell distribution width (RDW) (Cook, 2004; Munice and Campbell, 2009).

2.8.1 Serum Ferritin

The serum ferritin has been used for measuring the iron status (Cook, 2004).Serum ferritin is the best test for screening iron deficiency anemia (Munice and Campbell, 2009).

2.8.2 Mean Corpuscular Volume (MCV)

MCV is used to differentiate the thalassemia and iron deficiency anemia. The MCV is usually less than 75fl for thalassemia patients and is less than 80fl in iron deficiency anemia.

2.8.3 Red Blood Cell Distribution Width (RDW)

The RDW diagnosis is also for differentiating thalassemia and iron deficiency anemia. The RDW will be elevated in more than 90 percent of iron deficiency and only 50 percent of thalassemia patients (Munice and Campbell, 2009).

Iron deficiency anemia and thalassemia can be differentiating by various laboratory tests. These tests are time consuming and expensive. These tests do not differentiate at a very early level of disease. Because of the limitation of laboratory tests, research going in the field of biophotonics. Biophotonics provide very simple and easy technologies for infectious disease. It gives the microscopic view and the spectroscopic view of infectious diseases. Spectroscopy scans the sample at molecular level while microscopy scans samples for checking the pathophysicological changes. There are different types of microscopes such as Optical, Scanning Electron, Transmission Electron, Super Resolved Fluorescence Microscopy, Laser Scanning Confocal Microscopy (LSCM) and Atomic Force Microscopy (AFM). In the present study, Laser

Scanning Confocal Microscopy (LSCM) and Atomic Force Microscopy (AFM) are used for the detection of erythrocytes of blood.

2.9 LASER SCANNING CONFOCAL MICROSCOPE (LSCM)

In 1950s Maruin Minsky originally developed the confocal microscopy concept and patented in 1961. As by following the Minsky's work, M. David Egger and Mojmir Petran invented a multiple beam confocal microscopy in the late 1960s. In the 1970s Egger develops the scanned confocal laser microscopy and published the first recognizable images of cells in 1973. During the era of the late 1970s and 1980s computer and laser technology emerge to become the new algorithms for digital manipulation of images which can lead to confocal microscopy (Claxton *et al.*, 2006).

2.9.1 Principle of Laser Scanning Confocal Microscopy (LSCM)

A laser light is used to provide the light that passes through a pinhole aperture that is situated in conjugate plane with a scanning point on the sample. And a second pinhole aperture is placed in front of photomultipliers tube detector. The laser light is reflected by a dichormatic mirror which scans the light across the sample in a defined focal plane. The emitted light passes back through the dichromatic mirror and are focused as a confocal point at the pinhole aperture near the detector. The emission of light passed through the pinhole is measured by a photomultiplier tube detector. Then detector passed the signals to the computer and output can be displayed on a computer. A small fraction of the out of focus fluorescence emission is conveyed through the pinhole aperture near the photomultiplier tube and does not contribute to the resulting image (Claxton *et al.*, 2006: Paddock *et al.*, 2000).

2.9.2 Advantages of Laser Scanning Confocal Microscopy (LSCM)

The primary advantage of LSCM is the ability to produce thin optical sections through fluorescent sample having a thickness up to 100 micrometers (Claxton *et al.*, 2006). Confocal laser scanning microscopy (CLSM) is a non-invasive technique to generate images from cell or tissue samples by means of laser scanning on an optical platform. The images are obtained at a higher resolution with depth selectivity compared to conventional optical microscopy. The main function of CLSM is its ability for optical sectioning in which the images are reconstructed that

is based on the point-by-point scanning. The surface of the samples can be imaged. Overall, the quality of the images is greatly improved due to the unique sectioning method of the laser, and the information from the out-of-focus field is not superimposed on the image in focus (Zhang *et al.*, 2012).

The laser scanning confocal microscopy is a non-invasive method which enables the examination of both living and fixed sample under a variety of conditions with enhanced clarity. It is also useful to help to explain the interrelationships between structure and function of cells and tissues in biological examination. Advancement in laser scanning confocal microscopy have made multi-dimensional views of living cells and tissues which includes image information in x, y, and z dimensions. Another advantage of LSCM is that it has ability to adjust magnification electronically by varying the area scanned by the laser without having to change objectives (Claxton *et al.*, 2006).

2.9.3 Applications of Laser Scanning Confocal Microscopy (LSCM)

In biomedical sciences, confocal microscopy is used for imaging either fixed or living tissues which have been labeled with one or more fluorescent probes. It is important tool for many biomedical applications (Paddock *et al.*, 2000). It is a well-used microscopic tool which provides valuable morphological and functional information within cells and tissues (Zhang *et al.*, 2012). LSCM could give extensive information about localization or interaction of cytoskeletal elements in fixed and living cells. In bio-nanotechnology, laser scanning confocal microscopy is used to get detailed structural and spectroscopic information. And it is also used to study the biochemistry and functions pf protein as well (Meller *et al.*, 2005).

2.10 ATOMIC FORCE MICROSCOPY (AFM)

In 1985, Binnig got the idea for the atomic force microscopy. Then he talked about his ideas with Christoph Gerbes of IBM and Calvin Quate of Stanford. When they calculated the forces between atoms then they were surprised to find that they could easily make a cantilever with a spring constant weaker than the equivalent spring between atoms. After sometimes when Quate focus on this idea and he was able to build the first prototype. To working with first

prototype Binnig, Quate and Gerber started working with atomic force microscopy (Rugar and Hansma, 1990).

2.10.1 Construction of Atomic Force Microscopy (AFM)

AFM consists of a cantilever with a sharp tip which is used to scan the specimen surface. When the tip is placed near the sample surface, the force between the tip and the sample leads to a deflection of the cantilever (Fig. 2.6). Laser light is reflected off the back of the deflected cantilever and collected by photodiodes. This interaction between the probe tip and sample surface is then translated into an appropriate image (Jeong *et al.*, 2012).

2.10.2 Application of Atomic Force Microscopy (AFM) in Biomedical Science

Atomic Force Microscopy (AFM) is a powerful technique that allows for the non-invasive examination of specimens under natural conditions and with minimal preparation. This technique can be used for studying a variety of material in surface science, biochemistry and biology. AFM has an ability to acquire topographic information from the surface of a specimen in non-aqueous, aqueous or dry condition (Zhang *et al.*, 2012). It is used to investigate chromosomes (Hoshi and Ushiki, 2011), cell membranes (Suresh and Edwardson., 2010), proteins (Engel *et al.*, 2011), DNA (Di Bucchianico *et al.*, 2011), RNA structure (Heus *et al.*, 2011), nucleic acid–protein complexes (Miklaszewska *et al.*, 2004), ligand–receptor binding (Odorico *et al.*, 2007), carbohydrates (Lesoil *et al.*, 2010), lipids, living cells(Zhang *et al.*, 2012). AFM is different from other microscopy i.e conventional optical and electron microscopy because it is performed by sensing the interacting force between its probe tip and sample surface. Furthermore, it is important in defining the sample's physical property. An AFM force curve can be used to assess the rigidity and force of attraction between the cells living things as the tip moves to and from the sample surface. (Jeong *et al.*, 2012).

Differentiation between iron deficiency anemia and thalassemia microcytosis has important clinical implications which will likely aid in the reduction of morbidity and mortality in patients. These two diseases are typically distinguished by specific clinical symptoms or by various lab tests. For instance, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and RNA interference (RNAi) have been used to distinguish the two. Moreover, expensive chemical

analyses, such as the serum concentration of soluble transferrin receptor have been applied. However, these methods are expensive and time consuming (Zhang *et al.*, 2012).

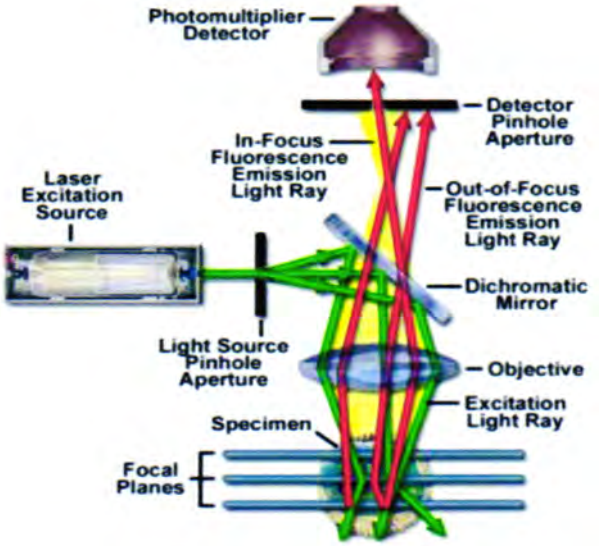

Figure 2.5 Schematic diagram of LSCM (Claxton *et al.*, 2006)

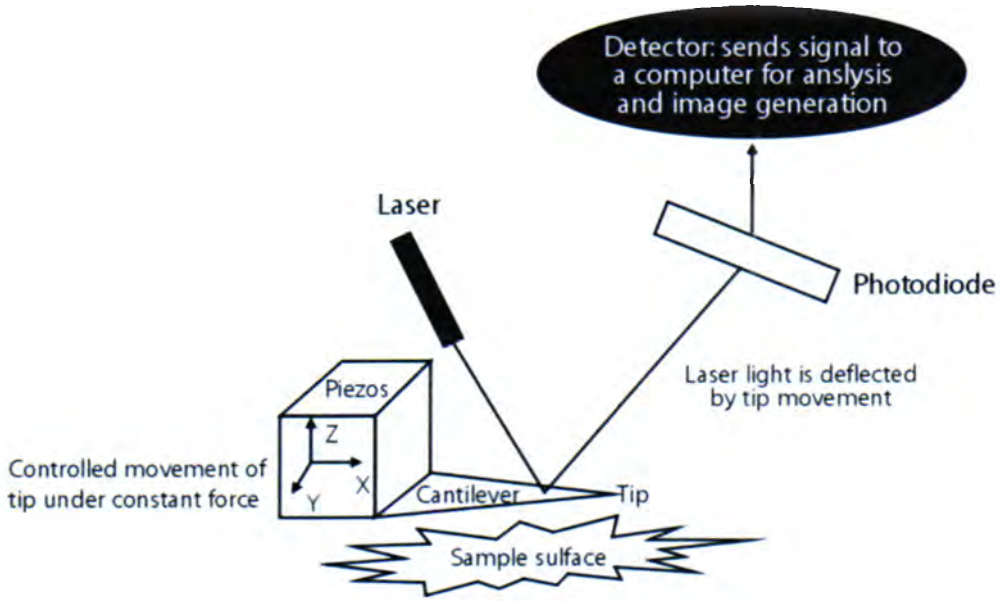

Figure 2.6 Atomic Force Microscopy Schematic Diagram (Jeong *et al.*, 2012)

CHAPTER 3

MATERIAL AND METHODS

MATERIAL AND METHODS

3.1 Study Approval

The current study approval was taken by the Ethical Review Committee of International Islamic University, Islamabad, from Pakistan Thalassemia Welfare Society (PTWS), Rawalpindi and Committee of National Institute of Laser and Optronics (NILOP), Islamabad before starting the research.

3.2 Blood Samples

The proper informed consent form was obtained from the patients before the start of research study. Fifty blood samples of thalassemia patients were collected from Pakistan Thalassemia Welfare Society (PTWS), fifty blood samples of iron deficiency anemia from different hosiptals of Rawalpindi and Islamabad and fifty blood samples of healthy volunteers were collected. These blood samples were collected with the help of the proper questionnaire which includes different parameters like name, age, sex, place, thalassemia start at what age, blood group, blood CP etc. The blood samples of 5cc were collected in EDTA filled vacutainers (HebeiXinle, Sci&tech Co.Ltd., China) by using disposable BD syringes. After collection, the blood samples had been transported to the National Institute of Laser and Optronics (NILOP) for preparation. The samples were stored at 4°C in the cooling system at International Islamic University, Islamabad.

3.3 Subjects and Protocols

The current study comprised three groups of the blood samples: Group 1 for iron deficiency anemia patients with confirmed clinical and pathological diagnosis from different hospitals, Group 2 for thalassemia patients with a confirmed clinical and pathological diagnosis from Pakistan Thalassemia Welfare Society (PTWS) and the group 3 includes an equal number of healthy volunteers which comprised of females and males having normal history.

3.4 Sample Preparation

Each blood sample was taken into two parts. One part was used for normal routine blood test and second part was used for that the cells were fixed with 2.5% paraformaldehyde and diluted in phosphate buffered saline solution containing pH 7.4. Then centrifuged at 3000rpms for 10mins and examined. Then an erythrocytes suspension was dropped on a glass cover slip and makes a very thin layer of suspension and then dried it. After that scan the suspension by using Atomic Force Microscopy (AFM) and Laser Scanning Confocal Microscopy (LSCM).

3.5 Materials

In the current study following materials were used:

- Blood samples from thalassemia patients
- Blood samples from iron deficiency anemia patients
- Blood samples from healthy people
- A laser scanning confocal microscopy (LS-510; Carls Zeiss, Germany)
- An atomic force microscopy (Alpha Contec, Germany)

3.6 Methods

3.6.1 Routine Blood Test

The Samples were sending to the diagnostic laboratory for routine blood test analysis and the results were collected for examining the disease after two days.

3.6.2 AFM measurement

The cells were fixed with 2.5% paraformaldehyde for all topographic images and imaged by an AFM (Alpha Contec, Germany) was used in the contact mode in air.

3.6.2.1 Radius, Diameter and Length Measurement

For all AFM experiments, silicon nitride tips (Alpha Contec Germany) were used. The radius of cantilever of tip is 7nm and a tip diameter is 14nm. The length of the cantilevers is 12.6μm,

thickness 3.52μm to 4.08μm, width is 30μ, to 31μm with the oscillation frequency of 287 kHz to 336 kHz and a force constant of 28N/m to 45N/m were used.

3.6.2.2 Usage of Optical Microscope

For the selection of the desired area, an optical microscope was used to help for selection and also direct the position of the AFM tip. Multiple cells imaging was performed for each condition.

3.6.2.3 Information of Topography of Cells

The images were analyzed for using the WSXM 4.0 Develop 12.1 software for gaining the information from the topography of the cells. The ratio of L/W is roughness analysis (RA). RA defines the average surface fluctuation of the erythrocyte. All of these paramaters were determined by using WSXM 4.0 Develop 12.1 software. The surface roughness value RA was determined for each sample. This value defined the mean value of the surface roughness in the area being identified. RA was determined by using WSXM 4.0 Develop 12.1 software.

3.6.3 LSCM Measurements

Fluorescence imaging was performed with a Laser Scanning Confocal Microscopy (LS-510; Carls Zeiss, Germany) which is equipped with argon ion lasers and helium neon lasers.

3.6.3.1 Laser Lines

The six laser lines are used for this purpose: four laser lines of argon ion laser and two laser lines of helium neon laser.

3.6.3.2 Argon Ion Laser Lines

The four argon ion laser lines are used. The laser lines are 458nm, 477nm, 488nm and 514nm.

3.6.3.3 Helium Neon Laser Lines

The two helium neon laser lines are used. The laser lines are 543nm and 633nm.

3.6.3.4 Resolution and Magnification

The resolution of LSCM is 200nm and the magnification of an objective lens of LSCM is 40X.

3.7 Statistical analysis

The demographic and clinical characteristics of patients like the name, age, sex, place, thalassemia start at what age, blood group, blood CP were also recorded. The mean and percentages were calculated for thalassemia and iron deficiency anemia and graphs were plotted for all the parameters.

CHAPTER 4

RESULTS

RESULTS

Thalassemia is one of the most common inherited disorders caused by variant or missing gene that affects the hemoglobin synthesis (Weatherall *et al.*, 2010). Due to a high rate of cousin marriages, high birth rate, and a large population size in Pakistan, thalassemia is the most widespread disease among children in Pakistan (Alwan and Modell, 1997).While Iron deficiency anemia is one of the most prevalent nutritional deficiency which leads to the frequent disability and death (Zhang *et al.*, 2012).

Erythrocytes are the simplest and smallest cells in the body (Sheeler *et al.*, 1997). They are the most abundant cells in the human body and have maximum mobility. RBCs are moving constantly throughout the human body and have a function of the oxygen carrier (Mukherjee *et al.*, 2015). The total volume of red blood cells is 90 to 95 cubic micrometers. The number of red blood cells per cubic millimeter is 5,200,000, in men while it is 4,700,000, in women. Life spans of red blood cells are 120 days (Guyton and Hall, 2006).

In this study, blood samples of thalassemia and iron deficiency anemia were analyzed through Atomic Force Microscopy (AFM) and Laser Scanning Confocal Microscopy (LSCM). The resolution and sensitivity of AFM used were 6nm and 10A. The resolution and the magnification of Laser Scanning Confocal Microscopy used were 200nm and 4000X. The main focus was on morphological changes of erythrocytes at nanometer scale which differ in healthy, thalassemia and iron deficiency anemia samples in order to make a good comparison.

4.1 ROUTINE BLOOD TEST

Blood test analysis for iron deficiency anemia, thalassemia and healthy individuals exhibited that erythrocytes derived from patients suffering from iron deficiency anemia and thalassemia showed reductions in the number of red blood cells (RBCs), hemoglobin (Hb), MCV, MCH, mean corpuscular hemoglobin concentration (MCHC) in comparison with healthy control individuals. The normal value of red blood cells in normal human was $3.8\times10^{6}\pm5.8\times10^{6}\mu L$ while the RBCs value was lower in thalassemia and iron deficiency anemia. The normal range of MCV in human was 76±96fl while in thalassemia it was 65.8±89fl and in iron deficiency anemia 6.27±67.50fl. The value of MCV shows reduction in thalassemia and iron deficiency anemia.

Table 4.1 Characteristics of the routine blood test of iron deficiency anemia and thalassemia

	RBC($\times 10^6$)/μL	Hb g/dL	MCV fL	MCH pg	MCHC g/dL
Iron deficiency anemia	0.47±4.0	1.5±10.2	6.27±67.50	16.5±26	25.5±33
Thalassemia	1.11±4.13	2.7±10.9	65.8±89	20.0±28.9	27.9±35.0
Healthy	3.8-5.8	12.0±18.0	76-96	26-32	32-35

MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin) , Hb (Hemoglobin), MCHC (Mean corpuscular hemoglobin concentration), RBCs (Red Blood Cells)

Likewise MCH, MCHC Hb values also decrease in thalassemia and iron deficiency anemia. The characteristics of the routine blood test showed that both iron deficiency anemia and thalassemia were types of microcytic anemia which were shown in table 4.1 In fact, all of these values were lower than those recorded for the healthy group.

4.2 ATOMIC FORCE MICROSCOPY (AFM)

4.2.1 Healthy Samples

In the present study, AFM (Alpha Contec, Germany) is used to detect the topographic information and morphological properties of blood samples of healthy humans. For healthy samples, the overall profiles of healthy erythrocytes or RBCs have been checked. Figure 4.1 (a, b and c) shows that the healthy erythrocytes are uniform and a single cell has the dimension of about 2 μm (Figure 4.1 a). The erythrocytes shapes were circular and biconcave and a very little amount of erythrocytes show irregular morphology. Figure 4.1 (d) shows the histogram of the healthy samples which depicts the particle size of the cell, i.e 8nm in size.

4.2.2 Thalassemia

The morphological properties and topographic information of thalassemia infected samples have been recorded by AFM (Alpha Contec, Germany). Figure 4.2 (a, b and c) shows the shape of erythrocytes in thalassemia patients. The shape of erythrocytes can be changed by squeezing through capillaries. The RBCs are anisocytosis and hypochromia in shape.

The results shows a little deformity in the shape of erythrocytes of thalassemia patient samples as shown in figure 4.2 (a,b and c). The shape of erythrocytes was crenated and other shapes but a very little amount of cells were biconcave disc which was shown in images. The images recoded by AFM also show that the cells were smaller in size and deformed with many large holes. These large holes show abnormal hemoglobin. Due to abnormal hemoglobin and large holes the shape of erythrocytes had been changed. The images also show that the center of cell surface is swelled i.e. the size of the center of a cell is more than 1 micrometer. Figure 4.2 (d) shows the histogram of the cell depicts thalassemic erythrocytes cell size is 20nm.

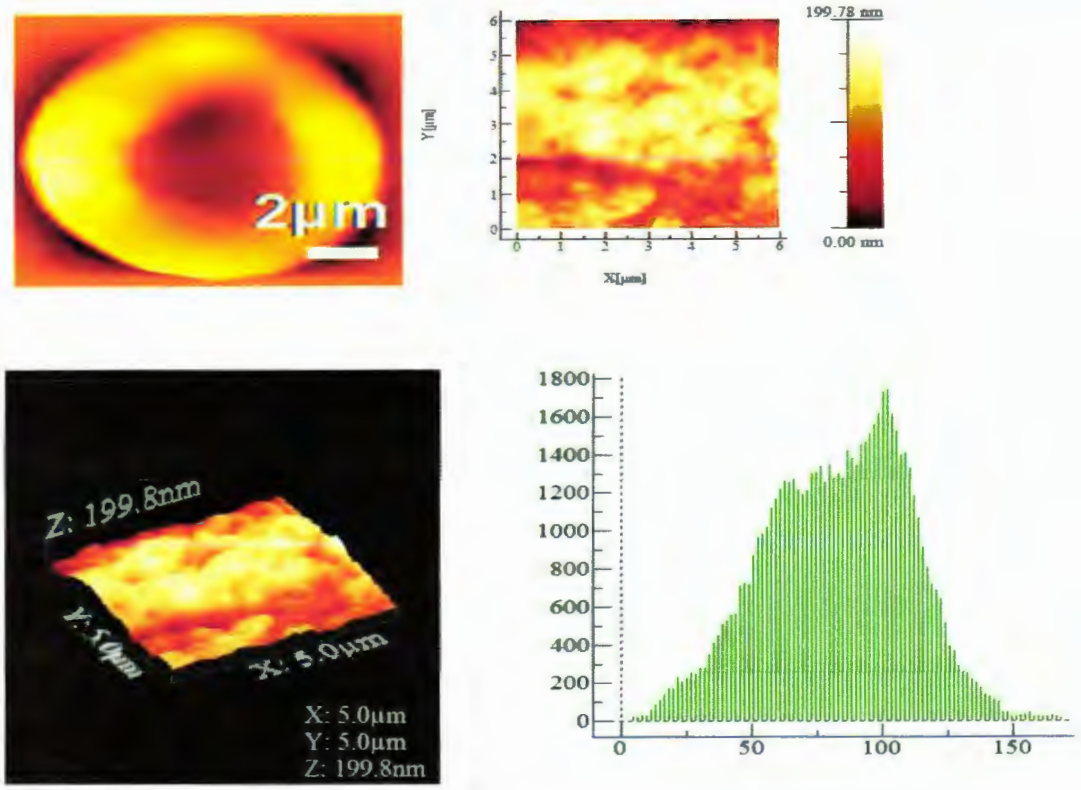

Figure 4.1 Atomic Force Microscopy

(a) Single Cell Image (b) 2-D view of healthy sample

(c) 3-D view of healthy sample (d) Histogram of healthy sample

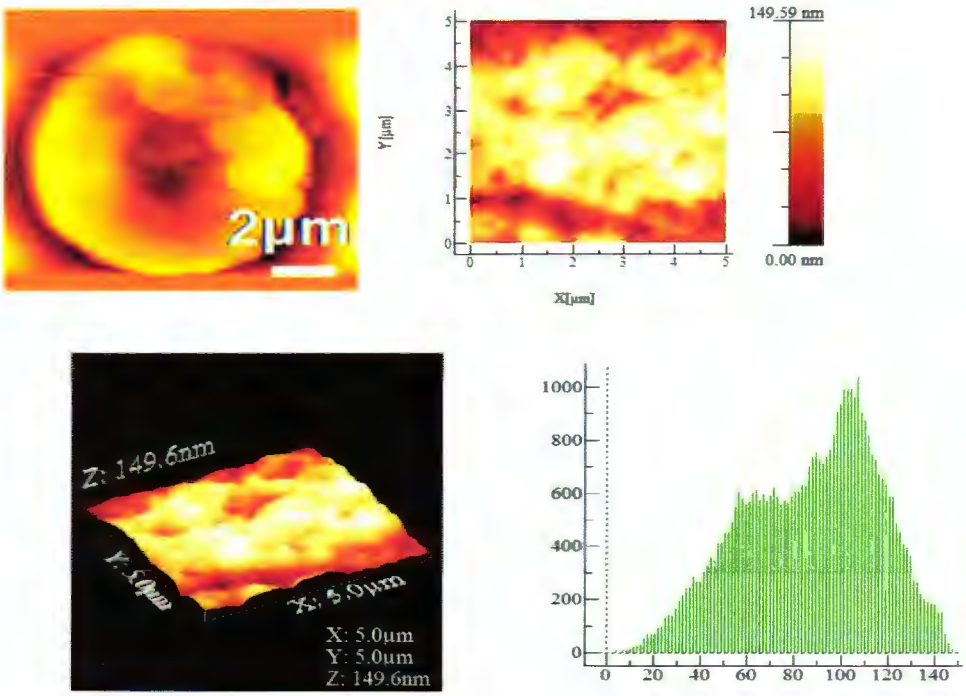

Figure 4.2 Atomic Force Microscopy

(a) Single Cell Image (b) 2-D view of thalassemia sample

(c) 3-D view of thalassemia sample (d) Histogram of thalassemia sample

The ultrastructure of thalassemia also shows a composite of membrane protein with a nanoscale network. In short, these cells had an abnormal hole on the cell surface, which make the cell rupture and appears flattened.

4.2.3 Iron Deficiency Anemia

The result of Atomic force Microscopy (AFM) gives topological information and morphological properties of the iron deficiency anemia patient. The erythrocytes of iron deficiency anemia show more deformed structure than thalassemia erythrocytes. Figure 4.3 (a) shows an irregular structure of a single cell. The figure 4.3 (b and c) shows oval shape, circular, elliptocytes and other shaped erythrocytes. The structures of cell surface were more deformed and had irregular shape. The center of cell surface was more swelled than thalassemia. Figure 4.3 (d) shows the histogram of iron deficiency anemia which depicts the size of the particle that how much it shrinks or squeeze in iron deficiency anemia. The IDA cell size was 140nm which shows erythrocytes of IDA were smaller in size as compared to thalassemia.

The membrane protein (hemoglobin) was reorganized into a stripe pattern in one direction which shows that it had been asymmetric and deformed structure of the erythrocytes. Actually anemic patient had abnormal hemoglobin which changes the shape of erythrocytes.

	Normal	Thalassemia	Iron Deficiency anemia
Shape	Circular, biconcave	Crenated	Elliptocytes, oval shaped
Size	8nm	20nm	140nm

4.3 LASER SCANNING CONFOCAL MICROSCOPY

4.3.1 Healthy Samples

The morphology of healthy RBCs were studied by Laser Scanning Confocal Microscopy (LSCM) (LS-510; Carls Zeiss, Germany) as shown in figure 4.4. LSCM allows us to visualize the cell surface properties at nanometer scale and also study the detailed structural information of the cell by using higher magnification. The main focus is on surface morphology of RBCs which depicts the shapes of erythrocytes was disc-shaped and most erythrocytes exhibited a typical

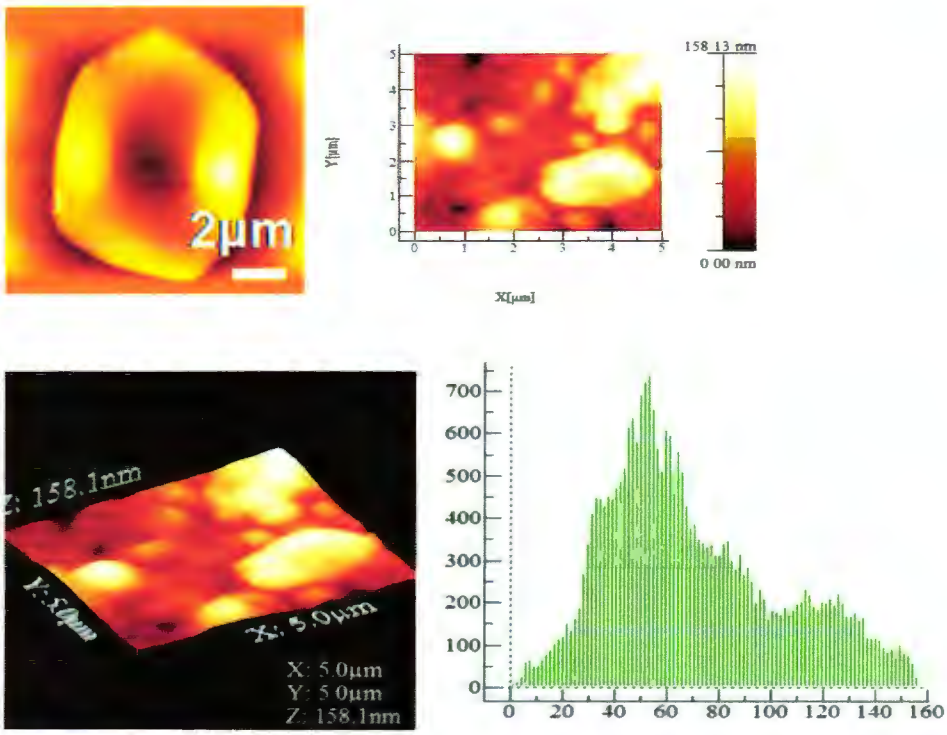

Figure 4.3 Atomic Force Microscopy

(a) Single Cell Image (b) 2-D view of iron deficiency anemia sample

(c) 3-D view of iron deficiency anemia sample (d) Histogram of iron deficiency anemia sample

circular and biconcave shape. Due to the normal hemoglobin, the healthy erythrocytes shapes were biconcave.

4.3.2 Thalassemia

The cell surface morphology of thalassemic RBCs was studied by Laser Scanning Confocal Microscopy (LSCM) as shown in figure 4.5. The image shows that the shapes of erythrocytes were smaller than healthy erythrocytes. It exhibited a typical crenated and had an irregular biconcave shape. The cell shapes of thalassemic erythrocytes were deformed, some were circular, crenated, anisocytosis, hypochromia and other shapes of RBCs were also formed. Due to abnormal hemoglobin the erythrocytes shape has been deformed as compared to healthy erythrocytes.

4.3.3 Iron deficiency anemia

The overall profiles of iron deficiency anemic RBCs were studied by Laser Scanning Confocal Microscopy (LSCM) as shown in figure 4.6. The shapes of erythrocytes were smaller as compared to thalassemia and healthy erythrocytes. The cell surface architecture had been more deformed than thalassemia erythrocytes. The cells were oval and typical elliptocytes shaped. Actually IDA cell shape changes by the binding of abnormal hemoglobin and erythrocytes shape had more deformed as compared to thalassemia and healthy erythrocytes.

	Normal	Thalassemia	Iron deficiency anemia
Shape	Circular, bioconcave and disc shaped	Crenated, anisocytosis and hypochormia	Elliptocytes and oval shaped
Volume	Normal range	Reduced than normal	More reduced than thalassemia

4.4 Statistical analysis of clinical and demographic parameters

The demographic and clinical characteristics of patients like age, sex, place, thalassemia start at what age, blood group, blood CP were also recorded. The mean and percentages were calculated for thalassemia and iron deficiency anemia and graphs were plotted. Figure 4.7 shows the

percentage distribution of thalassemia patients on the basis of gender. Samples contain 53%females and 47% males of thalassemia which shows that females are more prevalent as compared to males. Figure 4.8 shows the percentage distribution of iron deficiency anemia on the basis of gender. The samples contain 35%males and 65% females of iron deficiency anemia. It exhibited that females are more prevalent in IDA. The distribution of gender based age groups in thalassemia patients which indicate that more females were affected at the age group of 5-10years and more males were affected at the age group of 1-5years and 10-15years as shown in figure 4.9. Thalassemia is diagnosed before the age of 15 years. The distribution of gender based age groups in iron deficiency anemia patients which indicate that females were more anemic at the age group of 16-25yrs and 36-45yrs as shown in figure 4.10.

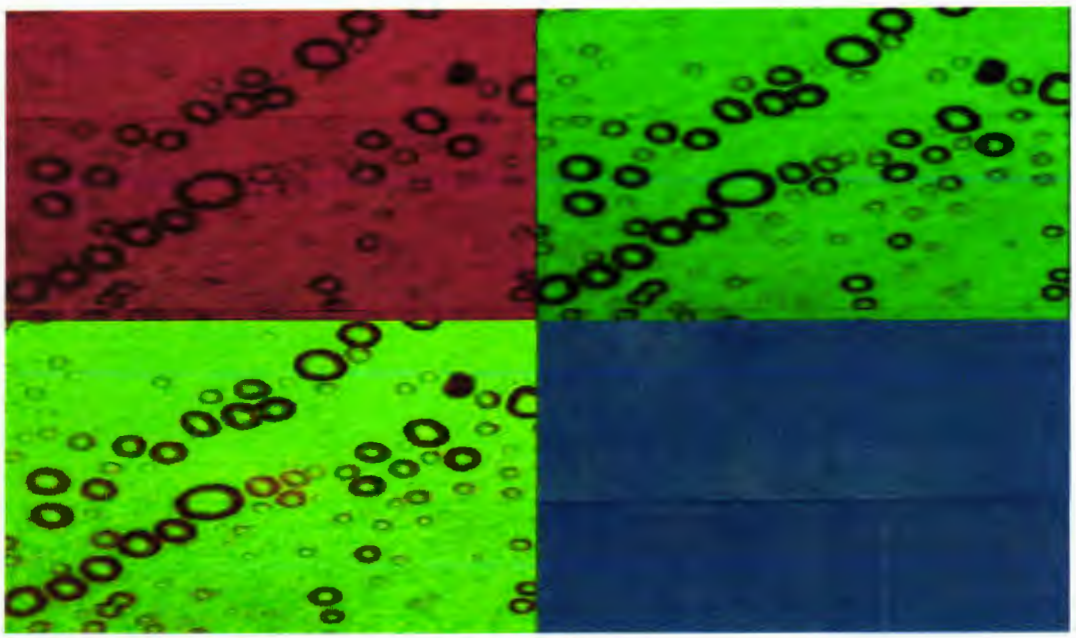

Figure 4.4 Laser scanning confocal microscopy images of the healthy RBCs

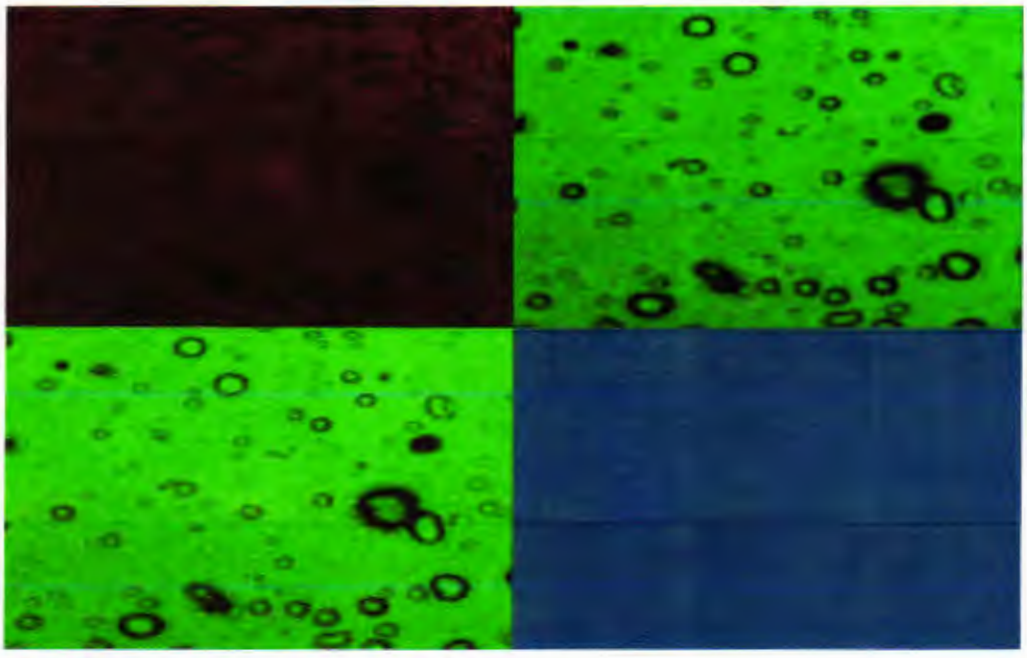

Figure 4.5 Laser Scanning Confocal microscopy images of the thalassemia RBCs.

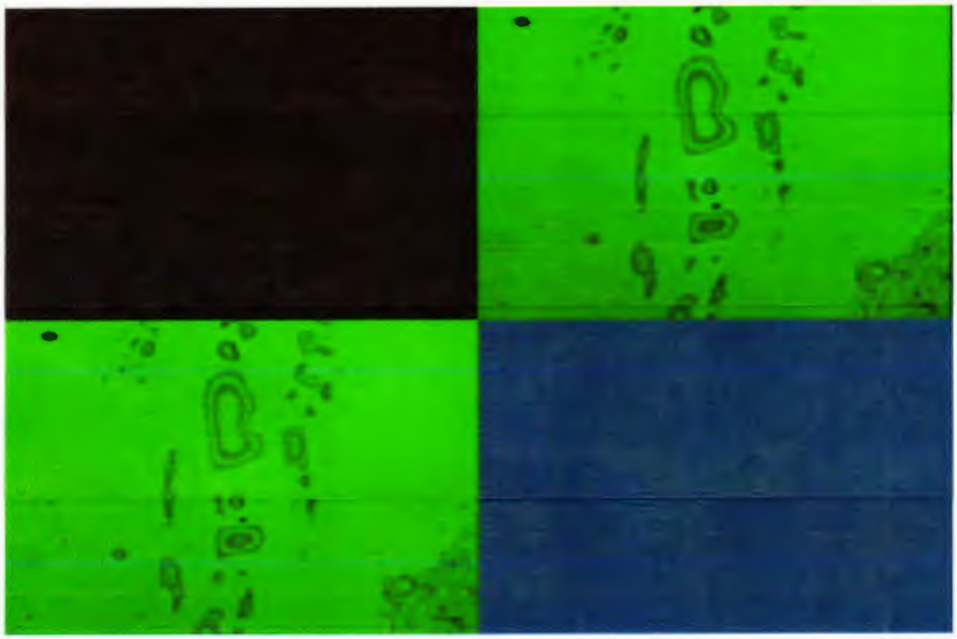

Figure 4.6 Laser Scanning Confocal microscopy image of the iron deficiency RBCs

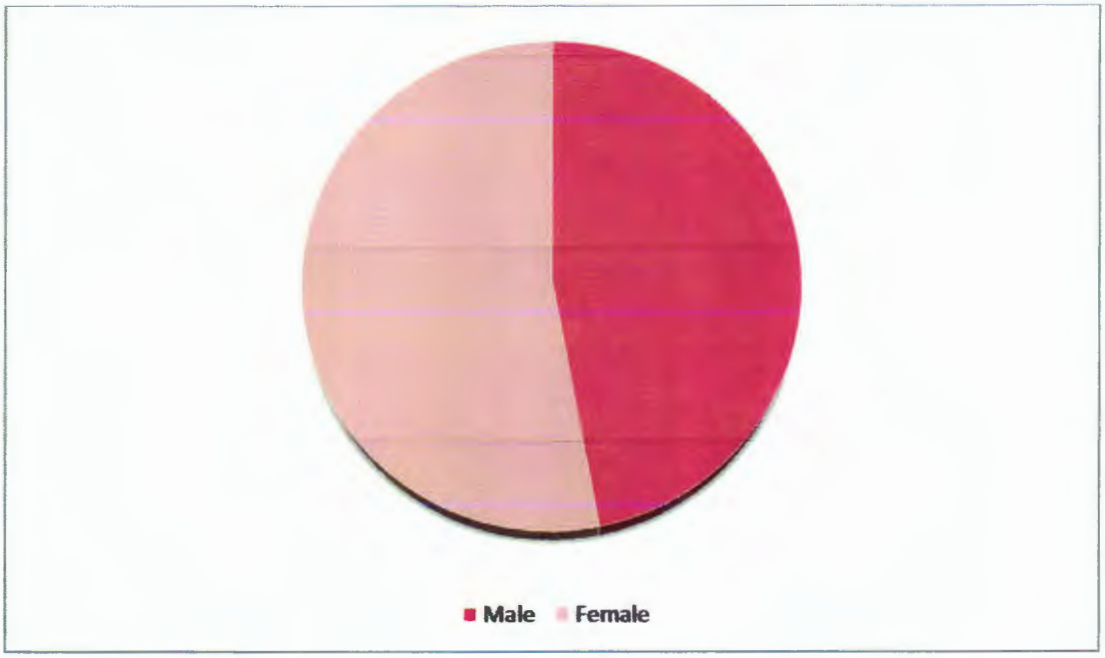

Figure 4.7 Percentage distributions of thalassemia patients on the basis of gender

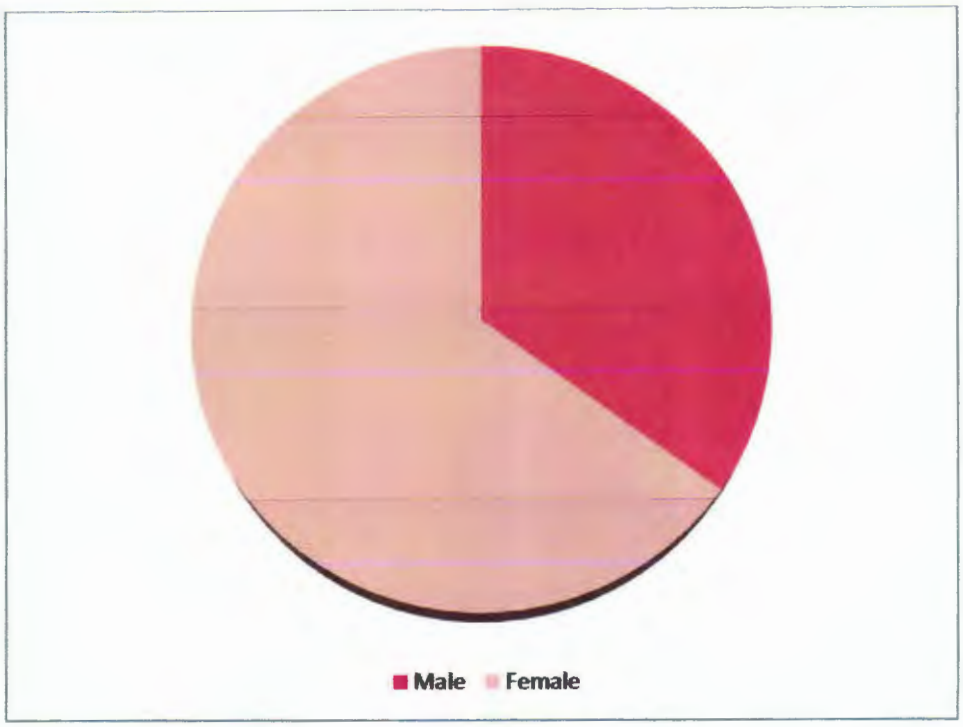

Figure 4.8 Percentage distributions of iron deficiency anemic patients on the basis of gender

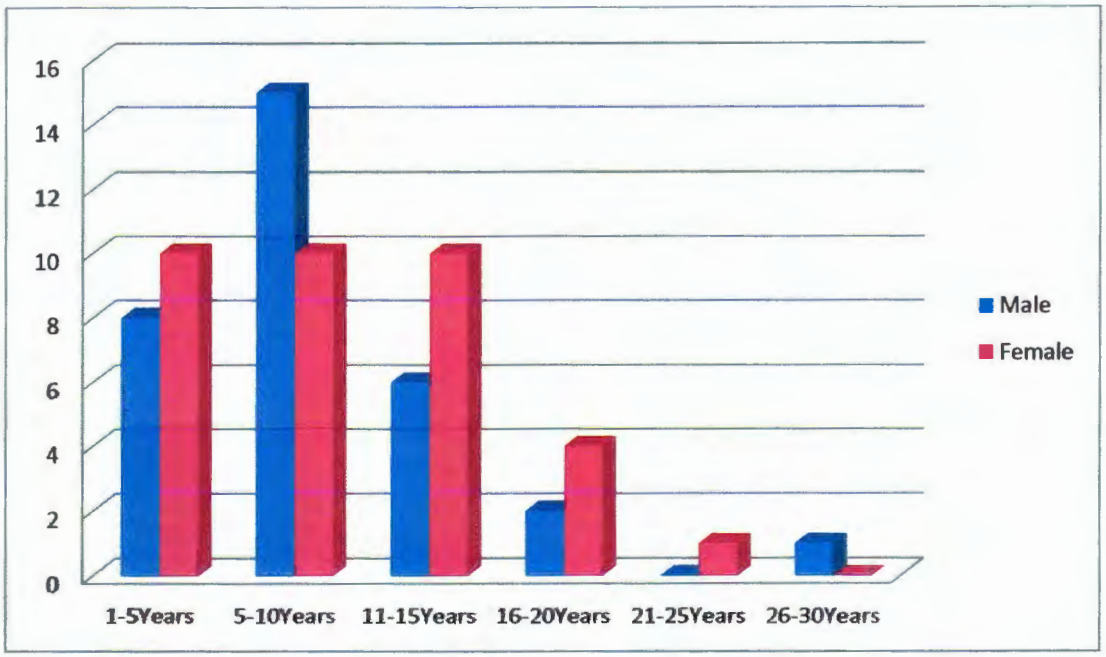

Figure 4.9 Distribution of gender based age groups in thalassemia patients

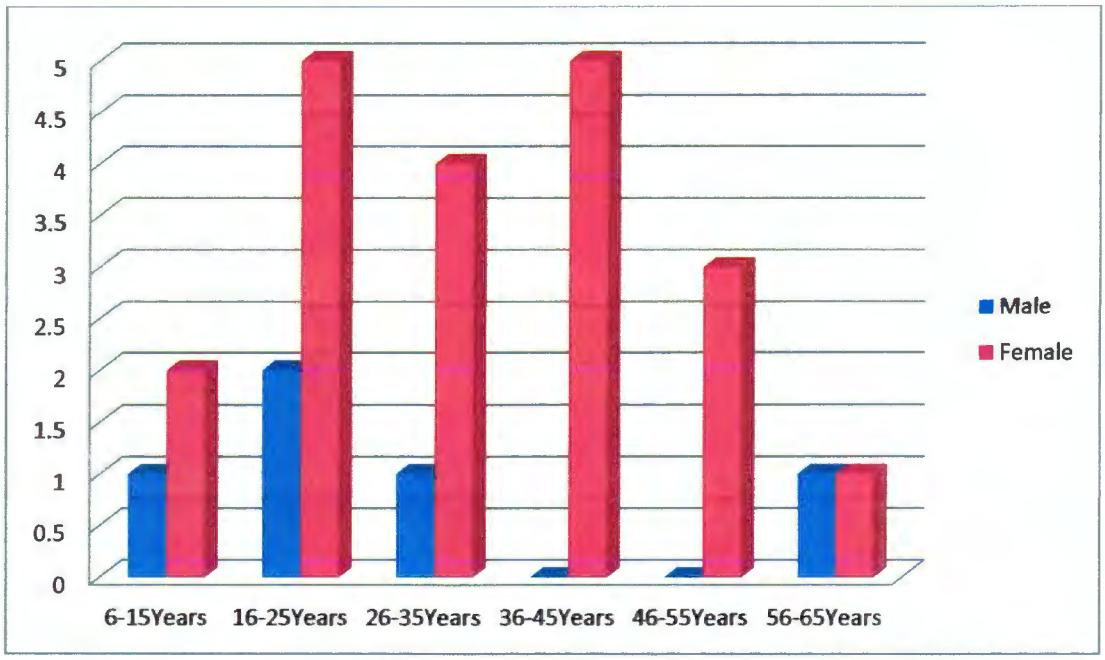

Figure 4.10 Distribution of gender based age groups in iron deficiency anemic patients

CHAPTER 5
DISCUSSION

DISCUSSION

According to World Health Organization (WHO), thalassemia is the widest spread monogenic and blood born disorder, found in more than 60 countries in the world with affectingupto150million people worldwide(Cao and Galanello, 2002).Annually, 5000 children are born with thalassemia in Pakistan (Saleem*et al.*, 1996). Multiple risk factors such as environmental and genetic are involved in disturbing the morphology of red blood cells.

Iron deficiencyanemia is the other most prevalent nutritional deficiency in the world, mostly affecting women and children (Zhang *et al.*, 2012; Tapiero*et al.*, 2001). In Pakistan, about 80%pregnant women are iron deficient, in which individual are deficient of iron as compared to normal range (Tapiero*et al.*, 2001).

Iron deficiency anemia and thalassemia has many clinical diagnosis which aids in the reduction of disease. These two diseases are categorized by specific clinical symptoms or by lab tests. Likewise mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), serum ferritin have been used for diagnosing and differentiating these two types of anemia. However, these methods are expensive, time consuming and cannot properly differentiate at a very early time of disease.Microscopy is one of the most important diagnostic/ characterization technique that allow scientists to study microorganisms, cells, crystalline structures and molecular structures.In this study AFM and LSCM are used which are much more sophisticated, and have higher magnifications. These technologies are not time consuming and can easily differentiate the iron deficiency anemia and thalassemia.

The main objective of the study was to make diagnosis of IDA and thalassemia through AFM and LSCM and to identify the morphological changes in the shape of RBCs at nanoscale level and compare with the healthy individuals. In this study, blood tests were evaluated for analysisofiron deficiency anemia, thalassemia and healthy control group..

The morphological properties of iron deficiency anemia and thalassemic erythrocytes differed from the healthy control erythrocytes. The structure of iron deficiency anemia

was deformed and the cells tended to swell or become oval and irregular shaped in the images of AFM. The cells morphology is very important in structure-function relationship and the size of cells is crucial for studying the detailed structure of anemic cells. By using AFM, the histogram depicts the cell size in iron deficiency anemia i.e. 140nm. The structure of thalassemic patient's cell was comparatively less deformed as compared to iron deficiency anemia in images obtained through AFM. The histogram depicts the size range of thalassemic patient cells as 20nm, which is significant for studying the detailed structure of cells. The structure of healthy control erythrocytes exhibited a biconcave disk shape with very little irregular morphology erythrocytes and size range 8nm in images of AFM.

According to Jin*et al.*, (2010) the changing occurs in the shape of cell and ultrastructure helps to understand the structure-function relationship. The ultrastructure of healthy erythrocytes shows a regular nanoscale network and represents a composite of plasma membrane. The ultrastructure of iron deficiency anemia erythrocytes was altered and exhibits some degree of aggregation in the membrane proteins. The ultrastructure of thalassemicerthrocytes had holes and cracked structure. According to Zhang *et al.*, (2012) the irregular morphology depicts the damagederythrocyte membrane. The histogram shows that the size of erythrocytes in healthy control group were 8nm whereas thalassemic erythrocytes were 20nm and iron deficiency erythrocytes were >140nm. The finding of erythrocytes size is nearly matched with the previous finding of Zhang *et al.*, (2012).

The morphological properties of iron deficiency anemic and thalassemic erythrocytes are also investigated through LSCM. The healthy erythrocytes were disc like and exhibited a typical circular and biconcave shape. Whereas the morphology of thalassemic erythrocytes exhibited a typical crenated and irregular biconcave shape and iron deficiency anemia erythrocytes were smaller, oval shaped and elliptocytes. It is a new technique that has the potential to improve the quality of research output. It gives detailed structural information of erythrocytes in healthy, thalassemia and iron deficiency anemia patients by using higher magnification.

In present study, total number of 50 thalassemia patients, 50 iron deficientanemia patient with equal number of age matched healthy controls were assessed for gender, age, sex, blood CP, blood group. According to the current study graphical representation total 90% of the patients have thalassemia at a very early age. Mostly thalassemia was found in females (53%) than in males (48%).Mostpatientsdiagnosed before the age of 10 years.According to Khan *et al.*, (2015)the proportion of male patients (56.9%) were higher than females (43.1%), and in one study from Turkey 56.7% maleshad higher rate ofthalassemia than 43.3% females (Rehman*et al.*, 2012). The results shows that mostly patient's parents were first cousins or close relatives. According to Arif*et al.*, (2008) the rate of consanguineous marriage were quite high which is 56.7% in the couples which were first cousins and 19.8% were relatives. Around 250 million individuals universally heterozygotes for β-thalassemia and at least 2,000,000 affected homozygotes are born yearly, whereasit is reported that 3% to 10% of the world's inhabitants carry a thalassemic gene (Gupta *et al.*, 2002;Premawardhena*et al.*, 2004). The carrier rate differs from state to state. Greece had a carrier rate of 6- 19%, Iran 4–5%, Cyprus 15-17%, Saudi Arabia 1-2% and Pakistan 1–8 % (Weatherall*et al.*, 2001;Khattak*et al.*, 1992).

According to present study, iron deficiency anemic females,especially in pregnant women (65%) presents higher ratio than males (35%). Mostly female patients are affected at the age group of 16 to 25 years and 36 to 45 years due to menstrual loss and pregnancy. This finding is nearly matched with the findings from earlier studies wherein 80% of the pregnant women were anemic at the age group of 15 to 44 in Pakistan (Khan *et al.*, 2007). It is also predicted that 26% urban area and 47% rural area women were anemic (Ansari *et al.*, 2008).

It is concluded that the clinical and pathologic results showed that IDA and Thalassemia is most prevalent in females. Thalassemia is most widespread in age group between 5-15 years whereas IDA is most prevalent between age group of 16-25 and 36-45years. Visualization of the cell surface properties at nanometer scale through AFM and LSCM clearly depict the morphological changes of erythrocytes in healthy, thalassemia and iron deficiency patients. The healthy erythrocytes shapes are circular and biconcave and a very little amount of erythrocytes show irregular morphology. While the shape of

thalassemia infected erythrocytes are crenated and of irregular shape when analyzed through AFM and LSCM. The erythrocytes of iron deficiency anemia show more deformed structure than thalassemia erythrocytes as depicted through results.

These techniques gave a new light into the structure-function relationship of erythrocytes with thalassemia and iron deficiency anemia. The results of Laser Scanning Confocal Microscopy (LSCM) and Atomic Force Microscopy (AFM) are quite convenient to distinguish between healthy and infected erythrocytes. The results are very much encouraging to employ these important techniques for diagnosis of diseases.

CHAPTER 6

REFERENCES

REFERENCES

Ahankari A.S., Myles P.R., Fogarty A.W., Dixit J.V., and Tata L.J., (2016) Prevalence of iron deficiency anemia and risk factors in 1010 adolescent girls from rural Maharashtra, India: a cross-sectional survey, *Public Health*, 1-8

Alwan A., and Modell B., (1997) Community control of genetics and congenital disorders.WHO regional office for the eastern Mediterranean.*Alexendria*, Egypt.

Al-Quaiz J.M., (2001) Iron deficiency anemia,*Saudi MedicalJornal*, 22(6): 490-496.

Anjum A., Manzoor M., Manzoor N., and Shakir H.A., (2015) Prevalence of anemia during pregnancy in district Faislabad, Pakistan, *PunjabUniversity Journal of Zoology*, 30(1): 15-20.

Ansari N.B., Badruddin S.H., Karmaliani R., Harris H., Jehan I., Pasha O., Moss N., McClure E.M., and Goldenberg R.L., (2008) Anemia prevalence and risk factors in pregnant women in an urban area of Pakistan, *Food and Nutrition Bulletin*, 29(2): 132-139.

Arif F., Fayyaz J., and Hamid A., (2008) Awareness among parents of children with thalassemia major,*Journal of the Pakistan Medical Association,* 58(11):621-624.

Baig M.S., Rabbi F., Hameed U., Qureshi J.A, Mahmood Z., Bokhari S.H., Kiani A., Hassan H., Baig J.M., Azhar A., and Zaman T., (2005) Molecular characterization of mutations causing beta thalassemia in Faislabad Pakistan using the amplification refractory mutation system (ARMS-PCR), *Indian Journal of Human Genetics*, 11(2): 80-83.

Borgna-pignatti C.,Rugolotto S., Stefano P.D., Zhoa H., Cappellini D.M., Vecchio G.C.D., Romeo M.A., Forni G.L., Gamberini M.R., Ghilardi R., Piga A., and Cnaan A., (2004) Survival and complications in patients with thalassemia major treated with transfusion and deferoxamine, *Haematologia*, 89:1187-1193.

Brelje T.C., Wessendorf M.W., and Sorenson R.L., (1993)Multicolor laser scanning confocal immunofluorescence microscopy: practical applications and limitations,*Methods in Cell Biology,*38:98-177.

Brismar H., TrepteO.,andUlfhake B., (1995) Spectra and fluorescence lifetimes of lissaminerhodaamine, tetramethylrhodamineisothiocyanate, texas red and cyanine 3.18 fluorophores- influences of some environmental factors recorded with a confocal laser scanning microscope,*Journal of Histochemistry and Cyto-chemistry.*, 43:699-707.

Cao A., and Galanello R., (2010) Beta thalassemia,*Genetics,*12(2):61-76.

Cao A., Galanello R., Monni G., and Rosatelli M.C., (2002) Screening for thalassemia: A model of success, *Obstetrics and Gynecology*, 29 (2): 305-328.

Claxton N.S., Fellers T.J., and Davidson M.W., (2006) Laser Scanning Confocal Microscopy,*Aptechnologies*,.

Cook J.D., (2005) Diagnosis and management of iron deficiency anemia,*Clinical haematology,* 18(2):319-322.

Cullander C., (1994) Imaging in the far-red with electronic light microscopy: requirements and limitations,*Journal of Microscopy.*,176:281-286.

Danjou F., Anni F., and Galanello R., (2011) Beta-thalassemia: from genotype to phenotype, *Haematologica*, 96(11):1573-1575.

Di Bucchianico S., Poma A.M., Giardi M.F., Di Leandro L., Valle F., Biscarini F., and Botti D., (2011) Atomic force microscope nanolithography on chromosomes to generate single-cell genetic probes,*Journal of Nanobiotechnology,*28: 9–27.

Dulinska I., Targoaz M., Strojny W., Lekka M., Czuba P., Balwierz W., and Szymonski M., (2006) Stiffness of normal and pathological erythrocytes studied by means of atomic force microscope,*Journal of Biochemical and Biophysical Methods,*31:1-11.

Engel A., (2011) Imaging and interrogating native membrane proteins using the atomic force microscope,*Methods in Molecular Biology.*,736: 153–167.

Ford J., (2013) Red blood cell morphology,*International Journal of Laboratory Hematology*,35:351-357.

Grant C.C., Wall C.R., Brewster D., Micholson R., Whitehall J., Super L., and Pitcher L., (2007) Policy statement on iron deficiency in pre-school aged children, *Journal of Paediatries and ChildHealth,*43:513-521.

Galanello R., and Origa R., (2010) Beta thalassemia,*OrphanetJournel of Rare diseases,*5(11): 1-15.

Galanello R., (2001) Iron chelation: New therapies,*Seminars in Hematology,* 38 (1): 73-76.

Gupta A., Hattori Y., and Agarwal S., (2002) Initiation codon mutation in an Asian Indian family, *American Journal of Hematology*, 71:134-136.

Guyton A.C., and Hall J.E.,(2006) Medical physicology.*Elsevier*, 11th Edition, pp 419-428.

Habib M.A., Black K., Soofi S.B., Hussain I., Bhatti Z., Bhutta Z.A., and Greenow C.R., (2016) Prevalence and Predicators of iron deficiency anemia in children under five years of age in Pakistan; A secondary analysis of National nutrition survey data 2011-2012, *PLOS one*, 10:1-13.

Hafeez M., Aslam M., Ali A., Rashid Y., and Jafri H., (2007) Regional and ethnic distribution of beta thalassemia mutations and effect of consanguinity in patients referred for prenatal diagnosis, *Journal of the College of Physicians and Surgeon Pak*istan, 17: 144-147.

Hansma H.G., Kim K.J., and Laney D.E., (1997) Properties of biomolecules measured from atomic force microscope images: A review,*Journal of structural biology,*119:99-108.

Heus H.A., Puchner E.M., van Vugt-Jonker A.J., Zimmermann J.L., and Gaub H.E.,(2011) Atomic force microscope-based single-molecule force spectroscopy of RNA unfolding,*Analytical Biochemistry*, 414: 1–6.

Hoshi O., and Ushiki T., (2011) Atomic force microscopy imaging of human metaphase chromosomes in liquid,*Methods in Molecular Biology.,*736:109–115.

Inoue S., and Spring K.S., (1997) Video microscopy: the fundamentals, *Plenum Press, New York*, 2nd edition.

Jeong K.H., and Lee S.H., (2012) A new technical approach to monitor the cellular physiology by atomic force microscopy,*Electrolyte Blood Press*, 10:7-11.

Jin H., Xing X., Zhao H., Chen Y., Huang X., Ma S., Ye H., and Cai J., (2010) Detection of erythrocytes influenced by aging and type 2 diabetes using atomic force microscope,*Biochemical and Biophysical Research Communication*, 391: 1698-1702.

Khattak M.F., and Saleem M., (1992) Prevalence of heterozygous B-thalassemia in Northern areas of Pakistan, *Journal of Pakistan Medical Association*, 42:32-36.

Kuypers F.A., Yuan J., Lewis R.A., Snyder L.M., Kiefer C.R., Bunyaratvej A., Fucharoen S., Ma L., Styles L., Jong K., and Schrier S.L., (1998) Membrane phospholipid asymmetry in human thalassemia,*Blood,*91(8):3044-3051.

Khan Z.M., Shoaib M. and Khalid M., (2007) Anemia during antenatal period; evaluation of different related parameters in pregnant women, *Professional Medical Journal,* 14(1): 1-6.

Khan M.S., Ahmed N., Khan R.A., Mushtaq N., and Shah M.W.U., (2015) Consanguinity ratio in beta thalassemia major patients in District Bannu, *Journal of Pakistan Medical Association*, 65(11): 1161-1163.

Lahiry P., Al-Attar S.A., and Hegele R.A., (2008) Understanding beta thalassemia with focus on the Indian Subcontinent and the Middle East, *Hematology*, 2: 5-13.

Lesoil C., Nonaka T., Sekiguchi H., Osada T., Miyata M., Afrin R.,and Ikai A., (2010) Molecular shape and binding force of Mycoplasma mobile's leg protein Gli349 revealed by an AFM study, *Biochemical and Biophysical Research Communication,*391:1312–1317.

Liu C., Liu X., Janes J., Stapley R., Patel R.P., Gladwin M.T., Daniel B., and Shapiro K., (2014) Mechanism of faster NO scavenging by older stored red blood cells, *Elsevier*, 2:211-219.

Munkherjee A., Barnett M.A., Venkatesh V., Verma S., and Sadler P.J., (2015) Human serum transferrin fibrils: Nanomineralisation in Bacteria and destruction of red blood cells, *ChemBioChem*, 16: 149-155.

Miklaszewska M., Targosz M., Pietrzyk J.A., Szymo´nski M., Rumian R., Krawentek L., and Sułowicz W., (2004) New measurement technics in biology and medicine: atomic force microscopy (part II),*Przegl.Lek,*61:126–133.

Muncie N.L., and Campbell J.S., (2009) Alpha and Beta Thalassemia,*American Family Physician,*80(4): 339-344.

Odorico M., Teulon J.M., Berthoumieu O., Chen S.W., Parot P., and Pellequer J.L, (2007) An integrated methodology for data processing in dynamic force spectroscopy of ligand–receptor binding, *Ultramicroscopy,*107:887–894.

Paddock S.W., (1999) Confocal Laser Scanning Microscopy,*Biotechniques,*27(5):992-1004.

Paddock S.W., (2000) Principles and practices of laser scanning confocal microscopy, *Molecular Biotechnology,* 16(2): 127-149.

Paddock S.W., (2001) Confocal Microscopy,*Methods in Molecular Biology,*1-12.

Percy L., Mansour D., and Fraser L., (2016) Iron deficiency and iron deficiency anemia in women's health, best practice and research,*Clinicial Obstetrics and Gynaecology*, 1-23.

Premawardhena A., Silva S., Arambepola M., Olivieri N., Merson L., Muraco J., Allen A., Fisher C., Peto C., Vichinsky E., and Weatherall D., (2004) Thalassemia in Srilanka: a progress report, *Human Molecular Genetics*, 13(2): 203-206

Rehman M.U., Bashirullah., Jan F., and Chisti T., (2011) Correlation of Hepatitis C with multiple blood transfusions in children of Thalassemia Major, *Pakistan Journal of Medical and Health Science*, 5(2): 323-326.

Rugar D., and Hansma P., (1990) Atomic force microscopy,*Physics Today*, 23-30.

Saleem M., Ahmad S., and Khattak M.F., (1996) An overview of thalassemia in Pakistan 4thintenational conference, Peshwar, *Pakistan Association of Pathologists* 76.

Schaller J., Gerber S., Kaempfer U., Lejon S., and Trachsel C., (2008) Human blood plasma proteins: structure and function, *John Wiley &sons,* 349-370.

Sheller P., (1996) Essential of Human Physicology, *Willam C Brown Publishers,* 2nd Edition, pp 189-214.

Suresh S., and Edwardson J.M., (2010) Phase separation in lipid bilayers triggered by low pH,*Biochemical and Biophysical Research Communication,*399:571–574.

Tapiero H., Gate L., and Tew K.D., (2001) Iron:deficiencies and requirements, *Biomedicine Pharmacotherapy,* 55: 324-332.

Telfer P., Coen P.G., Christou S., Hadjigavriel M., Kolnakou A., Pangalou E., Psiloines M., Simamonian K., Skordos G., Sitarou M., and Angastiniotis M., (2006) Survival of medically treated thalassemia patients in Crypus. Trends and risk factors over the period 1980-2004,*Haematologica,*91:1187-1192.

Urrechaga E., Borque L., and Escanero J.F., (2011) Erythrocyte and Reticulocyte parameters in iron deficiency and thalassemia,*Journal of Clinical Laboratory Analysis,*25:223-228.

Vander A.J., Sherman J.H., and Luciano D.S., (1990) Human physiology,*McGraw-Hill,* 5th edition, pp 349-425.

Weatherall D.J., Williams T.N., Allen S.J., and O'Donnell A., (2010)The population genetics and dynamics of the thalassemia, *Hematology/oncology clinics of North America*, 24(6):1021-1031.

Weatherall D.J., (2001) Phenotype-genotype relationships in monogenic disease: lessons from the thalassemias, *Nature Review Genetics*, 2: 245-255.

Weatherall D.J., (1998) Hemoglobin E beta thalassemia: An increasingly common disease with some diagnostics pitfalls,*The journal of pediatrics,*132(5): 765-767.

Weatherall D.J., William T.N., Maitland K., and Bowden D.K., (1996) Red blood cell phenotypes in the alpha thalassemias from early childhood to maturity, *British journal of haemotology*, 95: 266-272.

Weatherall M.W., Higgs D.R., Weiss H., Weatherall D.J., and Serjeant G.R.,(2005) Genotype/phenotype relationships in sickle cell disease: A pilot twin study, *Clinical Laboratory of Haematology*, 27: 384–390.

Winichagoon P., Thonglairoam V., Fucharoen S., Wilairat P., Fukumaki Y., and Wasi P., (1993) Severity differences in beta thalassemia/haemoglobin E syndromes:implications of genetic factors,*British Journal of Haematology,*83:633.

Wentrup-Byrne E., Anusorn W.C., Pierrre T.G.S., Webb J., Ramsay A., and Rintoul L., (1997) A Spectroscopic study of thalassemic gallstones,*John Wiley and Sons,* 97:1-8.

Xing X., Jin H., Lu Y., Wang Q., Pan Y., Cai J., and Wang H., (2011) Detection of erythrocytes in patient with elliptocytosis complicating ITP using atomic force microscopy,*micron*, 42:42-46.

Zeidan A., and Yelin D., (2015) Reflectance confocal microscopy of red blood cells: Simulation and experiment,*Biomedical Optics Express,*6(11):4335-4341.

Zhang Y., Zhang W., Wang S., Wang C., Xie J., Chen X., Xu Y., and Mao P., (2012) Detection of human erythrocytes influenced by iron deficiency anemia and thalassemia using atomic force microscope,*micron,*43:1287-1292.

Zimmermann M.B., and Hurrell R.F., (2007) Nutritional iron deficiency, *Lancet,* 370: 511-520

Annex 1

Diagnosis of Thalassemia Patients using Confocol and Atomic Force Microscopy

Informed: I am donating blood for research purpose only and not for commercial use. میں خون کا عطیہ صرف تحقیقی مقاصد کے لئے کر رہا ہوں.	
Signature:	**Date:**

Personal Information of Patient:

1. Name: _____
2. Age: _____
3. Sex: _____
4. Martial Status: _____
5. Weight: _____
6. Residence: _____
7. Ethnic Group: _____
8. Contact No. : _____

About thalassemia:

9. Disease diagnosed at what age: _____
10. Type of Thalassemia:
    - Minor
    - Intermediate
    - Major
11. Consanguineous marriage of parents Yes / No

12. Knowledge whether consanguinity has a role in thalassemia Yes/ No

13. Curability of disease Yes /No

14. Family history of thalassemia: Yes/No

15. Transfusion history: _____

16. Drug history: Yes/No

If Yes_____

17. Body Pain Yes/No

18. Fatigue Yes/No

19. Jaundice Yes/No

20. Fever Yes/No

21. Anaemic: Yes/No

22. Spleen Enlarged: Yes/No

If Yes then removed: Yes/No

23. Any other comorbidity: Yes/No

Blood CP:

24. Blood group: _____

25. Hb level: _____

26. Red cell count: _____

27. MCV: _____

28. MCH: _____

29. MCHC: _____

30. Platelet count: _____

31. Reticulocyte count: _____

DLC

32. Neutrophils _____

33. Lymphocytes _____

34. Moocytes _____

35. Eosinophils _____

RBC Morphology

36. Anisocytosis _____

37. Poikilocytes _____

38. Microcytes _____

39. Macrocytes _____

40. Hypochromia _____

Annex 2

Diagnosis of Iron Deficiency Anemia using Confocal and Atomic Force Microscopy

Informed: I am donating blood for research purpose only and not for commercial use. میں خون کا عطیہ صرف تحقیقی مقاصد کے لئے کر رہا ہوں.	
Signature:	**Date:**

Personal Information of Patient:

1. Name: _____

2. Age: _____

3. Sex: _____

4. Martial Status: _____

5. Weight: _____

6. Residence: _____

7. Ethnic Group: _____

8. Contact No. :_____

About Iron Deficiency Anemia:

9. Disease diagnosed at what age: _____

10. General fatigue: Yes/No

11. Weakness: Yes/No

12. Pale skin: Yes/No

13. Shortness of breath: Yes/No

14. Dizziness: Yes/No

15. Strange cravings for non-food items, such as dirt, ice, and clay: Yes/No

16. Tingling or a crawling feeling in the legs: Yes/No

17. Swelling or soreness in the tongue: Yes/No

18. Cold hands and feet: Yes/No

19. Fast or irregular heartbeat: Yes/No

20. Brittle nails: Yes/No

21. Headaches: Yes/No

Blood CP:

22. Blood group: _____

23. Hb level: _____

24. Red cell count: _____

25. White cell count:_____

26. MCV: _____

27. MCH: _____

28. MCHC: _____

29. Platelet count: _____

30. Reticulocyte count: _____

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