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Immunological assessment of β-thalassemic major children aged 5-12 years old attending Abd El-Aziz El-Rantisy Hospital in Gaza strip

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Dedication

To Allah's most sacred religion 'Islam", which urged us to seek knowledge on its first revelation
To my mother who spared no effort to help me wholeheartedly
To my father's and husband's souls
To my beloved sons Anas, Yasmin and Kawther
To my brothers
To my sister Fatema
To all my friends
Special dedication to all Thalassemics in Gaza Strip

To all of them I dedicate this work

Declaration

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

Signature	Name	Date
Samah	**Samah Fawzi Hamed Elhams**	**2010-10-2**

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Immunological assessment of β-thalassemic major children aged 5-12 years old attending Abd El-Aziz El-Rantisy Hospital in Gaza strip

Abstract

Background: Beta-thalassemia major patients suffer from many problems rather than severe anemia. Immune abnormalities have been suggested as a precipitating factor for the fourth most common cause of death in β-thalassemia. These abnormalities have been attributed both to the disease itself and to the applied therapeutic intervention.

Objective: to assess some immunological parameters in children aged 5-12 years old with β-Thalassemia major in Gaza Strip.

Materials and methods: this case-control study comprised 43 β-thalassemic major children aged 5-12 years old attending Abd El-Aziz El-Rantisy hospital and 43 healthy children served as controls. Cases and controls were matched for age and sex. Blood samples were collected by a well-trained nurse from thalassemic children just before a scheduled transfusion of packed red blood cells and also from controls. Complete blood count (CBC) was done in the same day of collection. Serum levels of interleukin-1-β (IL-1-β), interleukin-6 **(**IL-6) and tumor necrosis factor-α (TNF-α) cytokines, immunoglobulins IgG, IgA and IgM, complements C3,C4, ferritin and C-reactive protein(CRP) were determined.

Results: the average age of the study population was 7.9±2.2 years. Most patients 29 (67.4%) had allergic reactions. Discharge of desferrioxamine was intramuscular in 25 (58.1%) patients and subcutaneous in 17 (39.5%) patients, one patient (2.3%) refused iron chelation therapy. In general primary and secondary blood indices were significantly decreased in thalassemic children compared to controls. The total white blood cell (WBCs) and lymphocytes (LYMP) were significantly increased in patients compared to controls (8.9±2.1 V.s 7.9±2.0 $X10^3$cell/µl, p=0.025 and 3.6±0.9 V.s 3.1±0.79 $X10^3$cell/µl, p=0.003, respectively). WBCs and LYMP significantly decreased with allergic reactions (8.5±2.2 V.s 9.9±1.6 $X10^3$cell/µl, p=0.042 and 3.4±0.9 V.s 4.0±0.9 $X10^3$cell/µl, p=0.050, respectively). Ferritin level in patients was markedly

higher than in controls (3138.0±1041.5 V.s 17.3±2.5 ng/ml, p=0.000). Ferritin level showed positive association with age and allergic reaction. Complements 3 and 4 reduced significantly in thalessemic children (118.7±12.4 V.s 136.6 ±23.3 mg/dL, p=0.000 and 49.3±13.4 V.s 62.3±21.6 mg/dL, p=0.001, respectively), regardless of allergic reaction, age, ferritin level and CRP. IgM and IgA were within the normal levels compared to control. However lower level of IgG was found in patients. Higher levels of IgG, IgM and IgA were found with increasing age and in positive CRP patients ($p<0.05$). On the other hand, immunoglobulins neither had a relation with ferritin nor with allergic reactions. Cytokines were normal that they almost showed undetected levels in controls and patients (TNF-α: 56.1 and 97.7%, IL-6: 95% and 88.4%, and IL-I-β: 100% for both controls and patients). C-reactive protein status didn't differ significantly between controls and cases.

Keywords: β-thalassemia, Children, Gaza strip, Immunological assessment.

تقييم الحالة المناعية لدى أطفال مرضى البيتا ثلاسيميا العظمى من 5-12 سنة المسجلين في مستشفى عبد العزيز الرنتيسي في قطاع غزة

ملخص الدراسة

المقدمة: يعاني مرضى البيتا ثلاسيميا الكبرى إضافة إلى مخاطر الأنيميا العديد من المشكلات و تعد اختلالات المناعة العامل الرابع المسبب للوفاة، ويمكن إيعاز هذه الاختلالات المناعية لفعل المرض ذاته و لتأثير العلاج الإجرائي له.

الهدف: تقييم بعض المتغيرات المناعية لدى أطفال مرضى الثلاسيميا الذين تتراوح أعمارهم من 5 -12 سنة في قطاع غزة.

الطرق و الأدوات: هذه الدراسة تشمل 43 طفل ثلاسيميا كبرى تتراوح أعمارهم من 5-12 سنة مسجلين في مستشفى عبد العزيز الرنتيسي، يقابلهم 43 طفلا سليما كعينة ضابطة. عملية سحب الدم تمت بواسطة ممرضين ذوي كفاءة وكانت قبل القيام بنقل الدم للمرضى مباشرة ثم تم تحليل هذه العينة أولا للحصول على العدد الكلي لكريات الدم الحمراء إضافة إلى استخدام السيرم لفحص مستوى التيومر نكروسز فاكتور الفا، الانترلوكين-6 و الانترلوكين-1-بيتا، كذلك تم فحص الأجسام المضادة ج ، أ و م ، أيضا مستوى المكمل 3 و المكمل 4 ، و مستوى الفريتين و سي البروتين الفعال.

النتائج: كان متوسط أعمار العينة الدراسية 7.9±2.2 و كان المعظم يعاني من تفاعل تحسسي من نقل الدم 29 . (67.4%) استخدم هؤلاء المرضى ديسفيروكسامين للتخلص من الحديد الزائد وذلك بالحقن العضلي 25 (58.1%) أو عن طريق ضخه تحت الجلد في 17 (39.5%) من المرضى، أما بالنسبة لنتائج الفحوصات التي أجريت فقد وجد أن المؤشرات الأولية و الثانوية لفحص الدم كلها تقل بدلالة إحصائية واضحة. في المقابل وجد أن العدد الكلي لكريات الدم البيضاء و اللمفاوية ازداد عن المجموعة الضابطة زيادة ذات دلالة إحصائية كالتالي(8.9±2.1 مقابل 7.9±2.0، p=0.025 ، 3.6±0.9 مقابل 3.1±0.79، p=0.003) على التوالي لكن عند تحديد مستواها بالنسبة للحساسية وجد أنها تنقص بدلالة إحصائية واضحة مع الحساسية (8.5±2.2 مقابل 9.9±1.6 ، p=0.042، 3.4±0.9 مقابل 4.0±0.9، p=0.050) على التوالي و كما أوضحت النتائج نسبة الفريتين ازدادت زيادة هائلة عند المرضى (3138.0±1041.5 ng/ml مقابل 17.3±2.5، p=0.000) و هذه الزيادة كانت مضطردة مع و جود الحساسية و زيادة العمر، أما بالنسبة لمستوى المكمل 3 و المكمل 4 فيقل بفرق ذي دلالة إحصائية واضحة دون أن يتأثر بالحساسية، العمر، مستوى الفيريتين وسي البروتين الفعال كما وجد أن مستوى الجسم المضاد م و الجسم المضاد أ لدى المرضى يحاكي مستواه لدى الأطفال السليمين وبالمقابل كانت فحوصات الجسم المضاد ج لدى المرضى اقل منها لدى السليمين وهذا النقصان كان ذا دلالة إحصائية كما بينت النتائج أيضا أن مستوى هذه الأجسام المضادة الثلاثة سجل زيادة ذات دلالة إحصائية مع الزيادة في العمر و مع الحالات الموجبة للسي بروتين الفعال ($p<0.05$) لكنها لم تتأثر عند مقارنتها مع مستوى الفيريتين ولا مع الحساسية، أما بالنسبة للسيتوكين فكانت نتائجهم طبيعية حيث لم يتم رصدها في معظم المرضى و السليمين إلا في بعض الحالات

(TNF-α: 56.1% مقابل 97.7% ، IL-6 : 95% مقابل 88.4% ،IL-I-β : 100%) ولم تكن هناك أي فروقات إحصائية بين المرضى و المجموعة الضابطة بالنسبة لكشف سي البروتين الفعال.

الكلمات المفتاحية: البيتا الثلاسيميا العظمى، الأطفال، قطاع غزة، تقييم الحالة المناعية.

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Chapter 1
Introduction

1.1 Overview

Thalassemia is a hereditary anemia resulting from defects in hemoglobin production (Higgs *et al*., 2001). It results from quantitative reductions in globin chain synthesis, so it can be classified according to which globin chains is produced in reduced amount. Those with diminished β-globin chains are termed β -thalassemias, whereas those with decreased α-chain production are called α-thalassemias. Severity of clinical manifestations in these disorders relates to the amount of globin chain produced and the stability of residual chains present in excess. The thalassemia minor syndromes are characterized clinically by mild anemia with persistent microcytosis. Thalassemia intermedia is typified by a moderate, variably compensated hemolytic anemia that may present with clinical symptoms during a period of physiologic stress such as infection, pregnancy, or surgery. The thalassemia major syndromes produce severe, life-threatening anemia (Clarke and Higgins, 2000). β-thalassemia is the most common type of thalassemia major (Chik *et al*., 1998). It can be caused by homozygosity or compound heterozygosity for β-globin gene mutations *(HBB gene*). Most cases are inherited from parents who both have diseased alleles of the HBB gene (Chang *et al*., 2008).

Management of thalassemia major requires hypertransfusion therapy to maintain nearly normal hemoglobin levels and partially suppresses the increased, but ineffective, erythropoiesis (Giardina *et al*., 1998 and Cao and Galanello, 2010). Unfortunately, red blood cell transfusions are associated with alloimmunization, risk of exposure to infectious pathogens, and the accumulation of iron. So iron chelation therapy is critical to avoid iron toxicity (Grady, 2002 and Singer *et al*., 2000). Increased susceptibility to infectious disease is observed in persons with transfusion-dependent thalassemia and iron overload who experience increased exposure to pathogens and chronic immune stimulation (Cunningham-Rundles *et al*., 2000).

Infectious complications constitute the second most common cause of mortality and a main cause of morbidity in β-thalassemia (Vento *et al*., 2006), these factors and others such as splenectomy (Ahluwalia *et al*., 2000), zinc deficiency which is an immune regulator (Consolini *et al*., 2001) and the use of the chelating agent, deferoxamine (DFO) have profound effects on the immune system (Farmakis *et al*., 2003).

Immune abnormalities have been suggested as a precipitating factor for the fourth most common cause of death in β-thalassemia, i.e. malignancies, especially leukemia and lymphomas (Zurlo *et al*., 1989). Several studies on immune competence in β-thalassemia have revealed numerous quantitative and functional defects, involving T and B lymphocytes, increased immunoglobulin production particularly immunoglobulin G (IgG), immunoglobulin M (IgM) and immunoglobulin A (IgA), deficient activity of the complement system with reduced levels of complement 3 (C3) and complement 4 (C4), decreased opzonization, changing the pattern of cytokine production such as tumor necrosis factor-alpha (TNF-α) and interlukine-6 (IL-6), and granulocyte phagocytosis. (Sinniah *et al*., 1981; Tovo *et al*., 1981; Quintiliani *et al*., 1983; Weatherall and Clegg, 2000; Weatherall *et al*., 2000; Farmakis *et al*., 2003 and Vichinsky, 2005).

Thalassemia is among the most common single-gene disorders worldwide (Urbinati *et al*., 2006). It is prevalent in the Mediterranean populations, the Middle East, India and Southern China through Thailand and the Malay Peninsula into the Island populations of the pacific (Weatherall, 2001). Palestine is one of the Mediterranean basin countries in which thalassemia disease is prevalent. The average incidence of thalassemia trait in the Gaza Strip is 3.0 – 4.5% (Sirdah *et al*., 1998). However, no previous study was carried out about immunological aspects associated with β-thalassemic patients in Gaza Strip. This is the first study to investigate some immunological parameters in β-Thalassemia major in Gaza City.

1.2 General objective

The main objective of this study was to assess some immunological parameters in children aged 5-12 years old with β-Thalassemia major in Gaza City.

1.3 The specific objectives are

1. To determine complete blood count (CBC).
2. To measure the concentration of serum complement C3 and C4.
3. To evaluate the levels of immunoglobulins G, M and A.
4. To determine the level of serum ferritin.
5. To estimate the serum levels of cytokines TNF-α, IL-6 and IL-1β.
6. To examine serum C- reactive protein (CRP) in patients and controls
7. To investigate the possible relationship between allergic reaction, age, ferritin level and CRP and immunological parameters.

1.4 Significance

β-Thalassemia disease is prevalent in Gaza Strip since very long time, and the costs to care for patients with β-thalassemia major are high. Although immune abnormalities have been suggested as a precipitating factor for the fourth most common cause of death in β-thalassemia, no studies were carried out about immunological aspects in Gaza Strip. Therefore, this will be the first study to assess immunological aspects associated with β-thalassemia major patients in Gaza Strip.

Chapter 2
Literature Review

2.1 Thalassemia

The thalassemias are a group of inherited hematologic disorders caused by defects in the synthesis of one or more of the hemoglobin chains. (Rund and Rachmilewitz, 2005). Human hemoglobin (Hb) is the molecule that carries and transports oxygen all through the body. It is made up of two parts – heme and globin. Heme is a porphyrin containing iron. Globin is made up of four polypeptide chains of two types two alpha (α) and two non-alpha chains (beta β, gamma γ and delta σ). Adult and fetal hemoglobins have α-chains combined with β-chains (Hb A, α2β2), δ-chains (HbA2, α2δ2) and γ-chains (Hb F, α2γ2). In normal adults, Hb A is the main type of hemoglobin (96–98%) while HbA2 and Hb F are only present in 2–3% and less than 1%, respectively (Weatherall and Clegg, 1981 and Hillman and Ault, 1995). During fetal life, the major part of hemoglobin is Hb F so that after birth, the production of gamma chains slows down and beta chains increases correspondingly. The failure in hemoglobin synthesis is a main cause of microcytosis and anemia in many population groups around the world (Clarke and Higgins, 2000). Inherited hemoglobin disorders fall into two main groups: the structural hemoglobin variants and the thalassemias which imbalances of globin chains cause hemolysis and impair erythropoiesis. Thalassemia affects men and women equally and occurs in approximately 4.4 of every 10,000 live births (Steinberg *et al*., 2001 and Rund and Rachmilewitz, 2005).

2.1.1 Types of thalassemia

The thalassemias are due to a large number of mutations causing abnormal globin gene expression and resulting in total absence or quantitative reduction of globin chain synthesis (Steinberg *et al*., 2001). They are divided according to which globin chain is produced in reduced amounts into the:

1. Reduced or absent α-globin chain: α-thalassemia
2. Reduced or absent β-globin chain: β-thalassemia
3. Reduced or absent δβ- globin chain: δβ-thalassemia
4. Reduced or absent γδβ- globin chain: γδβ- thalassemia

All types of thalassemias are considered quantitative hemoglobin disease. From a public health view point only the α and β thalassemias are sufficiently common to be of importance (Weatherall and Clegg, 2001 and Hoffbrand *et al*., 2005).

Alpha-thalassemia is usually due to deletions within the alpha-globin gene cluster, leading to loss of function of one or both alpha-globin genes in each locus leading to excess beta globin chains. α-thalassemia generally presents as a milder form of the disease. This is due to the fact that there are four α-globin genes, requiring multiple mutations to result in a clinical impact. Also, the unpaired β-globin chains are intrinsically less prone to precipitation as compared with unpaired α-globin chains in β-thalassemia (Weatherall, 1994 and Rund and Rachmilewitz, 2005).

Beta thalassemia is the result of deficient or absent synthesis of beta globin chains, leading to excess alpha chains. β-thalassemias are the most important among the thalassemia syndromes because they are so common and usually produce severe anemia in their homozygous and compound heterozygous states. β-thalassemia has become a worldwide clinical problem due to an increasing immigrant population. (Olivieri, 1999; Hahalis, 2005 and Cao and Galanello, 2010). The beta globin (*HBB*) gene maps in the short arm of chromosome 11, in a region containing also the delta globin gene, the embryonic epsilon gene, the fetal A-gamma and G-gamma genes, and a pseudogene (ψ β1). Beta-thalassemias are heterogeneous at the molecular level. More than 200 disease causing mutations have been so far identified. The majority of mutations are single nucleotide substitutions, deletions, or insertions of oligonucleotides leading to frameshift. Rarely, beta-thalassemia results from gross gene deletion (Cao and Galanello, 2010).

2.1.2 Classes of β- thalassemia

The clinical severity of beta-thalassemia is related to the extent of imbalance between the alpha and non alpha globin chains, so thalassemia can be categorized into three classes according to the severity of the symptoms:

The beta-thalassemia carrier state which results from heterozygosity for beta-thalassemia, is clinically asymptomatic and is defined by specific hematological features

The beta-thalassemia intermedia, comprehend a clinically and genotypically very heterogeneous group of thalassemia-like disorders, ranging in severity from the asymptomatic carrier state to the severe transfusion-dependent type (Cao and Galanello, 2010).

The beta-thalassemia major a severe transfusion-dependent anemia. The condition was first recognized in 1925 by Thomas Cooley and Pearl Lee who described a form of severe anemia occurring in children of Italian origin and associated with splenomegaly and characteristic bone changes. In 1936, Whipple and Bradfor, in describing the pathological changes of the condition for the first time, recognized that many of their patients came from the Mediterranean region, and hence invented the word ‘thalassemia’ from the Greek word for sea, *thalassa*. Over the next 20 years, it became apparent that Cooley and Lee had described the homozygous or compound heterozygous state for a recessive mendelian disorder not confined to the Mediterranean, but occurring widely throughout tropical countries (Weatherall and Clegg, 1996). Table: 2.1 summarized the different classes of β-thalassemia (Urbinati *et al*., 2006).

Table: 2.1. Different classes of β-thalassemia (Urbinati et al., 2006).

Type	Genotype*	Phenotype	HB Electrophoresis
β-Thalassemia minor	Heterozygous: β+/βwt, β0/βwt	Asymptomatic carrier mild microcytic anemia (Hb >10 g/dL)	Elevated HbA2 and HbF common**
β-Thalassemia intermedia	Homozygous β+/β+ Compound heterozygous: β+/β+, β0/β+***:	Intermediate (Hb 7–10 g/dL)	Variable
β-Thalassemia major	Compound heterozygous** β0/β+, β+/β+ (two distinct β+ mutations) Homozygous: β0/β0, β+/β+ (two identical mutations)	Severe anemia, transfusion dependence (Hb <7 g/dL)	Elevated HbA2 pronounced HbF elevation

***** βwt represents the normal β-globin allele with normal β-globin chain production; β+ denotes β-globin mutations that decrease β-globin chain production but do not entirely eliminate it; β0 denotes β-globin gene mutations that completely abolish β-globin chain production from the affected allele.

****** Types of hemoglobin (Hb) found on electrophoresis. HbA2, 'minor' adult Hb (α2δ2); HbF, fetal Hb (α2γ2).

******* Compound heterozygotes are individuals carrying different β0 or β+ mutations on the two alleles

2.1.3 Epidemiology of thalassemia

Approximately 5 percent of the world's population has a globin variant, but only 1.7 percent has alpha or beta thalassemia trait. Thalassemia affects men and women equally and occurs in approximately 4.4 of every 10,000 live births. β-thalassemia is most common in persons of Mediterranean, African, and Southeast Asian descent. Thalassemia trait affects 5 to 30 percent of persons in these ethnic groups (Rund and Rachmilewitz, 2005). Palestine is one of the Mediterranean basin countries in which thalassemia disease is prevalent. The carrier frequency for β-thalassemia in these areas ranges from 1% to 20%, rarely greater (WHO, 1989). Figure 2.1 shows geographical distribution of β-thalassemia around the world. In Gaza Strip the average incidence of thalassemia trait is 3.0 – 4.5% (Sirdah *et al*., 1998). The number of thalassemia patients in Gaza Strip is 325 patients. There are 246 confirmed β-

thalassemia major, 2 patients are α thalassemia and 77 individuals are thalassemia intermediate (Thalassemia center, 2010). Thalassemic patients get their treatment and health care in three hospitals in Gaza Strip. Patients living in both Rafah and Khan-Younis are treated at the European Hospital regardless of their age. Adult thalassemic patients living in Gaza City, Northern and Middle Governorates are treated at Al–Shifa Hospital in Gaza City while young thalassemic patients (<12 years) are treated at Abd El-Aziz El-Rantisy Hospital. Thalassemic children aged 5-12 represent 49 in Abd El-Aziz El-Rantisy Hospital and 33 in the European Hospital.

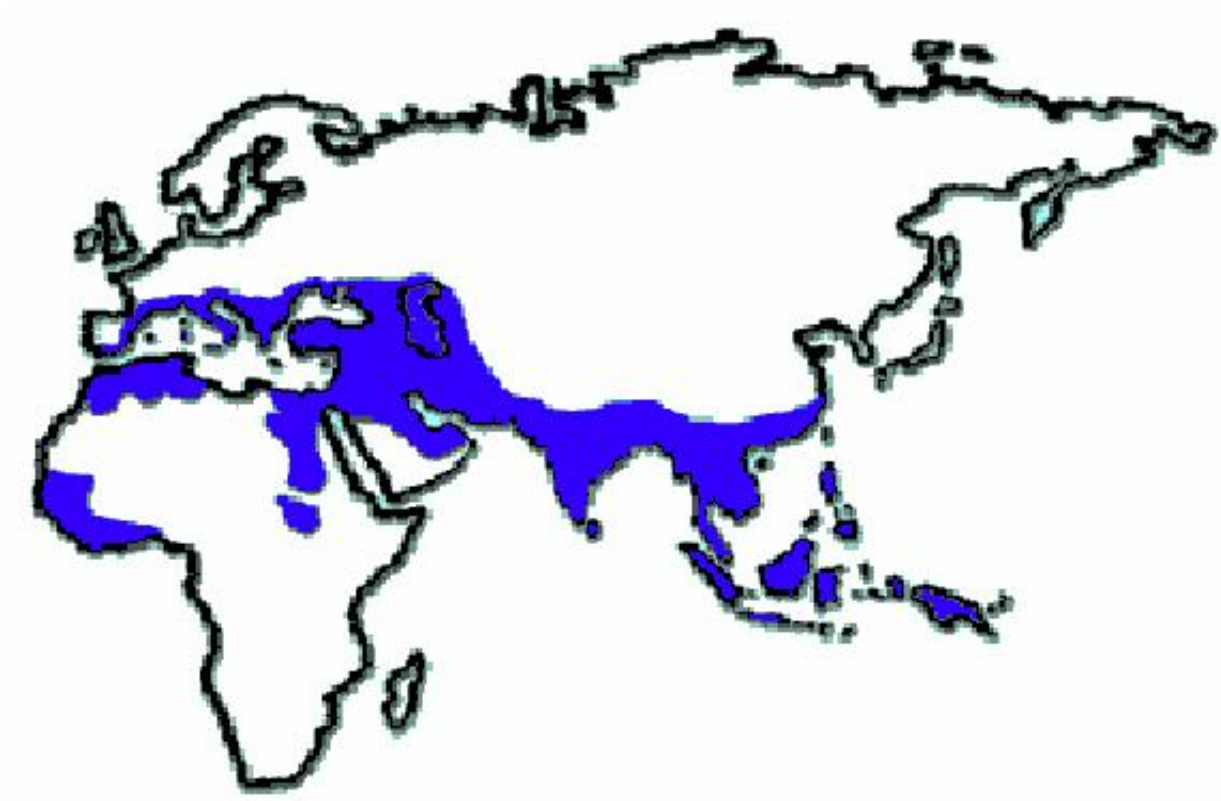

Figure 2.1: Geographical distribution of β-thalassemia around the world. Blue areas indicate the countries where thalassemia is prevalent.

2.1.4 Clinical aspects of thalassemia

Individuals with thalassemia major usually come to medical attention within the first 2 years and require regular blood transfusion to survive. Affected infants with thalassemia major appear well at birth but develop anemia that becomes

progressively worse. The first signs of the progressive anemia are pallor, listlessness, fail to thrive, feeding problems, diarrhea, irritability, recurrent bouts of fever, and enlargement of the abdomen, caused by splenomegaly. If a regular transfusion program that maintains a minimum Hb concentration of 9.5–10.5 g/L is initiated, then growth and development are normal until the age of 10–11 years (Anionwu and Atkin, 2001 and Cao and Galanello, 2010).

2.1.5 Complications of thalassemia

Over the past three decades the quality of life has dramatically improved and transformed thalassemia from a rapidly fatal disease in early childhood to a chronic disease compatible with prolonged life. Today life expectancy varies between 25-55 years, depending on the compliance with medical treatment. Despite increased life expectancy, complications keep arising. After the age of 10–11 years, affected individuals are at risk of developing severe complications which can be grouped as: inadequate transfusions, transfusion-related infections, allosensitization, iron-overload and toxicities of iron chelators. However many of these problems are strongly age dependent (Modell *et al*, 2000; Cunningham *et al*., 2004 and Satwani *et al*., 2005).

1. Inadequate transfusions: patients suffer from the direct effects of anemia itself and from the effects of expansion of extramedullary haematopoiesis that results from the anemia, including skeletal abnormalities such as bossing of the skull, prominent maxilla and malar eminences and depression of the bridge of the nose, as well as splenomegaly, spinal cord compression, and growth retardation (Weatherall, 1997).

2. Transfusion-related infections: blood supply suggests that blood safety cannot be completely ensured and that despite sensitive and specific testing, transfusion-related infections are still major complications and constitute the second most common cause of mortality and a main cause of morbidity in patients with thalassemia. Severe anemia, iron overload, splenectomy, and a range of immune abnormalities consider predisposing factors for infections in thalassemic patients (Vento *et al*., 2006).

3. Allosensitization: one of the complications of blood transfusion is the formation by the recipients of alloantibodies and autoantibodies RBC antigen which can result in

difficulty obtaining compatible blood, transfusion reactions, haemolysis and occasionally life-threatening events (Wang *et al*., 2006 and Haslina *et al*., 2007).

4. **Iron-overload**: causes most of the mortality and morbidity associated with thalassemia. Iron deposition occurs in visceral organs (mainly in the heart, liver, and endocrine glands), causing tissue damage and ultimately organ dysfunction and failure. Cardiac events due to iron overload are still the primary cause of death (Olivieri, 1999).

5. Toxicities of iron chelators: some side effects of high-dose desferrioxamine (Desferal) are high frequency sensoneural hearing loss, retinal damage (night vision), abnormalities of bone growth such as vertebral dysplasia, agranulytosis (<1 %), neutropenia and arthralgia (Porter, 2001 and Piga *et al*., 2006).

2.1.6 Pathophysiology of thalassemia

The two main problems of thalassemias are decreased hemoglobin synthesis and an imbalance between the β- and α-globin chains. The former accounts for RBC hypochromia, microcytosis and erythrocytosis, but is of minor relevance. More important is the deleterious effect of the globin chain imbalance – a relative excess of unbound α- protein in the erythroid cells. In the absence of the complementary globin chains, the excess unbound α- globin protein aggregates and precipitates, damaging cell membranes and leading to premature destruction (Schrier, 2002).

Mechanisms of anemia

Precipitation of the excess unbound α- globin protein leads to cell membranes damage. When this occurs in immature erythroid precursors it results in ineffective erythropoiesis; in mature RBCs it results in hemolysis. Both result in anemia (Figure 2.2). Other factors such as abnormally rigid thalassemic RBCs, iron overload and oxidative stresses also contribute to the disease process (Shinar and Rachmilewitz, 1993 and Weatherall and Clegg, 2001).

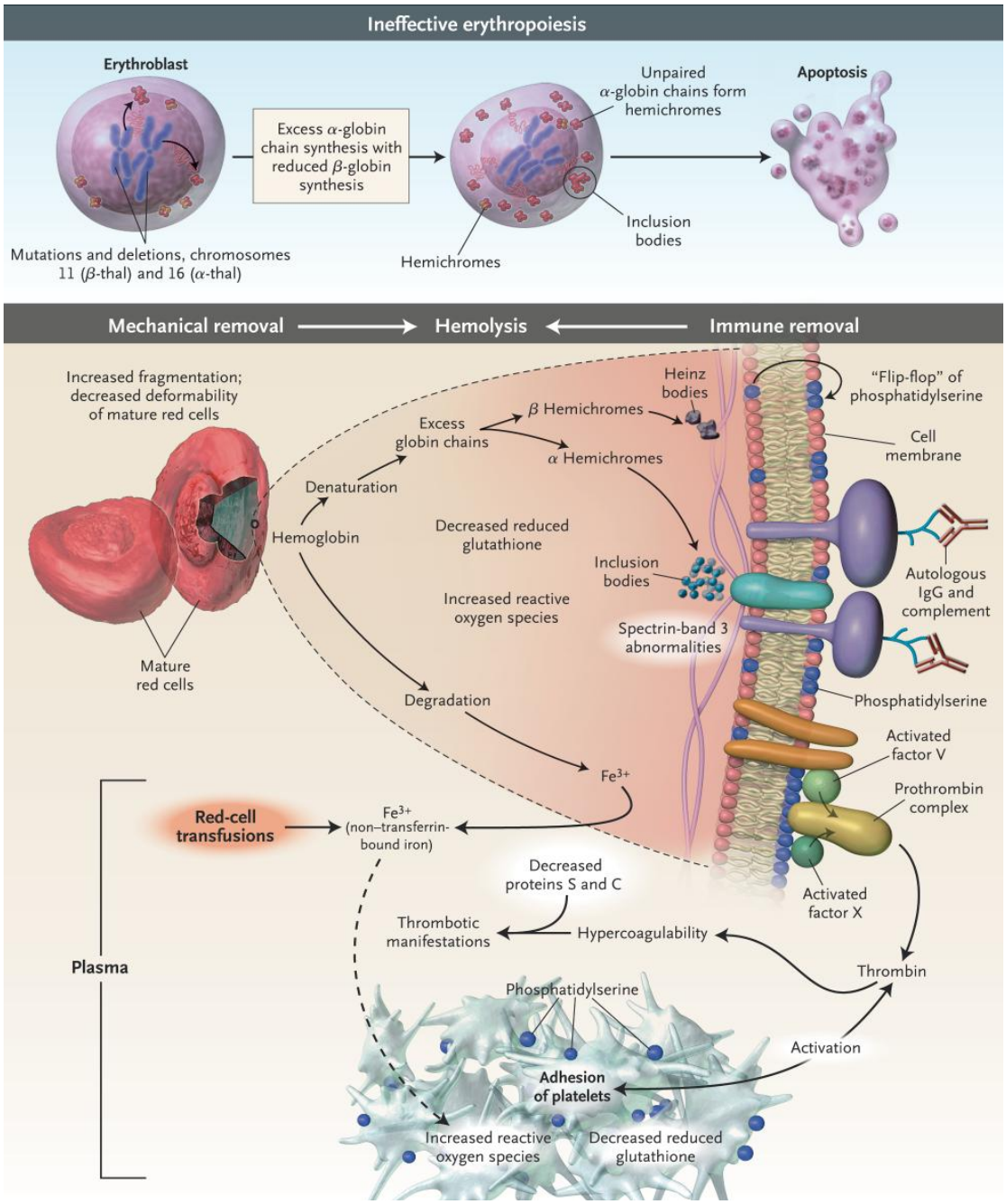

Figure 2.2. Mechanisms of anemia (Rund and Rachmilewitz, 2005)

Ineffective erythropoiesis

In severe untreated β-thalassemia, erythropoiesis may be increased by a factor of up to 10, more than 95 % of which may be ineffective. Ineffective erythropoiesis, the hallmark of β-thalassemia, unpaired α-globin chains are more insoluble and unstable and dissociate into monomers. They form hemichromes at a faster rate. Precipitates of the excess unbound α- globin protein leads to the formation of small multiple

inclusions. Inclusion bodies are seen in large amounts in the bone marrow and throughout the erythroid maturation pathway (Higgs *et al*, 2001). The bone marrow of patients with thalassemia contains five to six times the number of erythroid precursors as does the bone marrow of healthy controls, with 15 times the number of apoptotic cells in the polychromatophilic and orthochromic stages (Centis et al, 2000). Both intramedullary death of red-cell precursors through arrest in the G1 phase of the cell cycle and accelerated intramedullary apoptosis of late erythroblasts which is the major cause of ineffective erythropoiesis have been demonstrated (Yuan *et al*, 1993 and Schrier, 1997). Although the exact mechanism is not known, a death-receptor–mediated pathway seems to be involved with Fas–Fas ligand interactions (De Maria *et al*, 1999). Cells undergoing programmed cell death seem to signal this fact to macrophages, probably through the movement of phosphatidyl serine to the surface of the membrane, so are removed by macrophages -whose numbers are increased in thalassemic bone marrow- via phagocytosis (Angelucci *et al*, 2002a and Kuypers and Jong, 2004).

Hemolysis in mature RBCs

RBCs that enter the circulation contain inclusions that result in their damage as they pass through the microcirculation, and these cells undergo extravascular hemolysis, particularly in the spleen. One mechanism involves the oxidation of a hemoglobin subunits which leads to the formation of hemichromes, whose rate of formation determines the rate of hemolysis (Rachmilewitz and Schrier, 2001). Hemichromes bind to or modify various components of the mature red-cell membrane, such as protein band 3, protein 4.1, ankyrin, and spectrin(Figure 2.2). This protein changes increased membrane rigidity and instability by the binding of oxidised α-globin chains to the membrane structural protein 4.1. Consequently, there is a decreased binding of spectrin to actin to protien 4.1, which normally helps to stabilise RBC membranes (Shinar and Rachmilewitz, 1993 and Schrier, 1997).

Other factors

Reticuloendothelial clearance of RBCs

The effect of hemoglobin denaturation on the RBC membrane and hemichrome formation promote clustering of the membrane protein band 3, autologous antibody binding IgG and complement fixation triggering removal of erythrocytes from

circulation, by macrophages. (Yuan *et al*., 1992). Thalassemia and normal senescent RBCs also contain a reduced amount of sialic acid. This results in an increased exposure of β-galactosyl residues that become bound by antigalactosyl IgG antibodies, leading to sequestration of senescent normal RBCs and thalassemia RBCs by the reticuloendothelial system. These factors, together with the previously described alteration in cell membrane rigidity and deformability, alter the normal exposure and composition of antigens on the outer surface of the membrane, and consequently cause erythrophagocytosis by macrophages (Galili *et al*., 1983).

Iron overload and oxidative stress

Iron overload constitutes a significant problem for β-thalassemia patients. The precipitation of hemichromes, heme disintegrates, and toxic non–transferrin-bound iron (NTBI) species leads to the formation of toxic free radicals from superoxide and hydrogen peroxide. Lipid peroxidation then occurs, disrupting cell membranes (Hershko *et al*, 1998).

2.1.7 Diagnosis of thalassemia

Complete blood count (CBC)

The CBC is a very common test help to diagnose thalassemia. The thalassemias are generally classified as hypochromic and microcytic anemia. Thalassemic individuals have increased number of red blood cells, decreased Hb concentration and low mean corpuscular volume (MCV) and mean corpuscular Hb (MCH) without a concomitant increase in red cell distribution width (RDW).

Peripheral blood smear

It shows, in addition to microcytosis and hypochromia, anisocytosis, poikilocytosis, and nucleated red blood cells.

Quantification of Hb pattern

It is achieved by cellulose acetate electrophoresis (Hemoglobin electrophoresis tests) or high performance liquid chromatography (HPLC) for quantification of Hb A_2 and HbF. HbF concentration is usually elevated and it is the first diagnostic test (Clarke and Higgins, 2000 and Cao, and Galanello, 2010).

Molecular diagnosis of beta-thalassemia

Commonly occurring mutations of the *HBB* gene are detected by a number of polymerase chain reaction (PCR)-based procedures (Old *et al*., 2005).

2.1.8 Control and management of thalassemia

Prevention programs

Screening programs for carriers, aimed at prevention of the disease, and prenatal diagnosis have resulted in a marked reduction in the birth rate of affected children in many countries (Cao *et al*., 1998).

Medical therapy

Clinical management of thalassemia major consists in regular long-life red blood cell transfusions every 2–3 weeks to correct the anemia by maintaining a pre-transfusion Hb concentration of 9–10 g/dL, improvement of growth and development, suppress erythropoiesis, and therefore hepatosplenomegaly, hypersplenism and bone deformities and inhibit increased gastrointestinal absorption of iron. Before starting the transfusions, it is absolutely necessary to blood unit preparation before each transfusion. After 10–12 transfusions, chelation therapy is initiated to manage iron overload. Desferrioxamine (Desferal) is the most commonly used chelating agent. Recommended dosage depends on the individual's age and the serum ferritin concentration (Porter, 2001 and Borgna-Pignatti *et al*., 2004). Deferiprone, is another orally administered chelator. An encouraging to chelation therapy is the sequential combined administration of deferiprone and deferoxamine. The other complications such as endocrinopathies can be treated with hormone replacement. At present, therapies under investigation are the induction of fetal hemoglobin, antioxidants and stem cell gene therapy**.** The only definitive cure is bone marrow transplantation (Rund and Rachmilewitz, 2005 and Cao, and Galanello, 2010).

2.2 Thalassemia and immune system

Beta-thalassemia major patients suffer from many problems rather than severe anemia including increased susceptibility to bacterial infections. Infectious complications constitute the second most common cause of mortality and a main cause of morbidity in β-thalassemia after heart failure (Borgna-Pignatti *et al*., 2004 and Vento *et al*., 2006). This predisposing to infections is related to some factors such as multiple transfusions, iron overload and immune abnormalities (Dua *et al*., 1993 and Weinberg, 2000). Numerous immune abnormalities have been described in thalassaemic patients. Many studies have been done to evaluate the possible

changes of immune system in thalassemic patients, considering the humoral and cellular immune systems and various immunological abnormalities are reported. These abnormalities have been attributed both to the disease itself and the applied therapeutic intervention (Dwyer *et al*., 1987).

Iron overload: it has been implicated as the main precipitating factor of immune deficiency in β-thalassemia (Walker and Walker, 2000 and Weiss, 2002) that is due to the important immunoregulatory properties of iron and its binding proteins. It constitutes a primary complication of both thalassemia itself and the therapy, leading to numerous abnormalities. Immune system abnormalities that have been described include decreased phagocytosis by the monocyte –macrophage system, alteration in T-lymphocyte subsets, with enhancement of CD8 and suppression of CD4, impairment of immunoglobulin secretion and suppression of complement system function (De Sousa, 1989). It has been demonstrated that iron plays an important role in regulating the expression of T-lymphocyte cell surface markers, influencing the expansion of different T-cell subsets. The impaired phagocytosis activity observed in iron overload results from the deleterious effect of ferritin-associated iron. At the same time, the high plasma ferritin content in thalassemic patients may induce the development of anti-ferritin antibodies, which in turn leads to the production of circulating immune complexes (Walker and Walker, 2000). Iron excess also may derange the immune balance in favor of the growth of infectious organisms (Farmakis *et al*., 2003).

Other factors

Multiple transfusions: include multiple transfusions are thought to be another major pathogenetic mechanism of immune abnormalities, besides causing iron overload, repetitive transfusions lead to continuous allo-antigenic stimulation and have been associated with autoimmune hemolysis (Singer *et al*., 2000). At the same time, they are followed by the risk of transmission of viruses with immunosuppressive properties.

Splenectomy: resulting in increased susceptibility to immune system modifications. These include quantitative lymphocyte changes, though without functional impairment (Ahluwalia *et al*., 2000)

level of zinc: which is an immune regulator, the low zinc levels in thalassemic patients have been associated with alterations of lymphocyte subsets and thymulin deficiency (Consolini *et al*., 2001)

Iron chelation therapy: This predisposes to serious infections (Farmakis *et al*., 2003).

Immune defect

Several studies on immune competence in β-thalassemia have revealed numerous quantitative and functional defects, involving T and B lymphocytes, immunoglobulin production, changing the pattern of cytokine production, neutrophils and macrophages, chemotaxis, and phagocytosis, as well as the complement system (Weatherall and Clegg, 2000; Weatherall *et al*., 2000; Farmakis *et al*., 2003; Alidoost *et al*., 2006 and Morabito, 2007). Immune abnormalities have also been held responsible for the frequent occurrence of malignancies in β-thalassemia, especially leukemia and lymphomas. Thus, surveillance for infections in patients with β-thalassemia is crucial, while further studies are warranted on immune function abnormalities and the implicated mechanisms (Farmakis *et al*., 2003).

2.2.1 Immunoglobulins

The immune system generates billions of different antibody molecules by mature B cells which are capable of secreting antibodies and expressing B cell receptors on their cell surfaces (Rolink *et al*., 1999 and Brekke and Sandlie, 2003). Serum immunoglobulin levels provide key information on the humoral immune status. Immunoglobulin levels aid in the diagnosis of some disorders changes in the quantity and quality of antibodies occur in the course of an immune response (Buckley, 1986 and Dispenzieri *et al*, 2001).

Immunoglobulin A (Ig A)

Immunoglobulin A is the predominant immunoglobulin isotype on most mucosal surfaces, it functions as an inflammatory antibody (Snoeck *et al*., 2006).

Immunoglobulin M (IgM)

Immunoglobulin M is one major type of B cell antigen receptor (BCR) expressed on most of the B cells from immature to mature stages. During normal B cell ontogeny, signals transduced through the IgM. B cell antigen receptor (BCR) plays an important

role in regulating B cell maturation and survival at multiple checkpoints **(**Zheng *et al*., 2007)**.** Upon encountering Ag, the cells become activated and make a switch from IgM to other Ig classes (Wang and Wabl, 2004).

Immunoglobulin G, (IgG)

Immunoglobulin G is a major effector molecule of the humoral immune response in man, accounts for about 75% of the total immunoglobulins in plasma of healthy individuals. IgG antibodies represent a large vocabulary of antigen recognition molecules. There are four subgroups, currently labeled with number suffixes (IgG1 to 4). IgG express predominant activity during a secondary antibody response. IgG antibodies have a relatively high affinity and persist in the circulation for a long time (Allergy, 2010).

2.2.2 The complement system

The complement system consists of about two dozen plasma and cell membrane proteins which function as cofactor in defense against pathogenic microbes and in the generation of many immunopathogenic disorders (Colten and Rosen, 1992). Complement 3 and complement 4 are components of the complement system. Complement component C3 activation is the point of convergence in the initiation of the complement cascade by the lectin, alternative and classical pathways (Markiewski and Lambris, 2007). The fourth component C4 is an essential intermediary for the classical and lectin pathways of complement activation (Gasque, 2004).

2.2.3 Cytokines

Cytokines are a group of protein cell regulators variously called lymphokines, monokines, interleukins, interferons and chemokines produced by a wide variety of cells in the body that play an important role in many physiologic responses. Cytokines are involved in the pathophysiology of a broad range of diseases and also have therapeutic potential. The cytokines consist of more than 40 secreted factors involved in intercellular communication and their functions are fully developed after binding to specific receptors (Jiang and Chess, 2006; Maki-Petaja *et al*., 2006; Tedgui and Mallat, 2006 and Hafler, 2007). One of the current classifications divides cytokines on the basis of their functions: cytokines that regulate hematopoiesis interferons;

cytokines that mostly regulate the functions of B- and T lymphocyte systems; pluripotent inflammatory cytokines (IL-1 and IL-6); tumor necrosis factors; chemokines and growth factors (Thomson and Lotze, 2003).

Interleukin-1 (IL-1)

Interleukin-1 is a pleiotropic proinflammatory cytokine produced by both activated lymphoid and nonlymphoid cells originally described as a product released from activated macrophages. There are two known forms of IL-1, a membrane-bound IL-1α and a secretory form IL-1β (Durum *et al*., 1985 and Dinarello, 1989). Interleukin-1β is the most studied member of the IL-1 family because of its role in mediating autoinflammatory diseases (Dinarello, 2009).

Interleukin- 6 (IL-6)

The Interleukin-6 family is probably unique among cytokines because it was cloned almost inadvertently long before the discovery of its major biological activities. It is secreted by T cells and macrophages to stimulate immune response and acts as both a pro-inflammatory and anti-inflammatory cytokine. It is a potent inducer of the proliferation and production of immunoglobulins in B-lymphocytes (Van Snick, 1990 and Wikipedia, 2009).

The tumor necrosis factor (TNF)

Originally thought of as selective anti-tumor agents. TNF “family” includes two structurally and functionally related proteins, TNF-α and TNF-β or lymphotoxin. They are now grouped among the major inflammatory cytokines. They play a beneficial role as immunostimulants and important mediators of host resistance to many infectious agents and, probably, malignant tumors. TNF-α turned out to be identical to cachectin, postulated to mediate wasting during chronic infections. There is increasing evidence that overproduction of TNF-α during infections leads to severe systemic toxicity and even death (Beutler, 1985 and Wikipedia, 2010).

2.2.4 Ferritin

Ferritin is the primary iron storage protein and in serum reflects the state of the iron stores in the body. It provides a reserve of iron readily available for formation of hemoglobin and other iron-containing proteins and enzymes ((Tietz, 1994). Its analysing represents the easiest method of supervising the levels of total body iron (Angelucci *et al*., 2002 b).

2.2.5 C-reactive protein (CRP)

C-reactive protein is an established marker for the detection of acute and chronic inflammatory processes. The most potent stimulator for the hepatic synthesis of this protein is interleukin 6 (Archararit *et al*., 2000). There is increasing evidence that CRP may be directly involved in thrombogenesis. C-reactive protein present in the vessel wall induces expression of adhesion molecules by endothelial cells which serves as a chemoattractant for monocytes. C-reactive protein binds to plasma membrane of damaged cells and activates complement via the classical pathway (Koenig, 2003).

2.3 Related studies

Tovo *et al*. (1981) studied IgG, IgA and IgM levels in 187 homozygous β-thalassemic patients and compared them with age-matched normal control subjects. The not yet transfused and the polytransfused nonsplenectomized patients showed a significant increase of all Ig classes. The polytransfused splenectomized patients showed a significant increase only of IgG and IgA. The splenectomized patients, when compared to the nonsplenectomized ones, showed a significant increase of IgG, of IgA in the elder ones and a significant reduction of IgM. The high Ig levels in younger not yet transfused patients, with little iron storage and normal hepatic enzyme values, demonstrate that transfusion therapy and liver damage do not play a main role in hypergammaglobulinemia. The absence of antimitochondrial, anticonnective tissue and homogeneous antinuclear antibodies seems to exclude a deficient suppressor mechanism. The hemocatheteric "overworking' of RES may reduce the antigen.

James *et al*., (1981) examined the activity of the complement system in sera from 24 thalassemic patients in a study to explore the basis for suffering from frequent and serious infections in patients with thalassemia major especially after splenectomy. Mean concentrations of C3, factor B, properdin, and immunoglobulins were normal. The result showed deficient activity of the alternative pathway of complement in β-thalassemia major, especially in conjunction with asplenia .

Serum IgG, IgM and IgA were determined in 25 patients with homozygous β-thalassemia and 7 with the trait (Khalifa *et al*., 1983). The levels were increased in homozygous patients and increased further after splenectomy. Serum opsonic activity against Salmonella typhi and staphylococci was impaired in homozygous patients.

Splenectomy caused more impairment against salmonella only. Similarly, phagocytic power against both organisms was lower in β-thalassemia. Further decrease against salmonella occurred after splenectomy. Patients with thalassemia trait did not differ from normal controls.

Akbar *et al*. (1985) reported that multiply transfused patients with β-thalassaemia major had a significantly increased ($P<0.001$) proportion of B-cells that contain cytoplasmic immunoglobulin when visualized immediately upon isolation. On the other hand, the same cell populations do not exhibit an increase in the proportion of immunoglobulin-secreting cells as measured by a reverse haemolytic plaque assay. Their results suggested that the cells containing the cytoplasmic immunoglobulin are likely to be terminally differentiated B-cells which persist in the circulation. While the reason for this phenomenon was not yet known, They had found that the increase in these cells is transfusion-related.

Meliconi *et al*. (1992) determined serum TNF concentrations by enzyme linked immunoassay in paired samples from 71 patients with β-thalassemia before and after bone marrow transplantation (BMT). Serial samples from 13 patients were also studied for up to six months after BMT. Forty one normal healthy children matched for sex and age were studied as controls. Results showed that β-thalassemic patients had high serum TNF concentrations before transplantation compared with controls. These were not related to sex, age, duration of disease, number of blood transfusions, transferrin concentrations or splenectomy. Patients with severe liver fibrosis had significantly higher TNF concentrations. They conclude that about 50% of β-thalassemic patients have increased serum TNF, and the changes after BMT are related to the occurrence of immune mediate complications. The persistence of low TNF concentrations after successful engraftment may be due to the preparative regimen and the lack of adverse immune reactions.

Serum levels of IL-2, IL-6, TNF, soluble (s) CD4, sCD8, sCD23 and sCD25 were measured using immunoenzymatic assays in 45 transfusion-dependent patients affected by β-thalassemia major (Lombardi, 1994). The results showed increased TNF, sCD8, sCD23 and sCD25 and lower sCD4 values compared to normal controls. IL-2 and IL-6 were found to be undetectable or within the normal range in all patients. Splenectomized patients presented lower levels of sCD8 and sCD23 than those observed in unsplenectomized ones. A series of correlations involving TNF,sCD8,

sCD23, sCD25, serum immunoglobulins and some lymphocyte subpopulations was observed. In addition, serum markers of immune activation (TNF, sCD23, sCD25) correlated directly with the annual blood transfusion requirement. No patient had a history of repeated infectious episodes**.**

El Nawawy *et al*. (1996) measured blood levels of IL-1-β, TNF-α, and islet cell antibody (ICA) in 20 children with independent diabetes mellitus (IDDM), 20 of their non-diabetic siblings, 20 children with thalassemia major on long-term hypertransfusion therapy and iron chelation, and 10 normal age-matched children. In the non-diabetic and thalassemic children they investigated the early phase of insulin release after i.v. glucose (0.5 g/kg, 30% solution) and evaluated tolerance to oral glucose (1.75 g/ kg). Circulating IL-1-β and TNF-α concentrations were significantly higher in IDDM-siblings (33.7±12.7 pg/ml and 655±165 pg/ml, respectively) v. normal children (21.1±6.4 pg/ml and 383±122 pg/ml, respectively). Thalassemic children had no detectable circulating ICA. The prevalence of ICA was 30% in children with IDDM and 60 % of their siblings. Impaired oral glucose tolerance was detected in five children with thalassemia (25 %), but in none of the IDDM-siblings. The early phase of insulin release was significantly depressed in thalassemic children (peak insulin=29.2±5.1 mIU/ml) v. normal children (52.3±9.5 mIU/ml) and IDDM-siblings (45.3±12.4 mIU/ml). It appears that thalassemic children in their study had significantly decreased insulin secretion and impaired glucose tolerance, however, the mechanism of B-cell dysfunction is not mediated by ICA nor by cytokines.

The unstimulated and induced production of granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), IL-3, IL-6, stem cell factor (SCF), IL-1β, TNF-α, TNF-β, interferon-γ (IFN-γ) and transforming growth factor-beta (TGF-beta) were determined after culture of blood mononuclear cells from 22 patients with severe β-thalassaemia in a regular transfusion programme, five non-regularly transfused patients with β-thalassaemia intermedia and nine normal persons (Salsaa and Zoumbos, 1997). A distinct pattern of cytokine production in thalassaemic patients was detected, namely a low unstimulated production of all cytokines and a significant increase in the stimulated production of IFN- γ, TNF-α and IL- 1β; these abnormalities were more pronounced in the more heavily transfused older patients. The increased production of the above cytokines, which usually characterize the acute response to infectious agents and have a negative effect on

erythropoiesis, may explain the deterioration of anemia found in thalassaemic patients during acute infections**.**

A small scale screening study for β-thalassemia trail has been carried out in the Gaza Strip involving 1650 secondary schools healthy students, 16-18 years old and from both sexes (Sirdah *et al*., 1998). The overall prevalence of β-thalassemia in the Gaza Strip was 4.3%. The frequency of β-thalassemia trait in the microcytic (defined as MCV≤80fl and/or MCH≤26pg) subjects was 27.1%. The efficacies of some of the proposed discrimination functions in the differentiation between β-thalassemia trait and non thalassemic microcytosis were evaluated. The Mentzer index, MCV of ≤72 fl, England and fracer DF and the shine & La1 formula were found to correctly identify 91.6%, 82.4%, 81.3% and 62.6% of the studied cases of microcytosis as having or not having the β-thalassemia trait. It was concluded that both β-thalassemia and microcytosis anemias are major health problems in the Gaza Strip.

Wanachiwanawin *et al*. (1999) estimated serum levels of TNF-α, IL-1α, and interferon-γ (IFN-γ) by conventional ELISA kits in 60, 42, and 58 Thai patients, respectively, with beta(o)-thalassemia HbE and found to be above the normal range in 13%, 21%, and 33% of the patients, respectively. Using high-sensitivity ELISA systems, an additional 10 β(o)-thal/HbE patients were compared with 9 controls for concentrations of circulating TNF-α and IL-1β, but only 1 and none of the controls, respectively, showed values above the normal ranges. In patients with abnormally high IFN-γ levels, basal hemoglobin values were significantly lower than in those with normal levels of the cytokine (mean±SEM: 6.03±0.24 V.s 7.08±0.18, $p<0.05$), although circulating concentrations of soluble transferrin receptors (sTrF) and absolute reticulocyte counts were similar in the two groups. Patients with raised or normal levels of TNF-α, IL-1α, or IL-1β had similar basal hemoglobin values. In a phagocytosis assay, monocytes of patients with raised serum levels of IFN-γ showed significantly more attached or ingested IgG-coated red cells than those of patients with normal concentrations of the cytokine (mean±SEM: 192±22 v.s 140±14 per 100 monocytes, $p<0.05$). Moreover, in 3 of 4 of the former patients, the number of attached or ingested IgG-coated red cells per 100 monocytes was above the 95% reference limit for the latter patients.

Archararit *et al*. (2000) determined serum C-reactive protein concentration in 28 beta thal/HbE postsplenec, 22 βthal/HbE, 12 postsplenec, 23 reactive thrombocytosis RT, 21 chronic myeloproliferative disorders MPD, and 26 healthy adult volunteers. The values of CRP in beta thal/HbE postsplenec were significantly higher when compared with beta thal/HbE, and normal volunteers (4.1±0.7 V.s 1.6±0.4 mg/L P=0.006, and 4.1±0.7 V.s 0.45±0.09 mg/L, P<0.001). CRP levels in beta thal/HbE postsplenec were also higher than the postsplenec group (4.1±0.7 V.s 0.19±0.7 mg/L P = 0.095). On the contrary, C-reactive protein levels were significantly lower than those in RT (4.1±0.7 V.s 55.4±14.8 mg/L, P = 0.002). However, when compared to those with MPD, the values were not statistically different (4.1±0.7 V.s 17.1±12.3 mg/L, P=0.871). Interestingly, there was a trend towards increasing C-reactive protein levels in beta thal/HbE postsplenec patients with higher platelet count, although no correlation was observed. Besides the inflammatory process, platelet and/or factor(s) that control(s) thrombopoiesis seem(s) to play a role in the high serum C-reactive protein levels in the studied population.

The expression of some activation and adhesion molecules on peripheral blood monocytes, neutrophils lymphocytes in 68 transfusion-dependent thalassemics (group A), 10 transfusion non-dependent thalassemics (group B), 18 β-thalassemia carriers (group C), and 28 normal individuals was investigated (Kyriakou *et al*., 2001). Furthermore, soluble intercelullar adhesion molecule 1 (sICAM-1), soluble vascular adhesion molecule 1 (sVCAM-1), and E-selectin, TNF-α, and IL-1β were measured in the plasma of patients. Results showed that the expression of CD11b, CD18, and CD69 on the monocytes of group A was significantly greater than in groups B and C and in controls, while CD44 was significantly downregulated in group A. CD11b, CD18, CD35, CD44, and CD67 on the surface of neutrophils and CD38 and CD69 on the surface of lymphocytes were also overexpressed in group A. CD44 was downregulated on the monocytes and upregulated on the neutrophils of the patients compared to controls. The levels of sICAM-1, sVCAM-1, E-selectin, TNF-α, and IL-1β in the serum of patients in groups A and B were higher than those in group C and the controls.

Oztürk *et al*. (2001) studied 14 patients with thalassaemia major by evaluating body iron status, iron supply for erythropoiesis, and plasma IL-6 and IL-8 levels, together with 12 age-matched healthy controls. Patients with β-thalassemia were found to have higher IL-8 concentrations than normal controls (p<0.001) and plasma

IL-6 concentrations increased significantly in the beta-thalassemic patients compared with control subjects (p=0.01). Serum ferritin levels of beta-thalassemic patients were significantly higher than those of control groups (p<0.05). IL-8 levels correlated with ferritin levels (r=0.694; p<0.05) and the total number of transfusions (r= 0.64; p<0.05). Plasma IL-6 levels in beta-thalassemic patients did not correlate with any clinical, hematological or biochemical parameters. It was also found that plasma IL-8 levels in the patients who had blood transfusions over 100 times were significantly higher than those of under 100 times (p<0.05), whereas there was no statistical difference for IL-6.

Immune functions of peripheral blood lymphocytes have been studied in 38 β-thalassemia major, 12 β-thalassemia trait, and 17 healthy children (Ezer *et al*., 2002). Results showed decrease in CD4C/CD8C ratios in the β-thalassemia major group and no difference according to absolute T-lymphocyte numbers and activated T-cell numbers. The results did not correlate with the tendency to infection. No significant difference was found in humoral immunity that immunoglobulin G, A, M levels of all thalassemic patients were within normal limits and there is no difference between groups according to C3 levels.

Vecchio *et al*. (2002) evaluated the possible occurrence of immunological abnormalities in thalassaemia major patients treated with deferiprone (L1) by longitudinal observational cohort study. Results showed that the absolute number of CD8+ lymphocytes was high and the CD4/CD8 ratio low before L1 treatment; but returned to normal after 3 months of L1 treatment. TNF-α, IL-2 and IL-2sR α were elevated before L1 treatment (11.83±1.75, 11.75±3.91, 1409±621 pg/ml, respectively), while IL-6 was normal (2.58±0.79 pg/ml). After 12 months of treatment, IL-10 was higher than in other previous periods, although always within the normal range. TNF-α, IL-2 and IL-2sR α returned to normal after 12, 6, and 3 months of L1 treatment, respectively.

Endothelial function and serum levels of inflammatory mediators in 67 patients with homozygous β-thalassemia major (aged 24.6±0.7 years) and 71 healthy age and sex matched controls were examined (Aggeli, 2005). Serum levels of IL-6, soluble vascular cell adhesion molecule (sVCAM-1) and soluble intercellular adhesion molecule (sICAM-1) were determined with ELISA. IL-6 levels were significantly higher in patients (3.03±0.31 pg/ml) than controls (1.15±0.15 pg/ml, p<0.01). Similarly,

sVCAM-1 and sICAM-1 levels were significantly higher in patients (513±31 and 368±25.5 ng/ml, respectively) than controls (333±13.8 and 272±14.05 ng/ml, respectively, p<0.01 for both). Such changes suggest a potential role of inflammation and endothelial dysfunction in the complications of the thalassemia.

Amin *et al*. (2005) assessed serum levels of immunoglobulin and complement factors in 68 β-thalassemia major Iranian patients and the same number of controls were selected with matched age and sex without any history of recent or recurrent infections. Results showed that serum levels of IgG, IgM & IgA were significantly higher (P<0.01) and those of C3 and C4 were significantly lower (P<0.01) in thalassemic patients than the controls. It was revealed that, thalassemia patients show much more increase in serum immunoglobulin levels as they get older. Splenectomized patients had higher serum IgG and IgA levels than non-splenectomized patients but had no difference in serum IgM, C3 and C4. Serum ferritin level had no correlation with the changes of humoral immunity; however, patients with serum ferritin level >2500ng/ml had higher serum IgM level.

Morabito *et al*. (2007) investigated the potential relationships among IL-1 α, IL-6 and TNF-α cytokines, several markers of bone turnover and bone mineral density (BMD) in 30 well treated β-thalassemia major (βTM) patients and in 20 healthy subjects, matched for age, sex and BMD. βTM patients showed an altered bone turnover, with increased deoxypyridinoline levels, decreased osteocalcin concentrations and significantly lower lumbar and femoral BMD values as compared to controls. Circulating levels of IL-1α (P<0.0001), TNF-α (P<0.0001) and IL-6 (P<0.05) were all increased in βTM patients as compared with controls.

The immune and neural status of 44 β-thalassemic patients, aged 10–30 years (mean 19.4±4.9), receiving deferiprone L1 as a monotherapy (n=21), or in combination with DFO (n=23), has been followed for 2 years by monitoring the level of immunoglobulins (IgG, IgM, IgA), the level of T and B lymphocytes, the auto antibodies: anti nuclear, anti-double-stranded (anti-ds DNA), anti reticulin (anti-R1), anti-extra nuclear (anti-ENA), anti histone, anti liver-kidney-muscle, anti-smooth muscle, anti-thyroid, anti-mitochondrial antibodies and the C-reactive protein (Tourkantoni *et al*., 2008). Result showed that the percentage of patients with disorders of the immune and nervous system concerned very few cases. None of the patients with pathological findings in their immunological or neurophysiological

examinations presented any signs or symptoms of involvement of the immune or nervous system. The mean serum immunoglobulin concentrations (IgG, IgM, IgA) did not appear to differ between the treatment groups, either before or after treatment. Mean serum immunoglobulin concentrations in neither group differed compared to controls ($p > 0.5$).

Gharagozloo *et al*. (2009) demonstrated that multiple blood transfusion and continuous immune stimulation could be responsible for making a double-faced immune response. Serum samples and peripheral blood mononuclear cells were collected from 28 patients of Iranian β-thalassemia major and 30 age- and sex-matched healthy individuals. Patients with thalassemia showed significantly increased absolute lymphocyte counts compared with the control group. An increased number of activated T cells and higher levels of serum neopterin were also observed in thalassemic, which suggest chronic stimulation of immune system. On the contrary, T-cell proliferation and IL-2, interferon gamma (IFN-γ), and IL-4 production were suppressed in patients compared to controls. Patients with high serum ferritin levels produced significantly less IFN-γ and IL-2, indicating the immunosuppressive effect of iron overload in β- thalassemia patients. The serum levels of TNF-α and absolute counts and percentages of B and T cells were higher in splenectomized patients; however, serum levels of neopterin significantly decreased in splenectomized patients compared to the nonsplenectomized group. Serum TNF-α levels revealed no significant difference in the thalassemia and control groups. Taken together, T lymphocytes express activated phenotype in polytransfused β-thalassemia major patients, while T cell proliferation and effector function are significantly suppressed.

Chapter 3
Materials and methods

3.1 Study design

The present study is a case-control study.

3.2 Setting of the study

This study was carried out at the Hematology Department, Abd El-Aziz El-Rantisy Specialized Pediatric Hospital. Experimental work was carried out at Al Aqsa University laboratory and Palestinian Medical Relief Society in Gaza, Gaza Strip.

3.3 Target population

The target population is β-thalassemic major children from both genders aged 5-12 years old attending Hematology Department at Abd El-Aziz El-Rantisy Hospital. All patients' are in hypertransfusion and they received regular subcutaneous or intramuscular iron-chelating therapy with desferrioxamine according to their weight and level of serum ferritin, ranging between 25-50 mg/kg.

3.4 Study sample

Study sample comprised 86 subjects: all 43 β-thalassemic major children aged 5-12 years old attending Abd El-Aziz El-Rantisy hospital and 43 healthy children as a control group. Cases and controls were matched for age and sex.

3.5 Study period

The study was conducted in the period from December 2009 to September, 2010.

3.6 Exclusion and inclusion criteria

Exclusion criteria

- Patients with splenectomy: three patients were excluded
- Hepatitis B or C infection
- A history of a positive HIV test

- Chronic renal or heart failure
- Recent or active infection at the time of blood sampling

Inclusion criteria

β-thalassemic major children aged 5-12 years old attending Abd El-Aziz El-Rantisy Hospital.

3.7 Ethical consideration

The researcher obtained the necessary approval to conduct the study from Helsinki committee (Annex 1). Coordination with the Ministry of Health was fulfilled (Annex 2). Informed consent was obtained from parents of all the participants. A full explanation about the purpose of the study, assurance about the confidentiality of the blood analysis, and the right to refuse or to participate (Annex 3) in the present study were given.

3.8 Collection of blood Samples and processing

Blood samples were collected by a well trained nurse from each thalassemic children just before a scheduled transfusion of packed red blood cells. Various scientists differ with respect to the necessity for freshly obtained blood samples (Hall *et al*, 1995 and Clarke and Higgins, 2000). Therefore, freshly collected blood is not critical. Five ml venous blood samples were obtained from each subject and divided into EDTA tube (1.0 ml) and vacutainer plain tube (4.0 ml). Vacutainer plain tubes were left for short time to allow blood to clot, and then clear serum samples were obtained by centrifugation at 4000 rpm for 10 minutes. CBC was done in the same day of collection in the laboratory of Palestinian Medical Relief Society in Gaza. The separated serum was placed in five plain tubes, sealed and stored at -20 ºC until the time of performing the analysis. The frozen serum samples were thawed at 4-8˚C then mixed by gentle shaking at room temperature prior to use. Then, the serum samples were used to determine serum levels of IL-1-β, IL-6 and TNF-α cytokines using commercially available ELISA kits according to the instructions of the manufacturer. Immunoglobulins IgG, IgA, IgM, complements 3 and 4 ferritin level and CRP were also determined. All biochemical analyses were done in the laboratory of Palestinian Medical Relief Society in Gaza and Al Aqsa University laboratory.

3.9 Biochemical analysis

3.9.1 Determination of serum Interleukin-6 (IL-6)

Human IL-6 was determined quantitatively by enzyme immunoassay in serum using IBL International GmbH Kit Flughafenstr. 52A, D-22335 Hamburg, Germany (Mindray 96-A microplate reader, Shenzhen 2007-2010).

Principle of the test

1. An anti-human IL-6 coating antibody is adsorbed onto microwells.
2. Human IL-6 present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human IL-6 antibody is added and binds to human IL-6 captured by the first antibody.
3. Following incubation unbound biotinconjugated anti-human IL-6 antibody is removed during a wash step. Streptavidin- HRP is added and binds to the biotinconjugated anti-human IL-6 antibody.
4. Following incubation unbound Streptavidin- HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.
5. A coloured product is formed in proportion to the amount of human IL-6 present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human IL-6 standard dilutions and human IL-6 sample concentration determined.

Reagents provided

One aluminium pouch with a Microwell Plate coated with monoclonal antibody to human IL-6
One vial (100 µl) Biotin-Conjugate anti-human IL-6 monoclonal antibody
One vial (150 µl) Streptavidin-HRP
Two vials human IL-6 Standard, lyophilized, 200 pg/ml upon reconstitution
One vial Control high
One vial Control low
One vial (5 ml) Assay Buffer Concentrate 20x (PBS with 1% Tween 20 and 10% BSA)
One bottle (50 ml) Wash Buffer Concentrate 20x (PBS with 1% Tween 20)
One vial (15 ml) Substrate Solution (tetramethyl-benzidine)
One vial (15 ml) Stop Solution (1M Phosphoric acid)

One vial (0.4 ml) Blue-Dye

One vial (0.4 ml) Green-Dye

One vial (0.4 ml) Red-Dye

Four adhesive Films

Reagent preparation

1. Wash Buffer

Wash Buffer Concentrate 20x (50 ml) was added to 950 ml distilled water.

2. Assay Buffer

Assay Buffer Concentrate 20x (5 ml) was added to 95 ml distilled water.

3. Biotin-Conjugate

A 1:100 dilution of Biotin-conjugate was made in Assay Buffer

4. Streptavidin-HRP

A 1:200 dilution of Streptavidin-HRP was made in Assay Buffer

5. Human IL-6 Standard

lyophilized human IL-6 standard was reconstituted with distilled water.

6. Controls

Three hundred Microlitters distilled water was added to lyophilized controls.

Test protocol

The assay procedure sheets were available with the kit, the application of assay procedure mentioned below:

a. The microwell strips were washed twice with approximately 400 µl Wash Buffer per well with thorough aspiration of microwell contents between washes. The Wash Buffer was allowed to sit in the wells for about 10–15 seconds before aspiration. (An automatic microplate washer was used). After the last wash step, wells and tap microwell strips were emptied on absorbent pad or paper towel to remove excess Wash Buffer. The microwell strips were used immediately after washing.

b. Standard dilution on the microwell plate: one hundred microlitters assay buffer was added to all standard wells. One hundred prepared standard was pipetted into the first wells and standard dilutions were created by transferring one hundred from well to well. one hundred from the last wells was discard.

Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1		2
A	Standard 1	(100.00 pg/ml)	Sample 1
B	Standard 2	(50.00pg/ml)	Sample 2
C	Standard 3	(25.00pg/ml)	Sample 3
D	Standard 4	(12.50pg/ml)	Sample 4
E	Standard 5	(6.25pg/ml)	Sample 5
F	Standard 6	(3.13 pg/ml)	Sample 6
G	Standard 7	(1.56pg/ml)	Sample 7
H	Blank		Sample 8

c. One hundred microlitters assay buffer was added to the blank wells.

d. Fifty microlitters of assay buffer was added to the sample wells.

e. Fifty microlitters of each sample was added to the sample wells.

f. Biotin-Conjugate was prepared.

g. Fifty microlitters of Biotin-Conjugate was added to all wells.

h. Microwell strips were covered with an adhesive film and incubated at room temperature (18 to 25°C) for 2 hours, on a microplate shaker set at 100 rpm.

i. Streptavidin-HRP was prepared.

j. Adhesive film was removed and wells were emptied. Microwell strips were washed 4 times with wash buffer by using an automatic microplate washer.

k. One hundred microlitters of diluted streptavidin-HRP was added to all wells.

l. Microwell strips were covered with an adhesive film and incubated at room temperature (18° to 25°C) for 1 hour, on a microplate shaker set at 100 rpm.

m. Adhesive film was removed and wells were emptied. Microwell strips were washed 4 times with wash buffer by using an automatic microplate washer.

n. One hundred microlitters of TMB substrate solution was pipetted to all wells.

o. The microwell strips were incubated at room temperature (18° to 25°C) for about 10 min. Direct exposure to intense light was avoided.

p. The enzyme reaction was stopped by quickly pipetting one hundred of stop solution into each well.

q. Absorbance was read of each microwell on a spectro-photometer at 450 nm. The plate reader was blanked according to the manufacturer's instructions by using the blank wells. The absorbance of both the samples and the standards was determined.

Calculation of results

The concentration of circulating human IL-6 for each sample was determined from the standard curve (Figure 3.1):

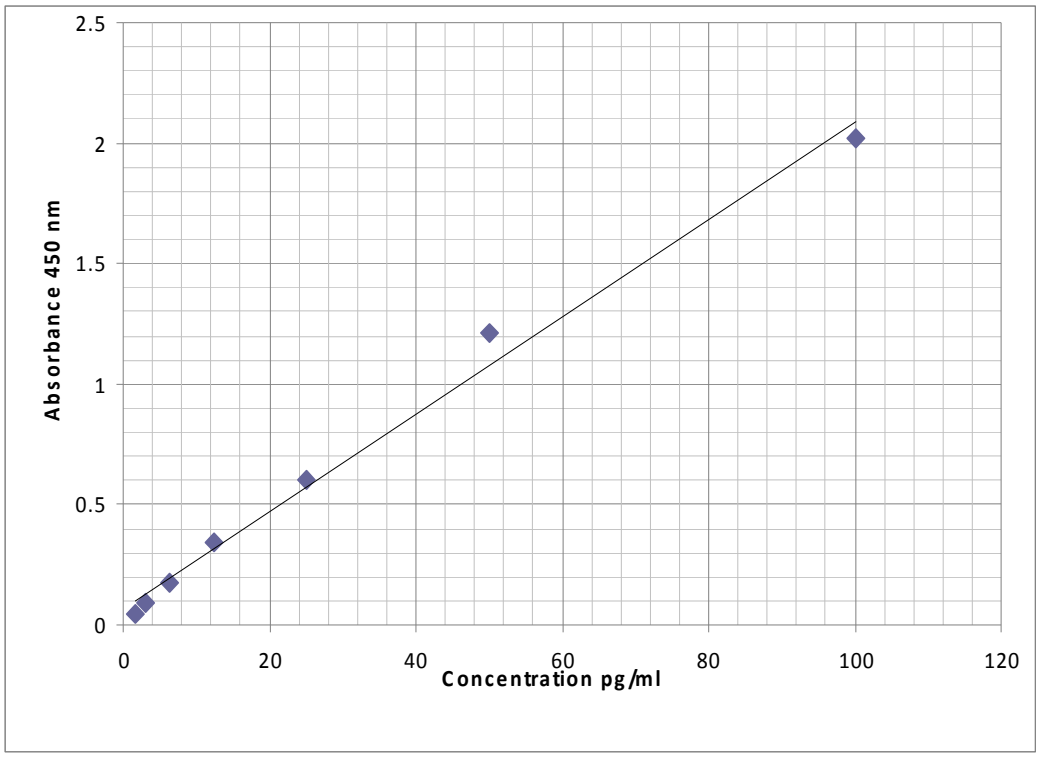

Figure 3.1: Interleukin-6 standard curve.

Expected values

A panel of samples from randomly selected apparently healthy donors (males and females) was tested for human IL-6. The levels measured may vary with the sample collection used. For detected human IL-6 levels see the following Table.

Sample matrix	Number of Samples evaluated	Range (pg/ml)	% Detectable	Mean of detectable (pg/ml)
Serum	40	nd *- 12.7	47.5	5.8

* n.d. = non-detectable, samples measured below the lowest standard point are considered to be non-detectable.

3.9.2 Determination of serum tumor necrosis factor-a (TNF-α)

Human tumor necrosis factor-a (TNF-α) was determined quantitatively by enzyme immunoassay in cell culture supernatants, serum, plasma using IBL International GmbH Kit Flughafenstr. 52A, D-22335 Hamburg, Germany (Mindray 96-A microplate reader ,Shenzhen 2007-2010)

Principle of the test

1. An anti-human TNF-α coating antibody is adsorbed onto microwells.
2. Human TNF-α present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human. TNF-α antibody is added and binds to human TNF-α captured by the first antibody.
3. Following incubation unbound biotin-conjugated anti-human TNF-α antibody is removed during a wash step. Streptavidin- HRP is added and binds to the biotin-conjugated anti-human TNF-α antibody.
4. Following incubation unbound Streptavidin- HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.
5. A coloured product is formed in proportion to the amount of human TNF-α present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human TNF-α standard dilutions and human TNF-α sample concentration determined.

Reagents provided

One aluminium pouch with a Microwell Plate coated with monoclonal antibody to human TNF-α

One vial (100 µl) Biotin-Conjugate anti-human TNF-α polyclonal antibody

One vial (150 µl) Streptavidin-HRP

Two vials human TNF-α Standard lyophilized, 3000 pg/ml upon reconstitution

One vial Control high, lyophilized

One vial Control low, lyophilized

One vial (12 ml) Sample Diluent

One vial (5 ml) Assay Buffer Concentrate 20x (PBS with 1% Tween 20 and 10% BSA)

One bottle (50 ml) Wash Buffer Concentrate 20x (PBS with 1% Tween 20)

One vial (15 ml) Substrate Solution (tetramethyl-benzidine)

One vial (15 ml) Stop Solution (1M Phosphoric acid)

One vial (0.4 ml) Blue-Dye

One vial (0.4 ml) Green-Dye

One vial (0.4 ml) Red-Dye

Four adhesive Films

Reagent Preparation

1. Wash Buffer

Wash Buffer Concentrate 20x (50 ml) was added to 950 ml distilled water.

2. Assay Buffer

Assay Buffer Concentrate 20x (5 ml) was added to 95 ml distilled water.

3. Biotin-Conjugate

A 1:100 dilution of Biotin-conjugate was made in Assay Buffer

4. Streptavidin-HRP

A 1:100 dilution of streptavidin-HRP was made in Assay Buffer

5. Human TNF-a Standard

Lyophilized human TNF-a standard was reconstituted with distilled water.

6. Controls

Five hundred microlitters distilled water was added to lyophilized controls.

Test protocol

The assay procedure sheets were available with the kit, the application of assay procedure mentioned below:

a. The microwell strips were washed twice with approximately 400 µl wash buffer per well with thorough aspiration of microwell contents between washes. The wash buffer was allowed to sit in the wells for about 10–15 seconds before aspiration. An automatic microplate washer was used. After the last wash step, wells and tap microwell strips were emptied on absorbent pad or paper towel to remove excess wash buffer. The microwell strips were used immediately after washing.

b. Standard dilution on the microwell plate. One hundred microlitters sample diluent was added to all standard wells. One hundred prepared standard was pipetted into the first wells and standard dilutions were created by transferring one hundred from well to well. One hundred from the last wells was discard.

Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	**2**
A	Standard (1500 pg/ml)	Sample 1
B	Standard 2 (750 pg/ml)	Sample 2
C	Standard 3 (375 pg/ml)	Sample 3
D	Standard 4 (188 pg/ml)	Sample 4
E	Standard 5 (94 pg/ml)	Sample 5
F	Standard 6 (47 pg/ml)	Sample 6
G	Standard 7 (23 pg/ml)	Sample 7
H	Blank	Sample 8

c. One hundred microlitters Sample Diluent was added to the blank wells.

d. Fifty microlitters of Sample Diluent was added to the sample wells.

e. Fifty microlitters of each sample was added to the sample wells.

f. Biotin-Conjugate was prepared.

g. Fifty microlitters of Biotin-Conjugate was added to all wells.

h. Microwell strips were covered with an adhesive film and incubated at room temperature (18 to 25°C) for 2 hours, on a microplate shaker set at 100 rpm.

i. Streptavidin-HRP was prepared.

j. Adhesive film was removed and wells were emptied. Microwell strips were washed 6 times with wash buffer by using an automatic microplate washer.

k. One hundred microlitters of diluted streptavidin-HRP was added to all wells.

l. Microwell strips were covered with an adhesive film and incubated at room temperature (18° to 25°C) for 1 hour, on a microplate shaker set at 100 rpm.

m. Adhesive film was removed and wells were emptied. Microwell strips were washed 6 times with Wash Buffer by using an automatic microplate washer.

n. One hundred microlitters of TMB Substrate Solution was pipetted to all wells.

o. The microwell strips were incubated at room temperature (18° to 25°C) for about 10 min. Direct exposure to intense light was avoided.

p. The enzyme reaction was stopped by quickly pipetting one hundred of Stop Solution into each well.

q. Absorbance was read of each microwell on a spectro-photometer at 450 nm. The plate reader was blanked according to the manufacturer's instructions by using the blank wells. The absorbance of both the samples and the standards was determined.

Calculation of results

The concentration of circulating human TNF-α for each sample was determined from the standard curve (Figure 3.2):

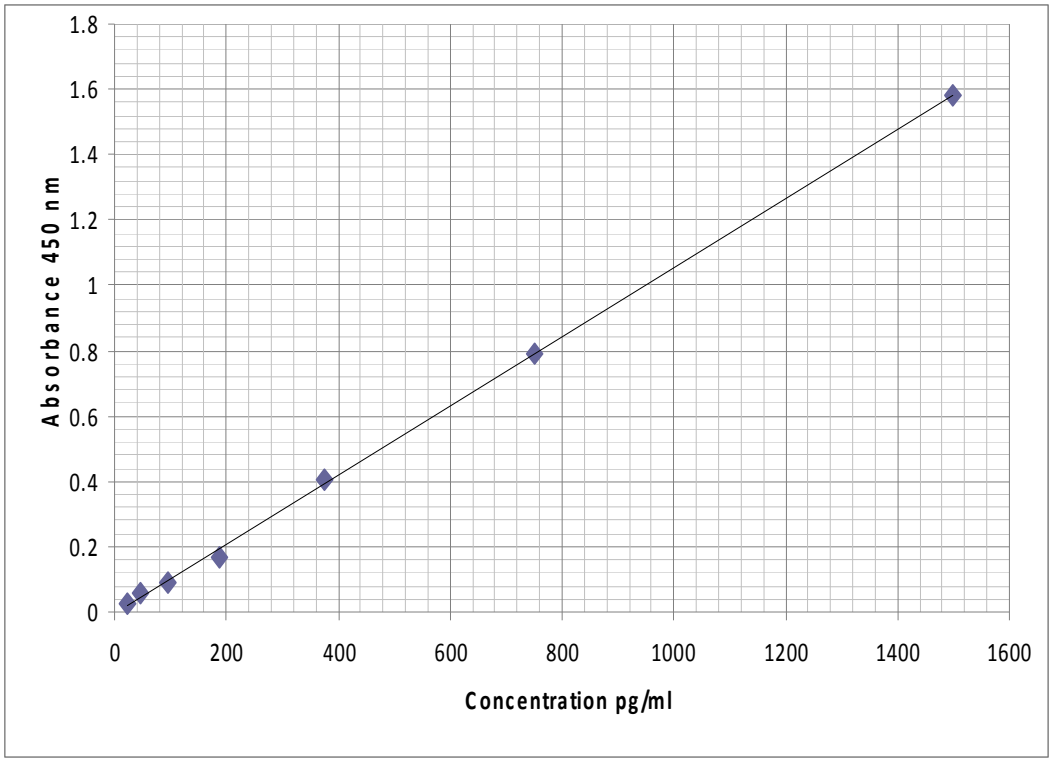

Figure 3.2 tumor necrosis factor-α standard curve.

Expected values

A panel of 40 sera samples from randomly selected apparently healthy donors (males and females) was tested for human TNF-α. There were no detectable human TNF-α levels found. Elevated human TNF-α levels depend on the type of immunological disorder.

3.9.3 Determination of serum interleukin-1β (IL-1β)

Human interleukin 1β was determined quantitatively by enzyme immunoassay in cell culture supernatants, urine and serum using IBL International GmbH Kit Flughafenstr. 52A, D-22335 Hamburg, Germany. (Mindray 96-A microplate reader, Shenzhen 2007-2010)

Principle of the test

1. An anti-human IL-1β coating antibody is adsorbed onto microwells.

2. Human IL-1β present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human IL-1 β antibody is added and binds to human IL-1 β captured by the first antibody.

3. Following incubation unbound biotin-conjugated anti-human IL-1 β antibody is removed during a wash step. Streptavidin- HRP is added and binds to the biotin-conjugated anti-human IL-1 β antibody.
4. Following incubation unbound Streptavidin- HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.
5. A coloured product is formed in proportion to the amount of human IL-1β present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human IL-1α standard dilutions and human IL-1β sample concentration determined.

Reagents provided

One aluminium pouch with a Microwell Plate coated with monoclonal
antibody to human IL-1 β
One vial (100 µl) Biotin-Conjugate anti-human IL-1 β polyclonal antibody
One vial (150 µl) Streptavidin-HRP
Two vials human IL-1 β Standard lyophilized, 500 pg/ml upon reconstitution
One vial Control high, lyophilized
One vial Control low, lyophilized
One vial (12 ml) Sample Diluent
One vial (5 ml) Assay Buffer Concentrate 20x (PBS with 1% Tween 20 and 10% BSA)
One bottle (50 ml) Wash Buffer Concentrate 20x (PBS with 1% Tween 20)
One vial (15 ml) Substrate Solution (tetramethyl-benzidine)
One vial (15 ml) Stop Solution (1M Phosphoric acid)
One vial (0.4 ml) Blue-Dye
One vial (0.4 ml) Green-Dye
One vial (0.4 ml) Red-Dye
Four adhesive Films

Reagent preparation

1. Wash Buffer

Wash Buffer Concentrate 20x (50 ml) was added to 950 ml distilled water.

2. Assay Buffer

Assay Buffer Concentrate 20x (5 ml) was added to 95 ml distilled water.

3. Biotin-Conjugate

A 1:100 dilution of Biotin-conjugate was made in Assay Buffer

4. Streptavidin-HRP

A 1:200 dilution of Streptavidin-HRP was made in Assay Buffer

5. Human IL-1β **Standard**

Lyophilized human IL-1β standard was reconstituted with distilled water.

6. Controls

Three hundred Microlitters distilled water was added to lyophilized controls.

Test protocol

The assay procedure sheets were available with the kit, the application of assay procedure mentioned below.

a. The microwell strips were washed twice with approximately 400 µl wash buffer per well with thorough aspiration of microwell contents between washes. The wash buffer was allowed to sit in the wells for about 10–5 seconds before aspiration. An automatic microplate washer was used. After the last wash step, wells and tap microwell strips were emptied on absorbent pad or paper towel to remove excess wash buffer. The microwell strips were used immediately after washing.

b. Standard dilution on the microwell plate: One hundred microlitters sample diluent was added to all standard wells. One hundred prepared standard was pipetted into the first wells and standard dilutions were created by transferring one hundred from well to well. One hundred from the last wells was discarded.

Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2
A	Standard 1 (250.0 pg/ml)	Sample 1
B	Standard 2 (125 pg/ml)	Sample 2
C	Standard 3 (62.5 pg/ml)	Sample 3
D	Standard 4 (31.3 pg/ml)	Sample 4
E	Standard 5 (15.6 pg/ml)	Sample 5
F	Standard 6 (7.8pg/ml)	Sample 6
G	Standard 7 (3.9 pg/ml)	Sample 7
H	Blank	Sample 8

c. One hundred microlitters sample diluent was added to the blank wells.

d. Fifty microlitters of sample diluent was added to the sample wells.

e. Fifty microlitters of each sample was added to the sample wells.

f. Biotin-Conjugate was prepared.

g. Fifty microlitters of Biotin-Conjugate was added to all wells.

h. Microwell strips were covered with an adhesive film and incubated at room temperature (18 to 25°C) for 2 hours, on a microplate shaker set at 100 rpm.

i. Streptavidin-HRP was prepared.

j. Adhesive film was removed and wells were emptied. Microwell strips were washed 3 times with wash buffer by using an automatic microplate washer.

k. One hundred microlitters of diluted Streptavidin-HRP was added to all wells.

l. Microwell strips were covered with an adhesive film and incubated at room temperature (18° to 25°C) for 1 hour, on a microplate shaker set at 100 rpm.

m. Adhesive film was removed and wells were emptied. Microwell strips were washed 3 times with Wash Buffer by using an automatic microplate washer.

n. One hundred Microlitters of TMB substrate solution was pipetted to all wells.

o. The microwell strips were incubated at room temperature (18° to 25°C) for about 10 min. Direct exposure to intense light was avoided.

p. The enzyme reaction was stopped by quickly pipetting 100 µl of stop solution into each well.

q. Absorbance was read of each microwell on a spectro-photometer at 450 nm.

The plate reader was blanked according to the manufacturer's instructions by using the blank wells. The absorbance of both the samples and the standards was determined.

Calculation of results

The concentration of circulating human IL-1β for each sample was determined from the standard curve (Figure 3.3):

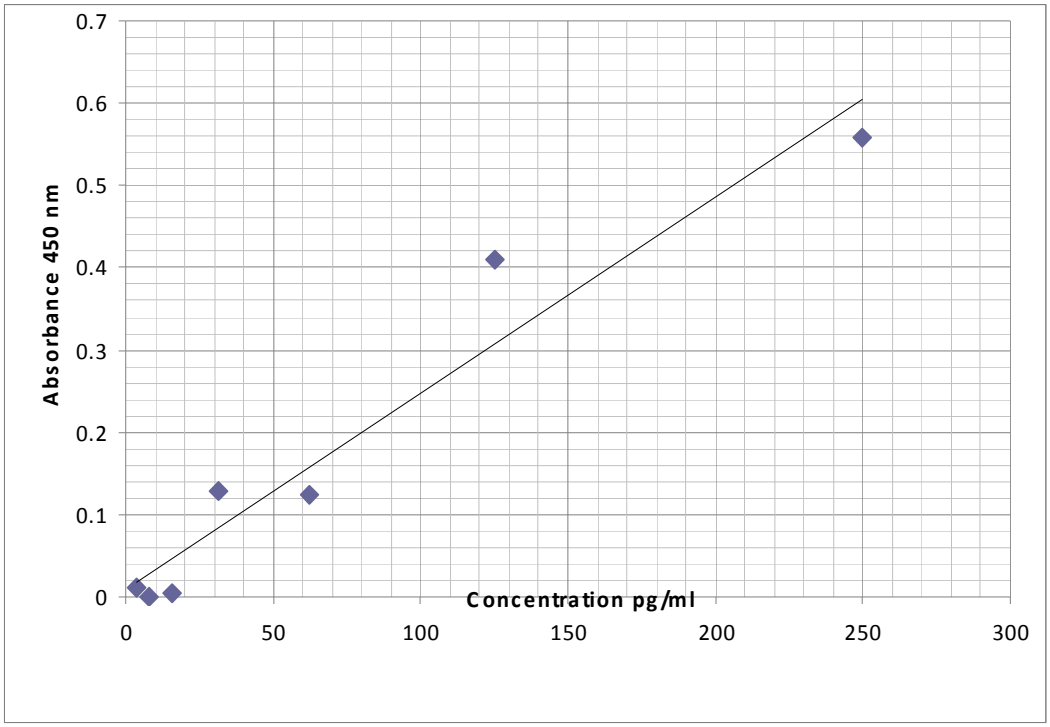

Figure 3.3: interleukin-1β standard curve.

Expected values

Panels of 40 serum as well as citrate and heparin plasma samples from randomly selected apparently healthy donors (males and females) were tested for human IL-1β. One positive citrate plasma sample was detected (8.7 pg/ml). Elevated human IL-1β levels depend on the type of immunological disorder.

3.9.4 Determination of serum immunoglobulin G (IgG)

Immunoglobulin G was determined by quantitative turbidimetric assay in human serum or plasma using Globe Diagnostics S.r.l. Via Galileo Galilei 38, 20096 Seggiano di Pioltello (Milan), Italy (Tietz, 1999).

Principle of the test

IgG is a quantitative turbidimetric assay for the measurement of IgG in human serum or plasma. Anti-human IgG antibodies form insoluble complexes when mixed with samples containing IgG. The scattering light of the immunocomplexes depends of the IgG concentration in the patient sample, and can be quantified by comparison from a calibrator of known IgG concentration.

Reagents provided

Reagent A : Goat antibodies anti-human IgG, Tris buffer 20 mmol/L, pH 8.2. Sodium azide 0.95 g/L. Reagent A is ready to use.

Test protocol

1. The reagent and the photometer (cuvette holder) were prewarmed to 37ºC.

2. The instrument was zeroed using distilled water at 540 nm.

3. Pipette into a cuvette:

Sample/Calibrator	7 µL
Reagent (RA)	1.0 mL

4. It was mixed well and the cuvette was inserted into the photometer. The absorbance (A) was recorded after 2 minutes of the sample or calibrator addition.

Calculation of results

The different absorbance values (A) were plotted against the IgG concentration of each calibrator dilution. IgG concentration in the sample is calculated by interpolation of its (A) value in the calibration curve.

Reference values

Adults	700-1600 mg/dL
Newborn	299-852 mg/dL

3.9.5 Determination of serum immunoglobulin M (IgM)

Immunoglobulin M was determined by quantitative turbidimetric assay in human serum or plasma using Globe Diagnostics S.r.l. Via Galileo Galilei 38, 20096 Seggiano di Pioltello (Milan), Italy (Tietz, 1999).

Principle of the test

IgM is a quantitative turbidimetric assay for the measurement of IgM in human serum or plasma. Anti-human IgM antibodies form insoluble complexes when mixed with samples containing IgM. The scattering light of the immunocomplexes depends of the IgM concentration in the patient sample, and can be quantified by comparison from a calibrator of known IgM concentration.

Reagents provided

Reagent A : Goat antibodies anti-human IgM, Tris buffer 20 mmol/L, pH 8.2. Sodium azide 0.95 g/L. Reagent A is ready to use.

Test protocol

1. The reagent and the photometer (cuvette holder) were Prewarmed to 37ºC.

2. The instrument was zeroed using distilled water at 340 nm.

3. Pipette into a cuvette:

Sample/Calibrator	10 µL
Reagent (RA)	1.0 mL

4. It was mixed well and the cuvette was inserted into the photometer. The absorbance (A) was recorded after 2 minutes of the sample or calibrator addition.

Calculation of results

The different absorbance values (A) were plotted against the IgM concentration of each calibrator dilution. IgM concentration in the sample is calculated by interpolation of its (A) value in the calibration curve.

Reference values

Adults	40-230 mg/dL
Newborn	5-30 mg/dL

3.9.6 Determination of serum immunoglobulin A (IgA)

Immunoglobulin A was determined by quantitative turbidimetric assay in human serum or plasma using Globe Diagnostics S.r.l. Via Galileo Galilei 38, 20096 Seggiano di Pioltello (Milan), Italy (Tietz, 1999).

Principle of the test

IgA is a quantitative turbidimetric assay for the measurement of IgA in human serum or plasma. Anti-human IgA antibodies form insoluble complexes when mixed with samples containing IgA. The scattering light of the immunocomplexes depends of the IgA concentration in the patient sample, and can be quantified by comparison from a calibrator of known IgA concentration.

Reagents provided

Reagent A: Goat antibodies anti-human IgA, Tris buffer 20 mmol/L, pH 8.2. Sodium azide 0.95 g/L. Reagent A is ready to use.

Test protocol

1. The reagent and the photometer (cuvette holder) were Prewarmed to 37ºC.

2. The instrument was zeroed using distilled water at 340 nm.

3. Pipette into a cuvette:

Sample/Calibrator	7 µL
Reagent (RA)	1.0 mL

4. It was mixed well and the cuvette was inserted into the photometer. The absorbance (A) was recorded after 2 minutes of the sample or calibrator addition.

Calculation of results

The different absorbance values (A) were plotted against the IgA concentration of each calibrator dilution. IgA concentration in the sample is calculated by interpolation of its (A) value in the calibration curve.

Reference values

Adults	70-400 mg/dL
Newborn	0-2.2 mg/dL

3.9.7 Determination of serum complement C3

Complement component 3 was determined by quantitative turbidimetric assay in human serum or plasma using Globe Diagnostics S.r.l. Via Galileo Galilei 38, 20096 Seggiano di Pioltello (Milan), Italy (Tietz, 1999).

Principles of the test

C3 is a quantitative turbidimetric assay for the measurement of the component complement C3 in human serum or plasma. Anti-human C3 antibodies form insoluble complexes when mixed with samples containing C3. The scattering light of the immunocomplexes depends of the C3 concentration in the patient sample, and can be quantified by comparison from a calibrator of known C3 concentration.

Reagents provided

Reagent A: Goat antibodies anti-human C3, Tris buffer 20 mmol/L, pH 8.2. Sodium azide 0.95 g/L. Reagent A is ready to use.

Test protocol

1. The reagent and the photometer (cuvette holder) were Prewarmed to 37ºC.

2. The instrument was zeroed using distilled water at 340 nm.

3. Pipette into a cuvette:

Sample/Calibrator	10 µL
Reagent (RA)	1.0 mL

4. It was mixed well and the cuvette was inserted into the photometer. The absorbance (A) was recorded after 2 minutes of the sample or calibrator addition.

Calculation of results

The different absorbance values (A) were plotted against the C3 concentration of each calibrator dilution. C3 concentration in the sample is calculated by interpolation of its (A) value in the calibration curve.

Reference values

Adults	90-180 mg/dL
Newborn	70-196 mg/dL

3.9.8 Determination of serum complement C4

Complement component 4 was determined by quantitative turbidimetric assay in human serum or plasma using Globe Diagnostics S.r.l. Via Galileo Galilei 38, 20096 Seggiano di Pioltello (Milan), Italy (Tietz, 1999).

Principle of the test

C4 is a quantitative turbidimetric assay for the measurement of the component complement C4 in human serum or plasma. Anti-human C4 antibodies form insoluble complexes when mixed with samples containing C4. The scattering light of the immunocomplexes depends of the C4 concentration in the patient sample, and can be quantified by comparison from a calibrator of known C4 concentration.

Reagents provided

Reagent A: Goat antibodies anti-human C4, Tris buffer 20 mmol/L, pH 8.2. Sodium azide 0.95 g/L. Reagent A is ready to use.

Test protocol

1. The reagent and the photometer (cuvette holder) were Prewarmed to 37ºC.

2. The instrument was zeroed using distilled water at 340 nm.

3. Pipette into a cuvette:

Sample/Calibrator	25 µL
Reagent (RA)	1.0 mL

4. It was mixed well and the cuvette was inserted into the photometer. The absorbance (A) was recorded after 2 minutes of the sample or calibrator addition.

Calculation of results

The different absorbance values (A) were plotted against the C4 concentration of each calibrator dilution. C4 concentration in the sample is calculated by interpolation of its (A) value in the calibration curve.

Reference values

Adults	10-40 mg/dL
Newborn	13-38 mg/dL

3.9.9 Determination of C-reactive protein (CRP)

CRP were determined using CRP latex kit (Teco Diagnstics).

Principle of the assay

The CRP reagent kit is based on an immunological reaction between CRP antisera bound to biologically inert latex particles and CRP In the test specimen. When serum

containing greater than 0.8 mg/ dl CRP is mixed with the latex reagent, visible agglutination occurs.

Reagents and materials provided

1- CRP latex Reagent: A suspension of uniform polystyrene particles coated with monospecific antihuman CRP In glycine buffer, pH 8.8 ±0.5,reagent sensitivity adjusted to approximately 0.8 mg/ dl. Mixed well before using .

2- CRP positive control serum: A stabilized prediluted human serum containing more than 0.8 mg/ dl CRP.

3- CRP negative Control serum: A stabilized prediluted human serum non-reactive with the test reagent.

4- Glycine-saline buffer (20x): pH 8.2 ± 0.1: A diluent containing 0.1M glycine and 0.15M NaCL. Buffer was diluted according to instructions on the label before using.

5- Reaction slide

6- Pipette/stir sticks

Procedure

Qualitative test

1. Reagent and samples wear allowed reaching to room temperature.

2. Fifty microlitters of the CRP positive and negative control was placed into separate circle field on the slide test. The remaining field was used for test specimens.

3. CRP-latex reagent was shacked gently and a drop was added to each test field.

4. Both drops were mixed and spread over the entire surface of the test field by stir stick.

5. The slide was rotated for 3 minutes and it was read immediately under direct light.

Quality Control

1- CRP positive and negative control were included in each test batch.

2- Acceptable performance was indicated when a uniform milky suspension with no agglutination was observed with the CRP negative control and agglutination with large aggregates was observed with the CRP positive control.

Interpretation

Negative result: A negative reaction is indicated by a uniform milky suspension with no agglutination as observed with the CRP negative control.

Positive result: A positive reaction is indicated by any observable agglutination in the reaction mixture. The specimen reaction was compared to the CRP of the negative control.

3.10 Hematological analysis

3.10.1 Complete blood count (CBC)

A complete system of reagents of control and calibrator, Cell-Dyn 1800 was used to determine complete blood count (CBC) of children in Palestinian Medical Relief Society laboratory in Gaza. (ABBOTT laboratories, 2006).

3.10.2 Determination of serum ferritin

In the present study serum ferritin was determined using a Microparticale Enzyme Immunoassay Technology. For this purpose Abbot fully–automated Axsym immunoassay analyzer ferritin assay system (Abbott laboratories, USA,1997) was used.

Sampling center

- Sampel and all AXYM Ferritin reagents required for one test are pipette by the sampling probe into various wells of a reaction vessel (RV).
- Sample is pipette into one well of the RV.
- Anti-Ferritin coated microparticles, anti-ferritin alkaline phosphatase conjugate, specimen diluents and tris buffer are pipetted into another well of the RV.

The RV is immediately transferred into the processing center. Further pipetting is done in the processing center with the processing probe.

Processing center

- An aliquot of the specimen diluent, conjugate, microparticles and TRIS buffer mixture is pipette and mixed with the sample.
- The ferritin, enzyme-labeled antibody and microparticles bind forming an antibody-antigen-antibody complex.

- An aliquot of the reaction mixture containing the antibody-antigen-antibody complex bound to the microparticles is transferred to the matrix cell. The microparticles bind irreversibly to the glass fiber matrix.
- The matrix cells washed to remove unbound materials.
- The substrate, 4-methylumbelliferyl phosphate, is added to the matrix cell and the fluorescent product is measured by the MEIA optical assembly.

3.11 Statistical analysis

Data were computer analyzed using SPSS/ PC (statistical package for social science Inc. Chicago, Illinois USA, version 13.0) statistical package. Simple distribution of the study variables and the cross tabulation were applied. Means were compared by independent-sample *t*-test. Chi-square (χ^2) was used. Percentage difference was calculated according to the formula:

$$\text{Percentage difference} = \frac{\text{mean of patients–mean of controls}}{\text{mean of control}}$$

Probability values (p) were obtained from the students table of "*t* " and significance was at $p < 0.05$.

Graphs were plotted using Microsoft Office Excel 2003.

Chapter 4
Results

4.1 General characteristics of the study population

The present study is a case-control included 86 children from both genders: 43 healthy controls and 43 β-thalassemic major on long-term hypertransfusion therapy and iron chelation with desferrioxamine either subcutaneous or intramuscular. The average age of the study population was 7.9±2.2 years. Study population comprised 24 (55.8%) males and 19 (44.2%) females.

4.2 Allergy and the way of iron chelation therapy in thalassemic patients

Table 4.1 illustrates allergic reaction and the way of discharge of iron chelator. Most of cases 29 (67.4%) had allergic reactions. Discharge of iron chelator was intramuscular in 25 (58.1%) patients and subcutaneous in 17 (39.5%) patients. However, only one patient (2.3%) refused iron chelation therapy.

Table 4.1. Allergic reaction and iron chelator therapy in the cases (n=43)

Character	Frequency	%
Allergic reaction		
Yes	29	67.4
No	14	32.6
Iron chelator		
Subcutaneous	17	39.5
Intramuscular	25	58.1
None	1	2.3

4.3 Hematological parameters

4.3.1 Primary and secondary blood indices

Primary and secondary blood indices of the cases and the controls are summarized in table 4.2. For the primary blood indices, the mean red blood cell counts (RBC) were significantly decreased in cases compared to controls (3.3±0.5 V.s 4.5±0.3X10^6 cell/µl, % difference=-26.7, p=0.000). In parallel, hemoglobin level was also significantly decreased in cases compared to controls (8.3±1.1 V.s 11.9±0.9 g/dl, % difference=-30.3, p=0.000). Hematocrit recorded significant decrease in the cases compared to the controls showing % difference of -34.7 (23.5±3.4 V.s 36.0±2.2 , p=0.000). Secondary blood indices including MCV, MCH, and RDW were also found to be significantly lower in cases compared to controls registering % differences of -12.0, -6.0, and -50.7, (70.5±5.6 fl, 24.9±1.6 pg, and 18.6±9.0 V.s 80.1±4.3 fl, 26.5±1.6 pg, and 37.7±7.0 respectively, p=0.000 each). In contrast MCHC was significantly higher in cases compared to controls (35.3±2.0 V.s 33.0±1.3 g/dl, % differences=7.0 and p=0.000).

Table 4.2. Primary and secondary blood indices of the study population

CBC profile	Control mean±SD	βTM mean±SD	% difference	t	P-value
RBCs (X10^{6}cell/µl)	4.5±0.3	3.3±0.5	-26.7	-13.609	0.000
Range *(min-max)*	*(3.87-5.09)*	*(2.29-4.44)*			
HGB (g/dl)	11.9±0.9	8.3±1.1	-30.3	-16.643	0.000
Range *(min-max)*	*(10.0-13.9)*	*(6.1-10.6)*			
HCT (%)	36.0±2.2	23.5±3.4	-34.7	-20.380	0.000
Range *(min-max)*	*(31.0-40.1)*	*(16.2-31.0)*			
MCV (fl)	80.1±4.3	70.5±5.6	-12.0	-8.873	0.000
Range *(min-max)*	*(71.0-88.5)*	*(55.7-81.2)*			
MCH (pg)	26.5±1.6	24.9±1.6	-6.0	-4.634	0.000
Range *(min-max)*	*(23.1-29.3)*	*(20.6-27.5)*			
MCHC (g/dl)	33.0±1.3	35.3±2.0	7.0	6.412	0.000
Range *(min-max)*	*(30.9-37.5)*	*(31.5-40.1)*			
RDW (fl)	37.7±7.0	18.6±9.0	-50.7	-10.918	0.000
Range *(min-max)*	*(13.1-44.2)*	*(12.5-55.9)*			

βTM: β-thalassemia major, RBCs: Red blood cells, HGB: Hemoglobin, Hct: Heamtocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, RDW: Red blood cell distribution width.
All values were expressed as mean ± SD.
P<0.05: significant.

4.3.2 White blood cells and platelets

Table 4.3 demonstrates total and differential white blood cells count **(**WBCs), and blood platelets **(**PLT) in cases and controls. White blood cell count was significantly increased in cases compared to the controls (8.9±2.1 V.s 7.9±2.0 X103cell/µl, % difference=12.7, p=0.025). For differential white blood cells, lymphocytes was also elevated in cases compared to controls (3.6±0.9 V.s 3.1±0.79, % difference=16.1, p=0.003). The other differential white blood cells (MID and neutrophils) were increased in cases compared to controls (0.8±0.3, 4.6±1.3 V.s 0.7±0.2, 4.1±1.5, % difference=14.3 and 12.2, respectively). However such changes were not significant (p=0.102 and p=0.277). Like differential white blood cells, blood platelets were slightly increased in cases compared to controls (370.3±107.2 V.s 345.9±58.6, % difference=7.1). However, this change was not also significant (p=0.192).

Table 4.3. Total and differential white blood cells and blood platelets in the study population

WBC profile	Control mean±SD	βTM mean±SD	% difference	t	P-value
WBCs ($X10^3$cell/µl)	7.9±2.0	8.9±2.1	12.7	2.278	0.025
Range *(min-max)*	*(3.7-11.7)*	*(4.4-13.5)*			
LYM ($X10^3$cell/µl)	3.1±0.79	3.6±0.9	16.1	3.044	0.003
Range *(min-max)*	*(1.6- 4.7)*	*(1.6-5.4)*			
MID ($X10^3$cell/µl)	0.7±0.2	0.8±0.3	14.3	1.654	0.102
Range *(min-max)*	*(0.3-1.1)*	*(0.3-1.6)*			
NEUT($X10^3$cell/µl)	4.1±1.5	4.6±1.3	12.2	1.094	0.277
Range *(min-max)*	*(1.5-7.2)*	*(1.9-7.3)*			
PLT ($X10^3$cell/µl)	345.9±58.6	370.3±107.2	7.1	1.315	0.192
Range *(min-max)*	*(204.0-459.0)*	*(203.0-803.0)*			

βTM: β-thalassemia major, WBCs: white blood cells, LYM: lymphocyte, MID: may include less frequently occurring and rare cells correlating to monoctes, eosinophils, basophils, blasts and other precursor white cells, NEUT: neutrophils, PLT: Blood platelets.
All values were expressed as mean ± SD.
p> 0.05: not significant, p<0.05: significant.

4.3.3 Serum ferritin level among the study population

Table 4.4. points out that the mean serum ferritin level in patients were significantly higher than that in controls (3138.0±1041.5 V.s 17.3±2.5 ng/ml, % difference=18038.7 and p=0.000).

Table 4.4. Serum ferritin level (ng/ml) in controls and cases

Ferritin level	Control mean±SD	βTM mean±SD	% difference	t	P-value
Ferritin	17.3±2.5	3138.0±1041.5	18038.7	19.648	0.000
Range *(min-max)*	*(13.0-20.7)*	*(1380.0-5244.0)*			

βTM: β-thalassemia major.
All values were expressed as mean ± SD.
P<0.05: significant.

4.4 Immunological status in β-thalassemia major patients

4.4.1 Complement 3 and complement 4 levels in the study population

The mean levels of C3 and C4 in cases and controls are presented in Table 4.5. The mean level of C3 in cases (118.7±12.4 mg/dL) was found to be significantly lower than that in controls (136.6 ±23.3 mg/dL) showing percentage difference of -13.1% p=0.000. Similarly, C4 showed a significant decrease in cases as compared to controls (49.3±13.4 V.s 62.3±21.6 mg/dL, % difference=-20.9 and p=0.001).

Table 4.5. Complement (C3) and complement (C4) levels (mg/dL) in study population

Complement	Control (n=43) mean±SD	βTM (n=43) mean±SD	% difference	t	P-value
C_3 Range *(min-max)*	136.6 ±23.3 *(101-245)*	118.7±12.4 *(93-153)*	- 13.1	-4.449	0.000
C_4 Range *(min-max)*	62.3±21.6 *(28.0-118.0)*	49.3±13.4 *(24-80)*	-20.9	-3.347	0.001

βTM β-thalassemia major.
All values were expressed as mean ± SD.
P<0.05: significant.

4.4.2 Immunoglobulins IgG, IgM and IgA concentrations in controls and cases

Table 4.6 illustrates the mean concentrations of IgG, IgM and IgA in cases and controls. The concentrations of IgG was significantly decreased in cases compared to controls showing percentage difference of -16.7 and p=0.002 (1783.6±335.7 V.s 2141.2±661.6). On the other hand, no significant change was observed in the mean levels of IgM and IgA between cases and controls (228.8±62.1 V.s 230.5±74.2 mg/dL and 297.9±121.9 V.s 307.9±106.3 mg/dL, p=0.909 and p=0.684, respectively).

Table 4.6. Immunoglobulins IgG, IgM and IgA concentrations (mg/dL) in controls and cases

Immunoglobulin	Control mean±SD	βTM mean±SD	% difference	t	P-value
IgG Range *(min-max)*	2141.2±661.6 *(1066.0-4796.0)*	1783.6±335.7 *(1178.0-3332.0)*	-16.7	-3.161	0.002
IgM Range *(min-max)*	230.5±74.2 *(64.0-492.0)*	228.8±62.1 *(120.0-377.0)*	-0.7	-0.115	0.909
IgA Range *(min-max)*	307.9±106.3 *(130.0-546.0)*	297.9±121.9 *(152.0-844.0)*	-3.3	-0.408	0.684

βTM: β-thalassemia major.
All values were expressed as mean ± SD.
P>0.05: not significant, P<0.05: significant.

4.4.3 Serum cytokines in the study population

As illustrated in Table 4.7, the tested cytokines were TNF-α, IL-6 and IL-1β of the cases and the controls. In general cytokines were mostly undetectable in both cases and controls. The numbers of cases with undetected TNF-α and IL-6 were 42 (97.7%) and 38 (88.4%) compared to the controls of 28 (65.1%) and 41 (95.3%). Interleukin-1β showed completely undetectable levels in both cases and controls.

Table 4.7. Cytokines levels (pg/ml) in the study population

Cytokine	Control (n=43)		βTM (n=43)	
	Detectable	Undetectable	Detectable	Undetectable
TNF-α No. (%) Mean *(min-max)*	15 (34.9) 27.9 *(23.6-36.8)*	28 (65.1)	1 (2.3) 26.6	42 (97.7)
IL-6 No. (%) Mean *(min-max)*	2 (4.7) 4.4 *(2.4-6.4)*	41 (95.3)	5 (11.6) 2.6 *(1.6-5.8)*	38 (88.4)
IL-1β No. (%) Mean *(min-max)*	-	43 (100)	-	43 (100)

βTM: β-thalassemia major, TNF-α: tumor necrosis factor-α, IL-6: interleukin-6, IL-1β: Interleukin-1β.

4.4.4 C-reactive protein (CRP) of study population

Positive and negative status of CRP are presented in Table 4.8. The number of cases showed positive status of CRP 12 (27.9%) was higher than controls 6 (14.0%). The controls and patients with negative CRP were 37 (86.0%) and 31 (72.1%), respectively. The chi square test showed no significant difference

between controls and cases in term of positive and negative CRP status (χ^2=2.529 and p=0.112, respectively).

Table 4. 8. CRP in controls and cases

CRP	Control (n=43)	βTM (n=43)	χ^2	P-value
Positive No. (%)	6 (14.0)	12 (27.9)	2.529	0.112
Negative No. (%)	37 (86.0)	31 (72.1)		

βTM: β-thalassemia major.
P>0.05: not significant.

4.5 Allergic reactions in relation to the studied immunological parameters

4.5.1 Allergic reactions in relation to total and differential white blood cells

The relation between allergic reactions and WBC, MID, LYMP and NEUT of the patients is provided in Table 4.9. The independent t-test showed that patients with allergic reactions had decreased mean levels of WBC, MID, LYMP and NEUT compared to non allergic ones (8.5±2.2, 0.74±0.3, 3.4±0.9 and 4.3±1.3 V.s 9.9±1.6, 0.8±0.2, 4.0±0.9, 5.1±1.1). These changes were significant for WBC and LYMP (t=2.098,p=0.042 and t=2.007, p=0.050, respectively) indicating that allergic reactions are mostly related to WBC and LYMP. The decrease in neutrophil was close to the significancy (t=1.816, p=0.077) and that of MID was not significant (t=0.943, p=0.351).

Table 4.9. Allergic reactions in relation to WBC, MID, LYMP and NEUT

	Allergic reaction			
WBC profile	**Yes (n= 29)**	**No (n= 14)**	**t**	**P-value**
WBC($X10^3$cell/µl) **Mean ±SD**	8.5±2.2	9.9±1.6	2.098	0.042
MID($X10^3$cell/µl) **Mean ±SD**	0.74±0.3	0.8±0.2	0.943	0.351
LYMP($X10^3$cell/µl) **Mean ±SD**	3.4±0.9	4.0±0.9	2.007	0.050
NEUT($X10^3$cell/µl) **Mean ±SD**	4.3±1.3	5.1±1.1	1.816	0.077

WBCs: white blood cells, MID: may include less frequently occurring and rare cells correlating to monocytes, eosinophils, basophils, blasts and other precursor white cells, LYM: lymphocyte, NEUT: neutrophil.
All values were expressed as mean ± SD.
p> 0.05: not significant.
p<0.05: significant.

4.5.2. Allergic reactions in relation to ferritin level

Allergic reactions in relation to ferritin level is shown in table 4.10. Although ferritin level is higher in patients who had allergy (3234.0±1115.2) more than those who had not (2939.1±873.5), its level showed no significant relation with allergy (p=0.391).

Table 4.10. Allergic reactions in relation to ferritin level

Allergic reaction	**Ferritin level Mean ±SD**	**t**	**P-value**
Yes (n=29)	3234.0±1115.2	-0.868	0.391
No (n=14)	2939.1±873.5		

All values are expressed as mean ±SD.
p> 0.05: not significant.

4.5.3. Allergic reactions in relation to complements C3 and C4

Table 4.11 present the relation between allergic reactions and complements C3 and C4. Independent sample t-test showed no relation between allergic reactions and the mean concentrations of C3 and C4 (t=-0.689, p=0.495 and t=-0.358, p=0.722, respectively).

Table 4.11. Allergic reactions in relation to complements C3 and C4 (mg/dL)

Allergic reaction				
Complement	**Yes (n= 29)**	**No (n= 14)**	**t**	**P-value**
C3 Mean ±SD	119.6±12.1	116.8±13.3	-0.689	0.495
C4 Mean ±SD	49.8±11.2	48.2±17.6	-0.358	0.722

All values are expressed as mean ±SD.
p> 0.05: not significant.

4.5.4. Allergic reactions in relation to immunoglobulins IgG, IgM and IgA

The relation between allergic reactions and IgG, IgM and IgA is summarized in Table 4.12. The mean concentrations of immunoglobulins showed a slightly decrease in cases who had allergic reactions in comparison to those who had not. These changes were not significant (t=1.002, p=0.322; t=0.326, p=0.746 and t=0.248, p=0.805, respectively) indicating no relation between allergic reactions and immunoglobulins.

Table 4.12. Allergic reactions in relation to IgG, IgM and IgA concentrations

Allergy reaction				
Immunoglobulin	**Yes (n= 29)**	**No (n= 14)**	**t**	**P-value**
IgG Mean ±SD	1747.9 ±249.1	1857.4±471.0	1.002	0.322
IgM Mean ±SD	226.6±63.4	233.3±61.6	0.326	0.746
IgA Mean ±SD	294.6±86.7	304.6±178.2	0.248	0.805

All values are expressed as mean ±SD.
p> 0.05: not significant.

4.6 Age of patients in relation to some immunological parameters

4.6.1 Age of patients in relation to total and differential white blood cells

As depicted from Table 4.13, there was no significant association between increasing age of patients and total white blood cells count (p=0.163). Similar results was observed for differential white blood cells including MID (p=0.386), lymphocytes (p=0.448) and neutrophils (p=0.104).

Table 4.13. Age of patients in relation to WBC, MID, LYMP and GRAN

Age (year)				
WBC profile	**5-8 (n= 25)**	**9-12 (n= 18)**	**t**	**P-value**
WBC Mean ±SD	9.3±2	8.4±2.2	1.419	0.163
MID Mean ±SD	0.8±0.3	0.7±0.3	0.876	0.386
LYMP Mean ±SD	3.7±0.8	3.5±1.0	0.766	0.448
NEUT Mean ±SD	4.8±1.3	4.2±1.1	1.661	0.104

WBC: white blood cell, MID: may include less frequently occurring and rare cells correlating to monoctes, eosinophils, basophils, blasts and other precursor white cells, LYM: lymphocyte, NEUT: neutrophil.
All values were expressed as mean ± SD.
p> 0.05: not significant.

4.6.2 Age of patient in relation to ferritin level

Table 4.14 presents age of patients in relation to ferritin level. The t-test showed an increase of ferritin level in age group 9-12 years compared to age group of 5-8 years (3495.1±1100.5 V.s 2880.8±935.9). This change was significant (t=1.973, p=0.050), indicating a positive association of ferritin level with age progression at least in our study patients.

Table 4.14. Age of patients in relation to ferritin level

Age	**Ferritin level Mean ±SD**	**t**	**P-value**
5-8 (n=25)	2880.8±935.9	1.973	0.050
9-12 (n=18)	3495.1±1100.5		

All values are expressed as mean ±SD.
P> 0.05: not significant.
p<0.05: significant.

4.6.3 Age of patients in relation to complements C3 and C4

Table 4.15. illustrates the mean concentrations of complements C3 and C4 of cases in relation to different age groups. The mean concentration of C3 didn't significantly change with increasing age with means of 117.6±13.5 and 120.1±10.9 at age groups of 5-8 and 9-12 years, respectively (t=-0.639 and p=0.526). Similarly the results showed that C4 mean concentrations didn't also significantly change with increasing age with means of 48.6±14 and 50.3±12.9 at age groups of 5-8 and 9-12 years, respectively (t=-0.410 and p=0.684). These results indicate

that complements C3 and C4 were not significantly related to age progression in such patients.

Table 4.15. Age of patients in relation to complement component C3 and C4

	Age/ year			
Complement	**5-8 (n= 25)**	**9-12 (n= 18)**	**T**	**P-value**
C3 Mean ±SD	117.6±13.5	120.1±10.9	-0.639	0.526
C4 Mean ±SD	48.6±14.0	50.3±12.9	-0.410	0.684

All values are expressed as mean ±SD.
p> 0.05: not significant.

4.6.4 Age of patients in relation to IgG, IgM and IgA

As indicated in table 4.16, independent t-test showed that the level of all immunoglobulin studied in age group 9-12 years were higher than that in age group 5-8 years. IgM and IgA showed significant increase (262.1±50.9 and 342.6±85.6 V.s 204.8±59.1 and 265.7±135.0, t=3.314, p=0.002 and t=2.124, p=0.040, respectively). However such increase in IgG was not significant (1868.3±199.6 V.s 1722.6±399.7, t=1.421, p=0.163). In general, we can suggest a positive association between age of patients and immunoglobulins concentrations.

Table 4.16. Age of patients in relation to IgG, IgM and IgA

	Age (year)			
Immunoglobulin	**5-8 (n= 25)**	**9-12 (n= 18)**	**t**	**P-value**
IgG Mean ±SD	1722.6±399.7	1868.3±199.6	1.421	0.163
IgM Mean ±SD	204.8±59.1	262.1±50.9	3.314	0.002
IgA Mean ±SD	265.7±135.0	342.6±85.6	2.124	0.040

All values are expressed as mean ±SD.
p> 0.05: not significant.
p<0.05: significant.

4.7 Ferritin level of patients in relation to immunological parameters

4.7.1 Ferritin level of patients in relation to complements C3 and C4

The relation between ferritin level and complement component C3 and C4 of the study population is pointed out in Table 4.17. The independent t-test showed that mean levels of C3 and C4 decreased slightly in patients who had ferritin level > 2000 than patients who had ferritin level ≤ 2000 (ng/ml). However , this difference was not significant (t=0.338, p=0.737 and t=0.428, p=0.671, respectively).

Table 4.17. The relation between ferritin level and complement component C3 and C4 by independent T- test

Ferritin level (ng/ml)				
Complement	**≤2000**	**> 2000**	**t**	**P-value**
C3 Mean ±SD	120.1±13.3	118.4±12.4	0.338	0.737
C4 Mean ±SD	51.3±11.5	48.9±13.9	0.428	0.671

All values are expressed as mean ±SD.
p> 0.05: not significant.

4.7.2 Ferritin level of patients in relation to IgG, IgM and IgA

Table 4.18. presents the relation between ferritin level and IgG, IgM and IgA. The results showed no significant difference in the level of immunoglobulins in patients with ferritin level ≤ 2000 and those with ferritin level > 2000 (ng/ml) p>0.05.

Table 4.18 . Ferritin level in relation to IgG, IgM and IgA

Ferritin level (ng/ml)				
Immunoglobulin	**≤ 2000**	**> 2000**	**t**	**P-value**
IgG Mean ±SD	1678.4±285.5	1804.0± 344.4	-0.904	0.371
IgM Mean ±SD	229.7±64.6	228.6±62.6	0.042	0.966
IgA Mean ±SD	307.1±61.4	296.1±131.0	0.218	0.829

All values are expressed as mean ±SD.
p> 0.05: not significant.

4.8 C-reactive protein of patients in relation to immunological parameters

4.8.1 C-reactive protein status in relation to complements C3 and C4

The status of CRP in relation to complements C3 and C4 is illustrated in table 4.19. There was no significant differences in the concentration of C3 and C4 between positive and negative CRP status p>0.05.

Table 4.19. C-reactive protein in relation to complement component C3 and C4

	CRP			
Complement	**Positive (n=12)**	**Negative (n=31)**	**t**	**P-value**
C3 Mean ±SD	123.3±9.9	116.9±12.98	-1.528	0 .134
C4 Mean ±SD	48.6±13.99	50.0±14.5	0.562	0.577

All values are expressed as mean ±SD.
p> 0.05: not significant.

4.8.2 C-reactive protein status in relation to IgG, IgM and IgA

Table 4.20 provides the relation between CRP and IgG, IgM and IgA. Patients who had positive CRP had higher levels of IgG, IgM and IgA than patients who had negative results (1974.7±498.8, 253.3±65.8 and 401.2±154.8 V.s 1709.6± 215.2, 219.3±59.1 and 257.9±78.1). These changes were found to be significant for IgG and IgA (p=0.018 and 0.000, respectively) whereas no statistical difference in IgM was detected p=0.109).

Table 4.20. C-reactive protein of patients in relation to IgG, IgM and IgA

	CRP			
Immunoglobulin	**Positive (n=12)**	**Negative (n=31)**	**t**	**P-value**
IgG Mean ±SD	1974.7±498.8	1709.6± 215.2	-2.457	0.018
IgM Mean ±SD	253.3±65.8	219.3±59.1	-1.638	0.109
IgA Mean ±SD	401.2±154.8	257.9±78.1	-4.040	0.000

All values are expressed as mean ±SD.
p> 0.05: not significant.
p<0.05: significant.

Chapter 5

Discussion

The immune system has evolved to survey the body constantly for potentially hazardous structures. Immune system provide a remarkably effective defense system by both the innate and adaptive immunity which their responses depend upon the activities of white blood cells. Innate immunity largely involves granulocytes and macrophages. Adaptive immune responses depend upon lymphocytes, which provide the lifelong immunity that can follow exposure to disease or vaccination (Matzinger, 2007). In the present study some immunological parameters in children with β-thalassemia major was investigated. Many studies have been done to evaluate the possible changes of immune system in thalassemic patients, considering the humoral and cellular immune systems. Various immunological abnormalities are reported such as functional defects, involving T and B lymphocytes, impairment production of immunoglobulin, deficient activity of the complement system with reduced levels of C3 and C4, decreased opzonization and granulocyte phagocytosis. (Quintiliani et al., 1983; Weatherall and Clegg, 2000; Weatherall et al., 2000; Farmakis et al., 2003). Although β-thalassemia disease is prevalent in Gaza Strip since very long time, and immune abnormalities have been suggested as a precipitating factor for the fourth most common cause of death in β-thalassemia after cardiomypathy, infection and liver disease (Zurlo *et al*., 1989), no studies were carried out about the associated immunological aspects. Therefore, this will be the first study to investigate the status of immunity and to evaluate the effects of age, allergy, and iron overload on the immune response of β-thalassemia major children in Gaza Strip. This could be useful in prevention and treatment strategies applied to thalassemic children.

5.1 General characteristics of the study population

Data presented in this study dealt with 86 children from both genders: 43 controls and 43 β-thalassemic major. The mean age of the study population, in the present study was 7.9±2.2 years. This mean age was close to that reported in El Nawawy *et al*. (1996) who measured blood levels of IL-l-β, TNF-α, and islet cell antibody

(ICA) in 20 children with IDDM, 20 of their non-diabetic siblings, 20 children with thalassemia major on long-term hypertransfusion therapy and iron chelation, and 10 normal age matched children (mean age 7.8 ± 1.6 years). IL-l-β and TNF-α concentrations did not differ significantly among the thalassemic group, diabetic group, and health controls. Males and females in the present study were 24 (55.8%) and 19 (44.2%), respectively .

5.2 Allergic reaction and iron chelation therapy

The thalassemic children in the present study were on long-term hypertransfusion therapy and on iron chelation with desferrioxamine. The present data showed that the majority of them had allergic reactions 29 (67.4%). This could be attributed to:1) hypersensitivity to soluble allergens found in the transfused blood component, 2) production of antibodies against RBCs by the recipients and 3) formation of multiple alloantibodies to common Rh, Kell, Kidd, or other blood group antigens. Several authors reported that regular blood transfusions are associated with alloimmunization (Spanos *et al*., 1990; Singer *et al*., 2000 and Wang *et al*., 2006). Furthermore, thalassemic children differ in the way of chelating the iron with desferrioxamine, they received it either subcutaneous 17 (39.5%) or intramuscular 25 (58.1%). One patient (2.3%) refused chelation therapy.

5.3 Hematological parameters

5.3.1 Primary and secondary blood indices

The present data showed that primary blood indices including red blood cell count, hemoglobin and hematocrit were significantly decreased in the cases compared to the controls. It is accepted that the number of red blood cells is proportional to the degree of decrease in hemoglobin concentration (Clarke and Higgins, 2000). Regarding secondary blood indices, MCV MCH and RDW were also found to be lower in the cases compared to controls. On the other hand, MCHC was higher in cases than controls. Hereditary spherocytosis (HS) is the only disease in which the the mean corpuscular hemoglobin concentration MCHC is increased (del Giudice *et al*.,1993). There are several reported cases of HS in combination with β-thalassemia and even with α -thalassemia (Uysal *et al*., 1998). Such results were

in agreement with the previously known findings that patients with thalassemia major have a severe microcytic and hypochromic anemia, associated with increased number of red blood cells and low MCV and MCH without a concomitant increase in RDW (Clarke and Higgins, 2000 and Cao and Galanello, 2010).

5.3.2 Total and differential white blood cells

Total white blood cell count was significantly elevated in the cases compared to controls. For differential white blood cells, lymphocytes, neutrophils and MID were also higher in the cases. However, only lymphocytes showed significant difference between cases and controls. Blood platelets were also increased in cases than controls but with no significant difference. The induction of white blood cell count observed in the present study indicates the activation of a defense mechanism and the immune system, which could be a positive response for survival (Wesseling *et al*., 1997). The observed increase in lymphocyte was in agreement with that found by Gharagozloo *et al*. (2009) who reported that absolute lymphocyte counts increased significantly in thalassemic patients compared with the controls, suggesting the presence of a chronic immunological stimulation due to multiple blood transfusion. Such immunological stimulation was attributed to allo-antigenic stimulation (Lombardi *et al*.,1994). This could be true in our patients who transfused with packed red blood cells contaminated with some leukocytes that might induce immunization to histocompatibility antigens in thalassemia patients. Even in the absence of donor leukocytes, antigens on donor RBCs are presented in the major histocompatibility complex class I pathway of recipient antigen-presenting cells (Zimring *et al*., 2006). It was pointed out that alloimmunization results in difficulty obtaining compatible blood, transfusion reactions, hemolysis and occationally life-threatining events (Wang *et al*., 2006).

5.3.3 Serum ferritin

Data revealed that the mean serum ferritin level in patients was markedly higher than that in controls (3138.0±1041.5 V.s 17.3±2.5, p=0.000). This is a logic finding in thalassemic patients where both transfusional iron overload and excess gastrointestinal absorption are contributory. Paradoxically, excess gastrointestinal iron absorption persists despite massive increases in total body iron load (Eldor and Rachmilewitz, 2002 and Rund and Rachmilewitz, 2005). Several studies

support such finding (Oztürk *et al*., 2001, Morabito *et al*., 2007, Gharagozloo *et al*., 2009). Serum ferritin reflects the state of the iron stores in the body (Burtis and Ashwood, 1994). This may be the case in our patients who suffer from iron overload even they used iron chelation with desferrioxamine. As indicated previously in our result iron chelation therapy was either subcutaneous through the pump or intramuscular injection. Both ways of therapy were not preferable by patients who find it painful. In addition, the pump was not always provided and sometimes broken down and need a long period of time to be repaired. All of the studied children said that they hope to use oral therapy to avoid the harmful pain. Despite that iron overload is the most important complication of *β*-thalassemia (Eldor and Rachmilewitz, 2002 and Cao and Galanello, 2010). Iron chelation therapy is still not efficient in Gaza Strip as indicated by the high ferritin level observed in our patients even after desferrioxamine chelation therapy. Strategies to improve chelation regimens should be of the highest priority. These strategies must includ development of novel oral iron chelators such as deferasirox (ICL670, Exjade) and deferiprone to improve compliance (Neufeld, 2006).

5.4 Immunological status in β-thalassemia major patients compared to controls

5.4.1 Complement levels

The present results revealed that the mean levels of complements 3 and 4 were significantly decreased in cases as compared to control. Similar result was found by James *et al*., (1981). The decrease in complements 3 and 4 can be attributed either to reduced their synthesis or increased their consumption; the latter is more probable with the increase rate of infection (Amin *et al*., 2005). In our patients the decrease noted in complements 3 and 4 may be due to reduced their synthesis rather than increased their consumption particularly in young children where the rate of infection is expected to be low.

5.4.2 Immunoglobulins IgG, IgM and IgA concentration

As depicted from the present result, the mean concentration of immunoglobulin G was significantly decrease in cases compared to controls. Amin *et al*., (2005)

showed an increase level in IgG in thalassemic patients with increasing age. They attributed this increase to repeated infection which was not the case of our patients. In another study Vergin *et al* (1997) showed that IgG level was within normal limit in thalassemic patients. This controversy may be due to marked heterogeneity of the patients in different studies. Immunoglobulin M and A concentrations were not significantly change in cases compared to controls, suggesting that anaphylactic reactions due to IgA deficiency is excluded (Cao and Galanello, 2010). Such finding was in agreement with that reported by Ezer *et al*., (2002) and Tourkantoni *et al*., (2008).

5.4.3 Serum Cytokine levels

In general cytokines were mostly undetectable in both cases and controls. The numbers of cases with undetected TNF-α and IL-6 were 42 (97.7%) and 38 (88.4%) compared to the controls of 28 (65.1%) and 41 (95.3%). Interleukin-1β showed completely undetectable levels in both cases and controls. Based on the procedure used in the present study the obtained undetectable result means that cytokines in both controls and patients are normal i.e. normally cytokines are undetectable. However, this depends on the immunological status of the patient implaying that our patients had low level of immunological complications related to cytokines. Elevated human cytokines levels depend on the type of immunological disorder. The present finding was in agreement with the study carried out by Gharagozloo *et al*. (2009) who revealed no significant difference in serum TNF-α levels in the thalassemia and control groups. Similarly El Nawawy *et al*. (1996) reported that IL-l-β and TNF-α concentrations did not differ significantly between patients and controls. In addition, Lombardi, (1994) showed no statistical differences between serum levels of IL-6 in β-thalassemic patients and in normal controls although most of patients showed undetectable levels of this cytokine. He considered the reason for the low or normal serum levels of IL-6 is the fact that IL-6 may be active locally at very low concentrations, without the need to reach high systemic levels.

In contrast to our findings, Kyriakou *et al*. (2001) and Aggeli *et al*. (2005) showed values of TNF-α , IL-1β and IL-6 were higher in thalassemic patients compared to controls. Jacob *et al.,* 1990 mentioned that the broad distribution of TNF-α concentrations accords with the significant differences among individuals in the

levels of TNF-α synthesis by stimulated mononuclear cells observed by Jacob *et al.,* 1990 in healthy subjects. Moreover the heterogeneity of thalassemia patients regarding genotype, age, number of transfusions, frequency of infections, and iron overload in the various reports makes comparison of the results in the various studies sometime unreliable (Kyriakou *et al.,* 2001).

5.5 C-reactive protein status

The outcome of CRP status was positive in 27.9% of the cases and in 14.0% of controls, but chi square test showed no significant difference. This may imply that patients display low level of inflammation. The above mentioned result that IL-6 was mostly undetectable in cases and controls may support this view. It was accepted that the most potent stimulator for the hepatic synthesis of this protein (CRP) is interleukin 6 (Archararit *et al*., 2000)

5.6 Allergic reactions in relation to the studied immunological parameters in patients

Data presented in this study showed that the WBC, MID, LYMP and GRAN decreased in the patients with allergic reactions with significant changes detected in the WBC and LYMP. Such decrease could be explained on the bases that allergic patients received immune suppressor drugs to avoid hypersensitivity resulting from blood transfusion. It is known that anti inflammatory drugs suppress the activity of the action of allergic mediators, or to prevent activation of cells and degranulation processes (Boumpas *et al*., 1993). When related to allergy, ferritin level was higher in allergic patients indicating that iron overload may play a role in immunological complication observed in the present study (Weiss, 2002). The complements 3, C4, IgG, IgM and IgA concentrations didn't significantly change between the allergic and none allergic patients. This means that allergic reactions may be related to immunoglobulin E rather than the studied ones (Zweiman, 1993)

5.7 Age of patients in relation to immunological parameters

Data presented in this study showed that there is no significant relationship between age and total white blood cells count and differential white blood cells including, MID, lymphocytes and neutrophils. Similar results were recorded for complement 3 and C4. Such results are in agreement with that reported by Amin

et al., (2005). On the other hand, there was a significant positive relation between ferritin levels and age progression i.e. ferritin level increased with increasing age. Evidence in support of this result has been reported by Salsaa and Zoumbos, (1997). High levels of IgG, IgM and IgA were also observed in age group 9-12 years compared to age group 5-8 years with significant difference for IgM and IgA. This result is in congruent to that of Amin *et al*. (2005) who found that the elder patients had significantly higher values of the three major classes of serum immunoglobulins (IgG, IgM and IgA) than younger group. They suggested that repeated blood transfusion in β-thalassemia patients will result in a continuous exposure to various antigens and will lead to increased levels of serum immunoglobulins (Amin *et al*. 2005).

5.8 Ferritin levels of patients in relation to immunological parameters

According to our results, there were no significant differences in complements 3 and 4, and immunoglobulins G, M and A between patients with ferritin level ≤ 2000 and those with ferritin level > 2000. This suggests that iron overload does not seem to play a major role in humeral immune system. Such results agreed with that found by Loebstein *et al*., (1997) and Amin *et al*., (2005). However, this finding doesn't exclude the idea that iron overload is an important factor in altering the immune system in thalassemia patients (Weatherland and Clegg., 2000) in term of high iron level in patients compared to healthy individuals.

5.9 C-reactive protein status in relation to immunological parameters

As indicated in our study there was no significant difference between serum concentration of C3 and C4 with the positive or negative status of C-reactive protein in thalassemia patients. On the other hand, the results generally showed significant increase in the level of immunoglobulins in positive CRP patients compared to negative ones. This positive relation of CRP with serum levels of IgG, IgM and IgA was expected since CRP is a marker for the detection of acute and chronic inflammatory processes (Archararit *et al*., 2000).

Chapter 6

Conclusions and Recommendations

6.1 Conclusions

1. The Study population comprised 24 (55.8%) males and 19 (44.2%) females with average age of 7.9±2.2 years.

2. Most of patients 29 (67.4%) had allergic reactions.

3. Desferrioxamine is the standard iron chelator used in Gaza Strip. Discharge of this chelator was intramuscular in 25 (58.1%) patients and subcutaneous in 17 (39.5%) patients.

4. In general primary and secondary blood indices were significantly decreased in thalassemic children compared to controls.

5. The total white blood cell and lymphocytes were significantly increased in patients compared to controls (8.9±2.1 V.s 7.9±2.0, p=0.025 and 3.6±0.9 V.s 3.1±0.79, p=0.003, respectively). When related to age, total white blood cell and lymphocytes showed no significant difference. However, they significantly decrease with allergic reactions (8.5±2.2 V.s 9.9±1.6, p=0.042 and 3.4±0.9 V.s 4.0±0.9, p=0.050, respectively).

6. Ferritin levels in patients were markedly higher than in controls (3138.0±1041.5 V.s 17.3±2.5 ng/ml, p=0.000), reflecting iron overload despite of desferrioxamine treatment. Ferritin levels showed positive relations with age and allergic reaction.

7. Complements 3 and 4 reduced significantly in thalessemic children (118.7±12.4 V.s 136.6 ±23.3 mg/dL, p=0.000 and 49.3±13.4 V.s 62.3±21.6 mg/dL, p=0.001, respectively), regardless of allergic reaction, age, ferritin level and CRP.

8. IgM and IgA of patients were in normal levels compared to controls. However, lower level of IgG was found in patients. Higher levels of IgG, IgM and IgA were found with increasing age and in positive CRP patients ($p<0.05$). On the other hand, immunoglobulins neither had a relation with ferritin nor with allergic reactions

9. Cytokines were normal that they almost showed undetected levels in controls and patients (TNF-α: 56.1 and 97.7%, IL-6: 95% and 88.4%, and IL-I-β: 100% for both controls and patients), reflecting the absence of acute immune abnormalities.

10. C-reactive protien status didn’t differ significantly between controls and cases.

6.2 Recommendations

1. The patients should be regularly tested for antibodies screening.

2. Transfusion transmissible infections (TTI) test should be carried out.

3. Some testes must be activated in blood transfusion centers as antibody screening for blood donors.

4. Washed red cells is highly recommended to be transfused to the blood transfusion dependent patients especially thalassemic patients.

5. New stratigies of chelation using other iron chelator drugs such as deferasirox (ICL670, Exjade) and deferiprone are recommended.

6. Providing easy access to the drug discharge pump for all patients and its instant repair when broken.

7. Improvement of blood transfusion program in terms of increase frequency and units of transfused blood to improve hemoglobin level in thalassemic patients.

8. Further studies are recommended on:

a) Cellular immunity including response of memory T cells and specific antigenic stimuli responsible for infections in thalassemic patients.

b) Immunological abnormalities in older thalassemic patients.

c) Assessment of IgE which could be useful in case of allergy.

d) Evaluation of other cytokine in thalassemic patients.

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Annex 1

Palestinian National Authority
Ministry of Health
Helsinki Committee

السلطة الوطنية الفلسطينية
وزارة الصحة
لجنة هلسنكي

التاريخ:2010/3/23

Name:

الاسم :سماح فوزي الهمص

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

نفيدكم علماً بأن اللجنة قد ناقشت مقترح دراستكم حول :

تقييم الحالة المناعية لاطفال التلاسيميا العظمى التي يتراوح اعمارهم من 6 –12 سنة و المسجلين بمستشفى النصر بقطاع غزة

.

In its meeting on March 2010

و ذلك في جلستها المنعقدة لشهر 3 2010

and decided the Following:-

و قد قررت ما يلي:-

To approve the above mention research study.

الموافقة على البحث المذكور عاليه.

Signature

توقيع

Member

عضو

Member

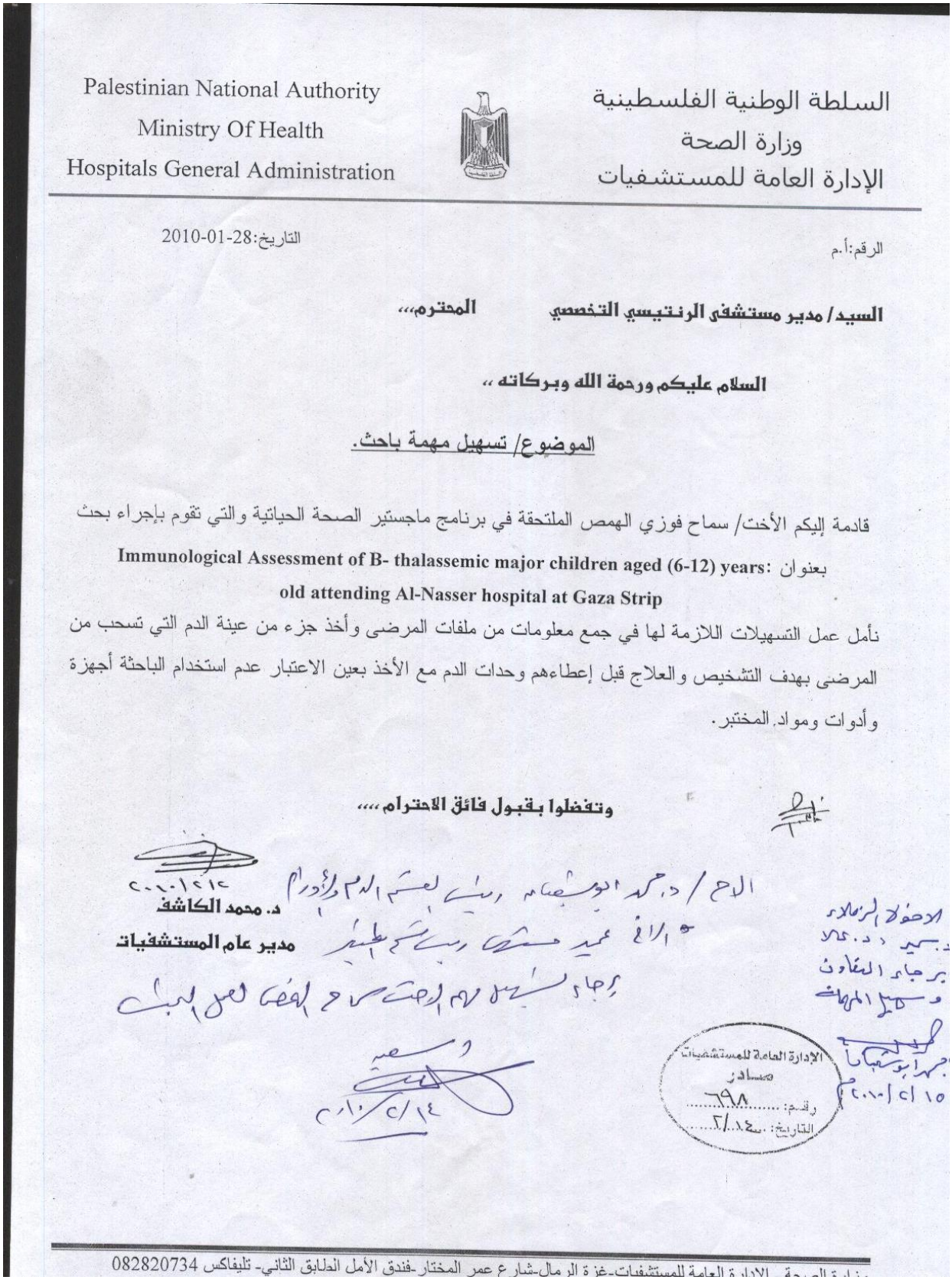
عضو

Chairperson

Conditions:-

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

Annex 2



Palestinian National Authority
Ministry Of Health
Hospitals General Administration

السلطة الوطنية الفلسطينية
وزارة الصحة
الإدارة العامة للمستشفيات

الرقم:أ.م

التاريخ:28-01-2010

السيد/ مدير مستشفى الرنتيسي التخصصي **المحترم،،،**

السلام عليكم ورحمة الله وبركاته ،،

الموضوع/ تسهيل مهمة باحث.

قادمة إليكم الأخت/ سماح فوزي الهمص الملتحقة في برنامج ماجستير الصحة الحياتية والتي تقوم بإجراء بحث بعنوان: **Immunological Assessment of B- thalassemic major children aged (6-12) years old attending Al-Nasser hospital at Gaza Strip**

نأمل عمل التسهيلات اللازمة لها في جمع معلومات من ملفات المرضى وأخذ جزء من عينة الدم التي تسحب من المرضى بهدف التشخيص والعلاج قبل إعطاءهم وحدات الدم مع الأخذ بعين الاعتبار عدم استخدام الباحثة أجهزة وأدوات ومواد المختبر.

وتفضلوا بقبول فائق الاحترام،،،

د. محمد الكاشف
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الإدارة العامة للمستشفيات
صادر
رقم: ٦٩٨
التاريخ: ١٤/١/٢٠١٠

وزارة الصحة - الإدارة العامة للمستشفيات-غزة الرمال-شارع عمر المختار-فندق الأمل الطابق الثاني- تليفاكس 082820734

الجـــــــامعة الإسلامية – غزة كلية العلوم
The Islamic University of Gaza
مدير برنامج ماجستير العلوم الحياتية

التاريخ/ ٢٠٠٩/١٠/٥م

الأخ الدكتور/ مدير عام مركز التلاسيميا حفظه الله ،،،

السلام عليكم ورحمة الله وبركاته ،،،

الموضوع / تسهيل مهمة باحثة

تشهد إدارة ماجستير العلوم الحياتية بالجامعة الإسلامية أن الطالبة: ***سماح فوزي الهمص*** طالبة في ماجستير العلوم الحياتية تقوم بإجراء البحث النهائي في برنامج الماجستير والذي بعنوان:

"الحالة المناعية عند أطفال التلاسيميا في غزة"

الباحثة بحاجة لأخذ معلومات وعينات.

لذا نرجو من سيادتكم مساعدة الباحثة.

ولكم منا جزيل الشكر والتقدير ،،،

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The Palestinian National Authority
Ministry of Health
Directorate General of Human Resources Development

السلطة الوطنية الفلسطينية
وزارة الصحة
الإدارة العامة لتنمية القوى البشرية

الرقم :..... التاريخ: 2010/01/20

الأخ الدكتور/ محمد الكاشف المحترم،،،
مدير عام المستشفيات

تحية طيبة وبعد،،،

الموضوع/ تسهيل مهمة باحث

بالإشارة إلي الموضوع أعلاه، يرجي تسهيل مهمة الباحثة / سماح فوزي الهمص، الملتحقة في برنامج ماجستير الصحة الحياتية تخصص علم حيوان، وتعمل معيدة في جامعة الاقصي، تقوم بإجراء بحث بعنوان:

" تقييم الحالة المناعية للأطفال الثلاسيما العظمي التي يتراوح أعمارهم من 6-12 سنة والمسجلين في مستشفي النصر التخصصي بقطاع غزة "

(Immunological Assessment of B-thalassemic major children aged (6-12) years old attending Al-Nasser hospital at GAZA-Strip)

وتشمل الدراسة إجراء جمع معلومات من ملفات المرضي وأخذ جزء من عينة الدم التي تسحب من المرضي قبل إعطاءهم وحدات الدم، بحيث لا تتعارض مع مصلحة العمل، وضمن ضوابط وأخلاقيات البحث العلمي دون تحمل الوزارة أي تكاليف.

ومن أجل حماية المريض يرجي التأكيد علي:

1. الحصول علي الموافقة الخطية من أهل المريض للمشاركة في البحث، قبل الاطلاع أو أخذ العينات.
2. عدم استخدام الباحث لأجهزة وأدوات المختبر.
3. الإشراف ومتابعة الباحث أثناء أخذ العينات.

وتفضلوا بقبول خالص الاحترام والتقدير،،،،،،،

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- الملف

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Annex 3

بسم الله الرحمن الرحيم

دراسة بحثية بعنوان

تقييم الحالة المناعية لدى مرضى الثلاسيميا العظمى الأطفال الذين تتراوح أعمارهم (5-12) سنة و المسجلين في مستشفى النصر التخصصي للأطفال

أولياء الأمور..... الكرام

السلام عليكم ورحمة الله و بركاته

تهدف هذه الدراسة إلى معرفة وتقيم الحالة المناعية لدى أطفال مرضى الثلاسيميا والذين تتراوح أعمارهم بين 5-12 سنة و المسجلين في مستشفى عبد العزيز الرنتيسي التخصصي للأطفال في مدينة غزة ، وعليه تتطلب هذه الدراسة الحصول على بعض البيانات و أخذ عينة دم
بسيطة من الأطفال قبل إجراء نقل الدم لهم لذا نأمل منك الموافقة على مشاركه طفلك في هذه الدراسة البحثية حيث قد تم اختيار طفلك كمشارك في هذا البحث نظرا لتكامل الشروط البحثية لحالته وعليه نرجو منك قراءة المعلومات الواردة بتأني وهدوء:

- إن هذه الدراسة هي متطلب من متطلبات الحصول على درجة الماجستير في العلوم الحياتية من الجامعة الإسلامية بغزة، كما أود إحاطتكم علما ان مثل هذه الدراسة لم تدرس من قبل في قطاع غزة .
- إن المشاركة في هذا البحث هي طوعيه وعدم الرغبة في المشاركة سوف لن تؤثر بأي شكل من الأشكال على الرعاية الطبية التي تتلقاها لذا لك الحق بالموافقة أو رفض المشاركة في هذا البحث.
- قد لا تنتفع من مشاركتك في هذا البحث نفعا مباشرا ولكن المعلومات والنتائج المستقاة من مشاركتك قد تكون ذات فائدة في المستقبل.
- يمكنك التوقف عن المشاركة في هذا البحث بأي وقت أو مرحله وبدون أن يترتب عليك أي شيء أو أن يؤثر على نوع الرعاية الطبية التي تتلقاها.
- إن هذا البحث ليس فيه أي ضرر يذكر سواء كان جسدي أو نفسي على هؤلاء الأطفال أو عوائلهم.

- تكون المعلومات الناتجة عن مشاركتك في البحث معامله بسريه تامة ولن يطلع عليها أي شخص أو جهة عدا المسؤولين عن إجراء الدراسة إلا بموافقتك ولن تكون هناك اشاره إلى شخصك أو عشيرتك في أي بحث ينشر عن هذه الدراسة.
- لك الحق بمعرفه طبيعة وأهداف البحث ومعرفه جميع التفاصيل المتعلقة بمشاركتك.
- لك الحق باستلام نسخه من نموذج الموافقة .
- لن تحصل على أي مردود مالي مقابل مشاركتك في الدراسة.
- كما بإمكانك طلب إيضاحات أو معلومات إضافية عن أي شيء مذكور في هذه الاستمارة أو عن هذه الدراسة ككل أو عن كل ما يتعلق بإجراءات البحث من طبيبك أو من الباحث نفسه.
- عند الموافقة على المشاركة بهذا البحث ،تكون جميع العينات ونتائج الفحوصات بتصرف الباحث والمؤسسة البحثيه ويتم التعامل معها وفقا للنظم العامة المراعاة في وزارة الصحة.

ولكم جزيل الشكر و العرفان

الطالبة سماح فوزي الهمص
ملتحقة ببرنامج الماجستير
للعلوم الحياتية الجامعة الإسلامية

مؤسسة فلسطين المستقبل
مركز الثلاسيميا
Palestine Avenir Foundation
Thalassaemia Center

إحصائية لعدد مرضى الثلاسيميا في قطاع غزة

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ملاحظة: - باقي المرضى شخصت حالاتهم كالتالي:-

Sickle cell - B.Thalass Trait - Pure cell aplasia.

PALESTINE AVENIR FOUNDATION THALASSAEMIA CENTER

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