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Immunophenotyping of ALL Palestinian Pediatric Cases in a single cancer center experience in southern West Bank Using Flow Cytometry

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M.Sc. Thesis

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Thesis Approval

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Jerusalem- Palestine

1438 - 2016

Declaration:

I Certify that this thesis submitted for the degree of Master, is the results of my own research, except where otherwise acknowledged , and that this study (or any part of the same) has not been submitted for a higher degree to any other university of institution.

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Date: 19/11/2016

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Abstract

Background: Acute Lymphoblastic Leukemia (ALL) is the most common cancer in children, represents approximately 75–80% of acute leukemia. Global incidence is about 3-4 cases per 100,000 population under 15 years old. Researches about pediatric ALL in Palestine are limited. This study provides the immunophenotyping of pediatric ALL patients at a single cancer center experience in southern west bank area in Palestine.

Objectives: The main objectives of this study are to characterize the immunophenotypic pattern of pediatric ALL subtypes among Palestinians, highlight the importance of some clinical features according to age and immunophenotypic pattern in patients prognosis and response to treatment, and to determine the response to therapy after induction phase using the minimal residual disease (MRD) in time point 1 (day 33).

Materials and Methods: This study is an incident study, was conducted from January 2015 to December 2015 at Huda Al-Masri pediatric cancer department at Beit-Jala hospital, which is located in the southern area of west bank in Palestine. A total of 15 consecutive children (7 females, 8 males) < than 14 years with newly diagnosed ALL were selected by pediatric oncologists at Huda Al-Masri pediatric cancer department at Beit-Jala hospital, this hospital is one of the largest pediatric centers for leukemia patients in Palestine.

Archived data of pediatric ALL patients in their medical files were reviewed with ethical consideration of patient privacy to obtain some clinical features of the given patient.

All cases either retrospective or incident were tested by College of American Pathologists (CAP) accreditation pathology department at King Hussein Cancer Center (KHCC) in Amman-Jordan on bone marrow aspirate samples collected in Ethylenediaminetetraacetic acid (EDTA) tubes according to a two step strategy using panels of monoclonal antibodies based on European Group for Immunological Characterization of Leukemia (EGIL). For the study purpose, the immunophenotyping of the incident target cases at 2015 was performed at Huda Al-Masri pediatric cancer department at Beit-Jala hospital in Bethlehem on bone marrow aspirate samples collected in EDTA tubes according to a two step strategy using Flow Cytometer, and for validation purpose, the achieved results were compared with the KHCC results of the same cases and no significant difference was noted.

Minimal Residual Disease results were evaluated by examining the most common antigens or CD markers through reactivity of fluorescent conjugated monoclonal antibodies directed against lymphoid associated antigens for each ALL type on the surface of bone marrow cells after the induction point 1 at day 33 of treatment by flow cytometry technique.

Results: Statistical analysis of the results was performed, and the study group comprised 15 newly diagnosed acute lymphoblastic leukemia cases (<14 years). There were 7 females and 8 males with a male/female ratio of 1.14:1. The age distribution of the precursor B-cell ALL subtype showed that precursor B-cell ALL patients were younger with peak incidence between 2 and 5 years. Geographical distribution of Palestinian pediatric ALL showed that about half of the cases (46.7%) from Hebron governorate, but with the same percentage of cases diagnosed in Bethlehem governorate depending on the number of inhabitants.

Immunophenotyping analysis showed that the precursor B-cell phenotype was encountered in 14 (93.3%) cases, mature B-cell in Zero (0%) cases, and T-cell in 1 (6.7%) case. Lineage Heterogeneity was found only in precursor B-cell ALL cases having one or two myeloid associated antigens (CD13,CD33) expressed on their blast cells. Lineage Heterogeneity for both antigens was found in 7.1% of precursor B-cell ALL cases.

By the examining of MRD, we found that 13 of pediatric ALL cases (86.7%) were subjected to complete remission after the end of induction point 1 at day 33 with the only case of T-cell ALL and 85.7% of precursor B-cell ALL cases, but not statistically significant.

Conclusion: This study is a unique study providing a clear idea about the munophenotyping of pediatric ALL cases at a single cancer center experience in southern west bank area in Palestine, and this immunophenotypic distribution was similar to the general immunophenotypic distribution pattern in developed countries. The age distribution showed a peak incidence between 2 and 5 years among the precursor B-cell ALL subtype as in developed countries. Geographical distribution of Palestinian pediatric ALL showed that about half of the cases (46.7%) from Hebron governorate, but with the same percentage of cases diagnosed in Bethlehem governorate depending on the number of inhabitants.

The response to treatment for ALL patients was very good as in developed countries, due to the negative MRD at the end of induction point 1 at day 33 was 86.7% of ALL patients.

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List of Abbreviations

ALL: Acute Lymphoblastic Leukemia.

nTdT: nuclear Terminal deoxynucleotidyl Transferase.

WBC: White Blood Cell.

MRD: Minimal Residual Disease.

PCR: Polymerase Chain Reaction.

SEER database: Surveillance, Epidemiology, and End Results database.

CNS: Central Nervous System.

CBC: Complete Blood Count.

FISH: Fluorescence in situ Hybridization.

FAB system: French American British system.

ITP: Immune Thrombocytopenia.

WHO: World Health Organization.

CD: Cluster of Differentiation.

MPO: Myeloperoxidase.

Ig: Immunoglobulin.

HLA-DR: Human Leukocyte Antigen-antigen D related.
UK: United Kingdom.

POG: Pediatric Oncology Group.

CCG: Children Cancer Group.

CTEP/NCI: Cancer Therapy Evaluation Program of the National Cancer Institute.

PEG: Polyethylene Glycol.

I.BFM.SG: International-Berlin Frankfurt Munster-Study Group.

AIEOP: Associazione Italiana di Ematologia ed Oncologia Pediatrica.

cCD3: cytoplasmic CD3.

TCR: T Cell Receptor.

FACS: Fluorescence Activated Cell Sorter.

FSC: Forward Scatter.

SSC: Side Scatter.

CAP: College of American Pathologists.

KHCC: King Hussein Cancer Center.

EDTA: Ethylenediaminetetraacetic acid.

EGIL: European Group for Immunological Characterization of Leukemia.

BD FACS: Becton Dickinson FACS.

cCD79a: cytoplasmic CD79a.

FITC: Fluorescein Isothiocyanate.

PE: Phycoerythrin.

Per CP: Peridinin Chlorophyll Protein.

APC: Allophycocyanin.

SPSS: Statistical Package for the Social Sciences.

AQU: Al-Quds University.

MOH: Palestinian Ministry Of Health.

Chapter one

Introduction

1.1. Background

Acute lymphoblastic leukemia (ALL) is a type of leukemia which is characterized by 20% or more lymphoblasts in the bone marrow. It is a rapidly developing, abnormal growth of the cells that are precursors of lymphoblasts (Neelkamal et al., 2013).

Acute lymphoblastic leukemia is the most common malignancy in children and represents 75–80% of acute leukemia. The incidence of pediatric ALL is about 3–4 cases per 100,000 under 15 years old. Although it affecting children of all ages, the incidence peaks between two and five years of age with a slightly higher among males over females (de Sousaa et al., 2015).

ALL arise from B-cell in 85% patients and from T-cell in 15% cases. However, B-cell leukemia occurs more frequently than T-cell leukemia as you seen in Figure 1.1 (Neelkamal et al., 2013, and Hunger and Mullighan, 2015). It is the most common type of leukemia found in children, nearly 75% of cases occur in children under six years of age (Neelkamal et al., 2013).

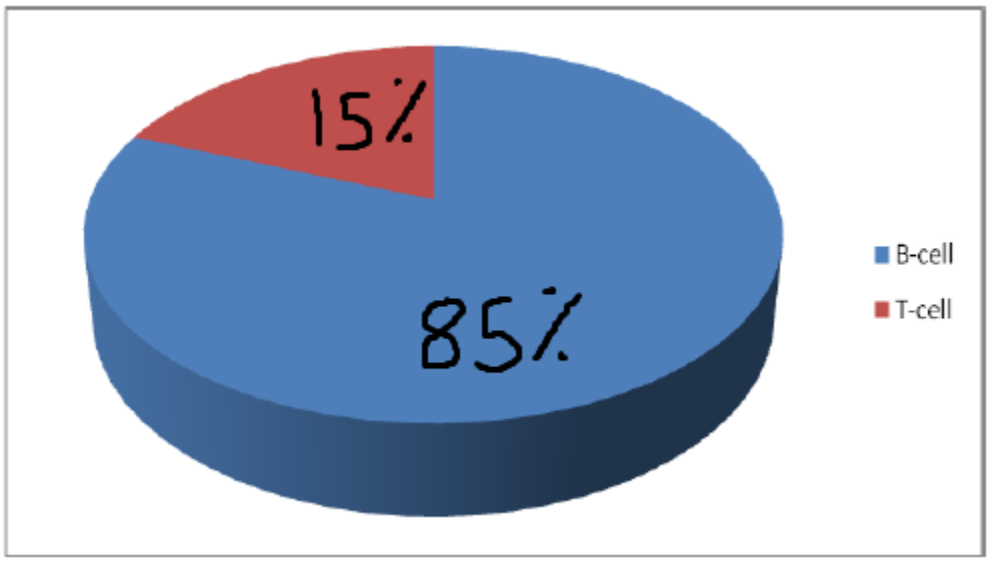


Figure 1.1: Percentage frequency of cell type in children of acute lymphocytic leukemia.

B-cell and T-cell lymphoblastic leukemia cells express surface antigens that parallel their respective lineage developments. Precursor B-cell ALL cells typically express CD10, CD19, and CD34 on their surface along with nuclear terminal deoxynucleotide transferase (nTdT), while precursor T-cell ALL cells commonly express CD2, CD3, CD7, CD34, and nTdT (Neelkamal et al., 2013). Usually T-lineage ALL is diagnosed in male adolescents and is frequently characterized by high white blood cells count at diagnosis, older age, mediastinal mass, and central nervous system involvement. Furthermore, children with T-ALL generally have a poorer prognosis than those with precursor B-lineage ALL (Goldberg et al., 2003; and Willemse et al., 2002).

Since the first description in 1948 of temporary remission of leukemia induced by chemotherapy, children of ALL has provided a model for refinement of survival among patients with cancer by progressive improvements in the efficacy of multi-agent chemotherapy regimens and by stratification of treatment intensity according to the clinical features of the patient, the biologic features of the leukemia cells, and the early response to treatment which are predictive of the risk of relapse. These advances have increased the survival rate from less than 10% in the 1960s to 90% nowadays (Figure 1.2) (Hunger and Mullighan, 2015).

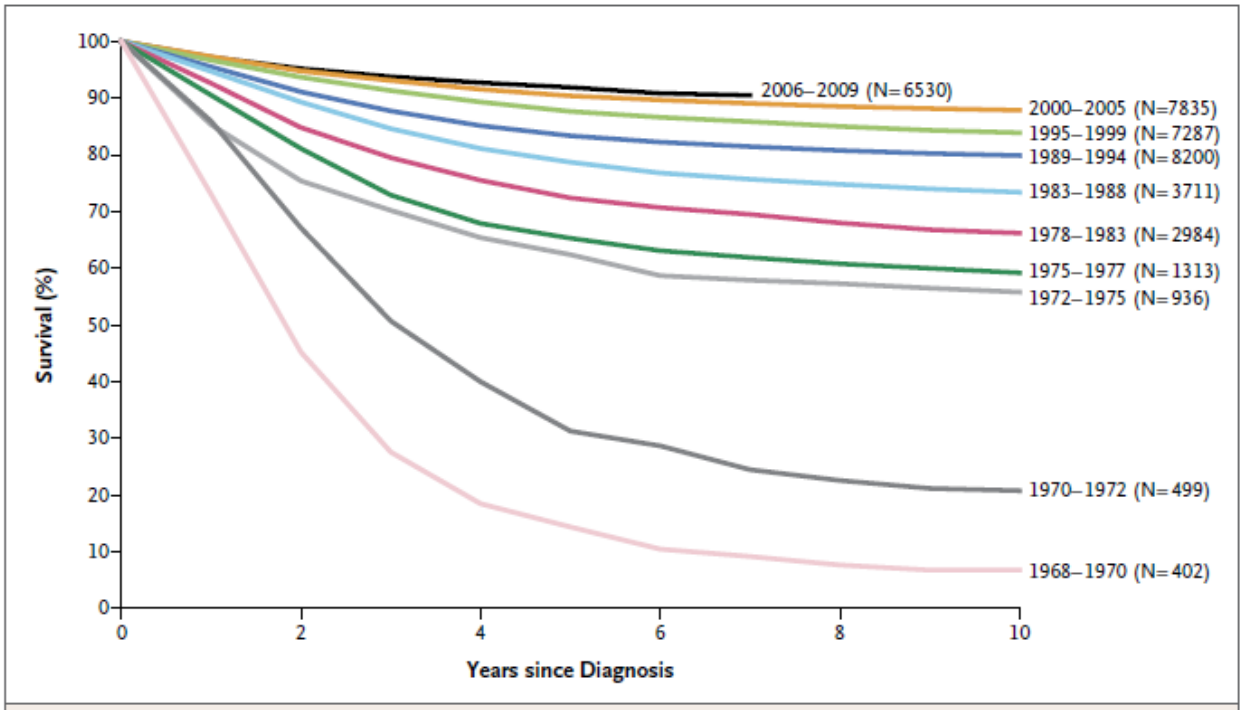


Figure 1.2: Overall survival among children with acute lymphoblastic leukemia, 1968-2009.

The survival rate of children ALL patients has improved to approximately 90% in recent years, especially for groups with good prognosis. This progress is mainly due to the stratification of patients into different risk groups. Stratification into risk groups is based on a range of clinical, and biological features such as age, gender, white blood cell (WBC) count at diagnosis, immunophenotype, and early response to induction therapy (de Sousaa et al., 2015).

Early response to treatment determined by the level of minimal residual disease (MRD) at the end of induction therapy (day 33) is actually the most important prognostic factor in patients with ALL (de Sousaa et al., 2015).

In a study at the time of morphologic remission at time point 1 at 5 weeks of treatment, 80% of patients with T-ALL were still MRD positive in contrast to 54% patients with precursor B-ALL. However, this study showed that MRD analysis allows a highly accurate prediction of clinical outcome within the 2 major immunophenotypic ALL categories (Willemse et al., 2002).

Nevertheless, the number of MRD positive patients and its level were higher in T-ALL than in precursor B-ALL, reflecting the more frequent occurrence of resistant disease in T-ALL patients (Willemse et al., 2002).

Furthermore, 23% of patients with T-ALL and 46% of patients with precursor B-ALL were classified in the low risk group, and had a 5-year relapse free survival rate of 98% or greater. In contrast, 28% of patients with T-ALL were classified in the MRD based high risk group compared to just 11% of patients with precursor B-ALL, and the relapse free survival rates were 0% and 25%, respectively (Willemse et al., 2002).

In another study on precursor B-ALL, 5-year event free survival was >90%. Therefore, MRD negativity at the end of induction point 1 at day 33 is the strongest predictor for excellent clinical outcome (Conter et al., 2009).

Minimal residual disease is a powerful predictor of the overall response (clinical outcome) of treatment in children ALL. The most reliable and validated methods to estimate MRD in ALL are flow cytometric analysis of leukemia associated immunophenotypes and polymerase chain reaction (PCR) amplification of antigen receptor gene rearrangements (Gaipa et al., 2013).

The causative factors of acute lymphoblastic leukemia may be like smoking, high birth weight, diet, and high socioeconomic status, electromagnetic field, being exposed to ionizing radiation,

pesticides, past treatment with chemotherapy or other drugs that weaken the immune system (Neelkamal et al., 2013).

Demonstration of seasonal variation in the incidence of acute lymphoblastic leukemia may provide insight into potential risk factors. Although the etiology of acute lymphocytic leukemia in pediatric remains largely unknown, hypotheses relating to a viral infections have gained support in recent years because viral infections have seasonal variations of onset (Badrinath et al., 1997, and Karimi and Yarmohammadi, 2003).

There have been a number of reports on the seasonality of leukemia, but the results are inconsistent due to some studies reported a significant late spring/early summer excess in the onset of leukemia, and another studies found an excess acute lymphoblastic leukemia patients in late autumn/early winter. However, several studies failed to demonstrate any significant seasonality in the onset of acute lymphoblastic leukemia cases (Badrinath et al., 1997, and Karimi and Yarmohammadi, 2003).

1.2. Acute lymphoblastic leukemia in the United States

ALL is the most common cancer among children and the most frequent cause of death from cancer before 20 years of age. Approximately 6000 cases of ALL are diagnosed in the United States annually, half of the cases occur in children and teenagers (Ward et al., 2014 and Hunger and Mullighan, 2015).

The incidence of ALL is about 30 cases per million persons younger than 20 years of age, with the peak incidence between 3 to 5 years of age, and develops more frequently in males than in females with (male: female ratio of 55% to 45%) (Hunger and Mullighan, 2015).

1.3. Acute lymphoblastic leukemia in the Middle East

In the Middle East, precursor B-cell ALL represented 84.2% and T-cell ALL represented 14.8% with 47.1% in low/standard risk, 16.9% with intermediate risk, and 36% with high risk according to clinical criteria. The peak age range at diagnosis between 3-6 years is similar to the ALL reported from the west with gender ratio of male to female (1.4:1) that may be slightly higher than reported from the west (AL-Mulla et al., 2014). Children had excellent induction response to chemotherapy with an overall complete remission rate of 96% (AL-Mulla et al., 2014).

However, the immunophenotypic distribution of ALL subtypes in the Middle East is similar with the general distribution pattern in developed countries (AL-Mulla et al., 2014).

1.4. Acute lymphoblastic leukemia in Palestine

The number of reported acute lymphoblastic leukemia cases in children by hospitals in West Bank in Palestine between 2009 to 2015 was 119 cases. However, 68 cases were registered at Huda Al-Masri pediatric cancer department at Beit-Jala Hospital. The number of reported acute lymphoblastic leukemia deaths in these Hospitals at west bank in Palestine from 2009 to 2015 was 9 cases, with a mortality rate of 7.6 per 100 (Palestinian Health Information Center, 2015).

There was a possibility that the immunological subtypes of pediatric acute lymphoblastic leukemia could include high risk of leukemia subtypes. To our knowledge, immunophenotyping analysis, clinical features, and risk stratification have not been previously tested in Palestine in previous studies.

1.5. Problem statement

Cancer incidence rates in Palestine have been increased; in addition, cancer has become the second leading cause of death. Leukemia has been reported to be the fourth most common cancer among Palestinians in the west bank both in 2014 and 2015 with ALL is the most common cancer among children. Many clinical and immunophenotypic characteristics have been identified as having prognostic significance in the outcome of patients with ALL, yet have never been studied in Palestine. In this study, we explored the clinical and immunophenotype pattern among children ALL patients diagnosed on 2015 at Huda Al-Masri pediatric cancer department at Beit-Jala Hospital; one of the largest oncology centers in Palestine.

1.6. Justification of the study

ALL has been associated with high morbidity and mortality among children worldwide and in Palestine. The clinical and the immunophenotype pattern of pediatric ALL have never been investigated among Palestinians although they are postulated to be associated with patients prognosis and complete remission of leukemia.

1.7. Study Questions

1:What are the clinical and the immunophenotypic characteristics of Palestinian pediatric ALL patients?
2: What are their associations with patients prognosis and complete remission of leukemia?

1.8. Study Aims

Immunophenotyping of pediatric ALL patients by Multicolor Flow Cytometric Analysis at a single cancer center experience in southern west bank in Palestine:

1: To characterize the immunophenotypic pattern of pediatric ALL subtypes among Palestinians.

2: To highlight the importance of some clinical features according to age and immunophenotypic pattern in patients prognosis and response to treatment.

3: To determine the response to therapy after induction phase using the MRD in time point 1 (day 33).

Chapter two

Literature review

2.1. Background

Acute lymphoblastic leukemia is a malignant clonal proliferation of lymphoid progenitor cells which differentiate into subtypes B and T, and is a fast growing cancer of a type of white blood cells called lymphoblasts (Figure 2.1). These cells are usually found in the bone marrow that produces a large number of immature lymphoblasts, prevent healthy blood cells from being made and do not work properly to fight infection (National Cancer Institute, 2016).

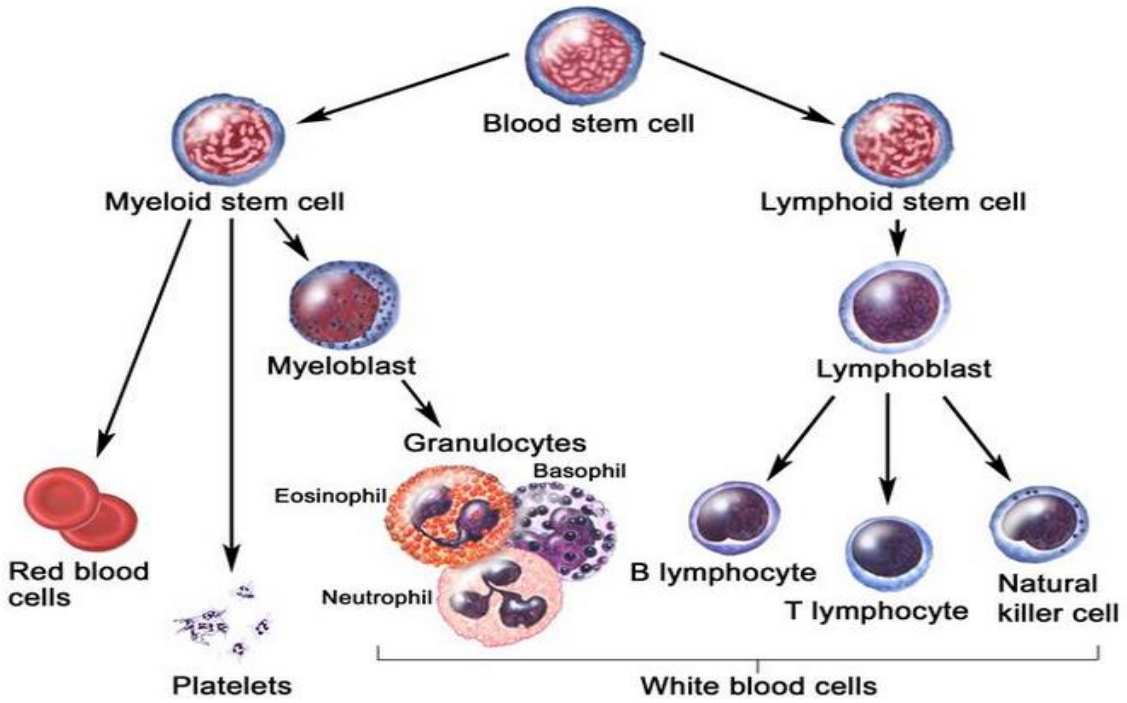


Figure 2.1: Blood cell development.

A blood stem cell goes through several steps to become a red blood cell, platelet, or white blood cell (National Cancer Institute, 2016).

2.2. Epidemiology of acute lymphoblastic leukemia

Annually, almost 3000 children in the United States are diagnosed with ALL, with annual incidence 3.7-4.9 cases per 100,000 children between 0-14 years of age with a similar estimated worldwide incidence (Ribera and Oriol, 2009). However, the improvements in diagnosis and treatment, overall survival rates for children with acute lymphoblastic leukemia have reached 90% (Hunger et al., 2012).

As explained by the following studies, it appears that the incidence of pediatric leukemia is increasing. In the analysis of Surveillance, Epidemiology, and End Results (SEER) database, there was a steady increase in ALL incidences from 1975 to 2010 with an average annual increase in incidence of almost 0.7 percent (Howlader et al., 2013). However, the estimated incidence increased from 25 cases per million in 1975 to 34 cases per million in 2010 (Ward et al., 2014).

Another study that used data from 63 European population based cancer registries of children diagnosed with cancer, the incidence of leukemia including ALL increased by a rate of 1.4 percent from 1970 to 1999 (Steliarova-Foucher et al., 2004). In Great Britain, the incidence of leukemia mostly attributable to ALL has steadily increased from 3.83 to 4.61 per 100,000 persons by sex and age from the five year period of 1971 to 1975 and 1996 to 2000 (Shah and Coleman, 2007).

On the other hand, a study from four Nordic countries (Denmark, Finland, Norway and Sweden) recorded that the incidence of pediatric ALL stayed stable at an approximate rate of 3.3 cases per 100,000 pediatrics below 15 years of age from 1983 to 2002 after an increase in incidence between 1975 and 1983 (Svendsen et al., 2007).

From 1992 to 2011, the incidence rate for acute lymphoblastic leukemia increased significantly among both Hispanic children and non-Hispanic children in USA as you see in Figure 2.2 (Howlader et al., 2013).

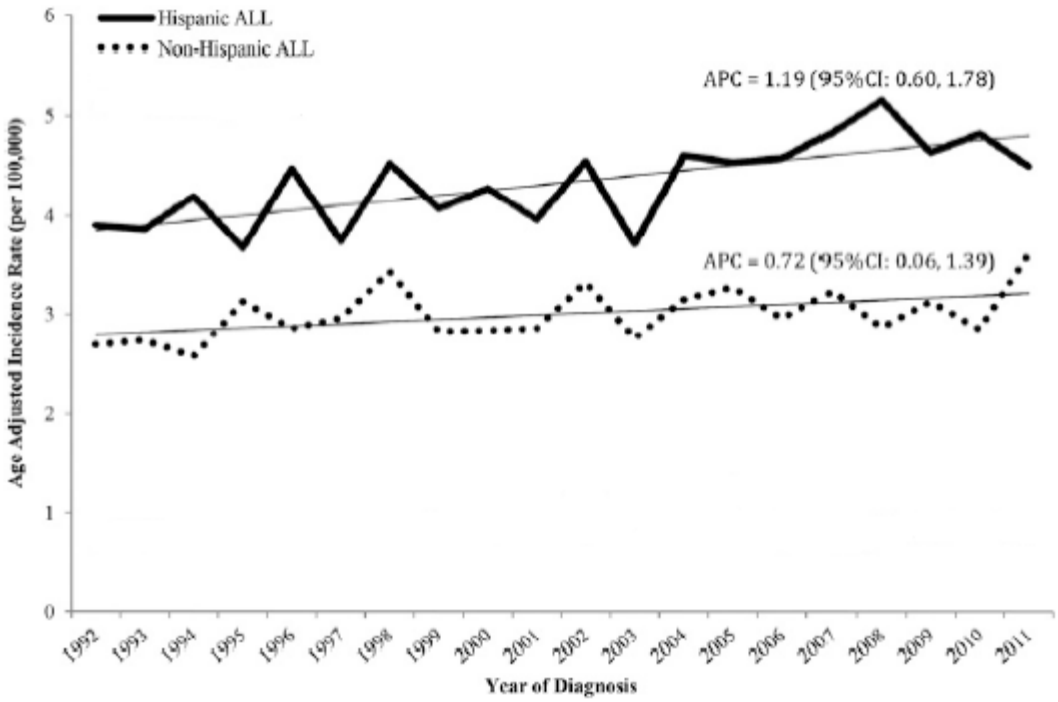


Figure 2.2: Age adjusted incidence rates of pediatric acute lymphocytic leukemia by ethnicity, SEER 13, 1992-2011.

The incidence of ALL appears to be highest in Hispanic children (43 cases per 1 million), and the incidence is substantially higher in white children than in black children, with a nearly three fold higher incidence of ALL from age 2 to 3 years in white over the black (National Cancer Institute, 2016).

However, there is no published data was found to explain the prevalence of pediatric ALL in Palestine.

2.3. Early clinical signs and symptoms of ALL

The most common presenting symptoms of ALL are nonspecific such as loss of appetite and weight loss, fever, night sweats, bleeding, bone pain, and lymphadenopathy (Margolin et al., 2001 and National Cancer Institute, 2016). Unexplained existence of any of these common signs or symptoms should be immediately considered of malignancy.

The most common presenting signs or symptoms of leukemia that are revealing of malignancy are:

1.Musculoskeletal pain: Early bone marrow screening should be considered in any child who has existence bone pain and peripheral blood abnormalities. Bone pain, specifically affecting the long bones and caused by leukemic involvement of the periosteum, is a presenting symptom in 21 to 38 percent of cases of acute leukemia (Rogalsky et al., 1986; Jonsson et al., 1990 and Sinigaglia et al., 2008).

2.Headache: Although uncommon in less than 5 percent of cases, leukemia involving the central nervous system (CNS) can exist with symptoms of increased intracranial pressure, including headache, vomiting, lethargy, and or nuchal rigidity (Bleyer, 1988, and National Cancer Institute, 2016).

3.Lymphadenopathy: Lymphadenopathy associated with malignancy usually is non-tender, firm, rubbery, and matted. Almost 50 percent of pediatric ALL exist with lymphadenopathy, which is considered to be one of the indications of extramedullary leukemic spread (Margolin et al., 2001, and National Cancer Institute, 2016).

4.Testicular enlargement: Although it is very rare, painless unilateral testicular enlargement can be a presenting sign of ALL. The persistent of painless solid testicular mass should be referred for biopsy by a pediatric surgeon after evaluation by ultrasound (National Cancer Institute, 2016).

5.Mediastinal mass: T-cell ALL commonly occurs in older boys (≥10 years old) who present with high initial white blood cell (WBC) counts (≥50,000/ul) and a large mediastinal mass (Margolin et al., 2001 and National Cancer Institute, 2016).

6.Peripheral blood abnormalities: Most pediatric ALL have anemia, thrombocytopenia with either normal or slightly increased WBC counts and lymphoblasts on peripheral smear (Chiaretti et al., 2013). Almost 50 percent of pediatric ALL have WBC counts <10,000/ul, and 20 percent have an initial WBC count >50,000/ul (Margolin et al., 2001 and Chiaretti et al., 2013). Furthermore, one-half of pediatric ALL have bleeding (including petechiae and purpura), and three quarters have a platelet count <100,000/ul at the time of diagnosis (Margolin et al., 2001).

2.4. Classical laboratory tests and clinical procedures for ALL

ALL can present clinically as either a mass lesion or as leukemia. Although the difference in some patients is qualitative, ALL is the preferred term when the bone marrow contains more than

25 percent lymphoblasts, whereas lymphoma is the best term when the process is restricted to a mass lesion with minimal or no blood and bone marrow involvement (Brunning et al., 2001).

The diagnosis and classification of leukemia by using specialized tests that are performed on cells derived from a bone marrow aspirate or tissue biopsy specimens. When clinical situations excludes bone marrow examination, the diagnosis can be occur from cells obtained from the peripheral blood or pleural effusions to diagnose ALL and find out if leukemia cells have spread to other parts of the body such as the brain or testicles (Margolin et al., 2001 and National Cancer Institute, 2016).

A physical exam that may detect bruising, hepatosplenomegaly (Swollen of liver or spleen), swollen of lymph nodes, and petechiae or purpura. Complete Blood Count (CBC) test that measures the number of red blood cells and platelets, the number and type of white blood cells, the amount of hemoglobin in the red blood cells, and the portion of the sample made up of red blood cells. Blood chemistry tests that used to measure the amounts of certain substances released into the blood by organs and tissues in the body. An uncommon either higher or lower than normal amount of a substance can be a sign of disease. Bone marrow aspiration for flow cytometry diagnosis and biopsy for morphological diagnosis of leukemic cells (National Cancer Institute, 2016).

Several tests used to characterize and stratify leukemic cells such as immunophenotyping test and molecular cytogenetic test. Immunophenotyping analysis that used antigens or markers on the surface of a bone marrow cells are characterized into lymphoid cells or myeloid cells, and then if the cells are malignant lymphocytes, they are also distributed into B lymphocytes or T lymphocytes by these specific antigens.
Molecular cytogenetic analysis used to characterize the changes of chromosomes in the lymphocytes under an microscope. For instance, in Philadelphia chromosome positive ALL, part of one chromosome is replaced to another chromosome (National Cancer Institute, 2016).

Another tests such as Fluorescence In Situ Hybridization (FISH) may also be used to diagnose certain changes in the chromosomes (Margolin et al., 2001). Moreover, another tests used to detect relapse and metastasis of leukemic cells such as lumbar puncture test, chest X-ray test, and testicular biopsy test (National Cancer Institute, 2016).

2.5. Differential diagnosis

A different malignant and nonmalignant situations must be considered in the differential diagnosis of ALL, because the existing signs and symptoms are nonspecific. They include (Simone et al.,1975 and Margolin et al., 2001):

- Juvenile idiopathic arthritis.
- Osteomyelitis.
- Epstein-Barr virus.
- Immune thrombocytopenia (ITP).
- Aplastic anemia.
- Acute infectious lymphocytosis.
- Other malignancies with bone marrow involvement (neuroblastoma, retinoblastoma, rhabdomyosarcoma, and Ewing sarcoma).
- Hypereosinophilic syndrome.

2.6. Pathological features of ALL

Historically, ALL has been classified morphologically using the French-American-British (FAB) system that integrates information regarding the size, amount of cytoplasm, and importance of nucleoli of tumor cells from the bone marrow aspirate (Bennett et al., 1976 and National Cancer Institute, 2016).

Nevertheless, some clinicians still use this system to characterize and describe the phenotype of the tumor cells, but it has lost its prognostic value as our knowledge of disease biology has improved and treatment regimens have begin to be more intense and successful. However, FAB criteria is not currently used in either diagnosis or treatment decisions (Bennett et al., 1976 and National Cancer Institute, 2016).

According to the FAB system:

●L1 lymphoblasts are small cells with weak cytoplasm, condensed nuclear chromatin, and unclear nucleoli (Figure 2.3). Most pediatric ALL cases almost 85 to 89 percent are classified as having FAB L1 (Miller et al., 1981 and Lilleyman et al., 1992).

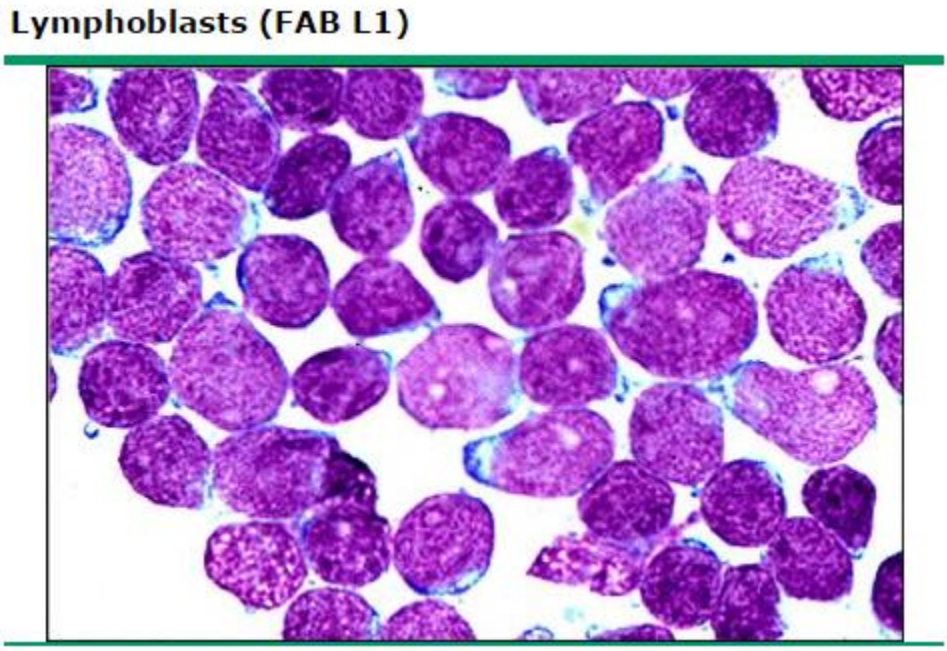


Figure 2.3: L1 morphology of bone marrow aspirate from a patient with ALL.

●L2 lymphoblasts are larger cells with a moderate amount of cytoplasm, dispersed chromatin, and multiple nucleoli (Figure 2.4). In several studies, L2 has been correlated with poor prognosis than has L1 (Miller et al., 1981 and Lilleyman et al., 1986). While, when patients are stratified according to age, sex, and initial WBC, differences in prognosis between L1 and L2 are no longer observed (Lilleyman et al., 1992). Eleven to fourteen percent of cases of pediatric ALL are classified as FAB L2 (Miller et al., 1981 and Lilleyman et al., 1992).

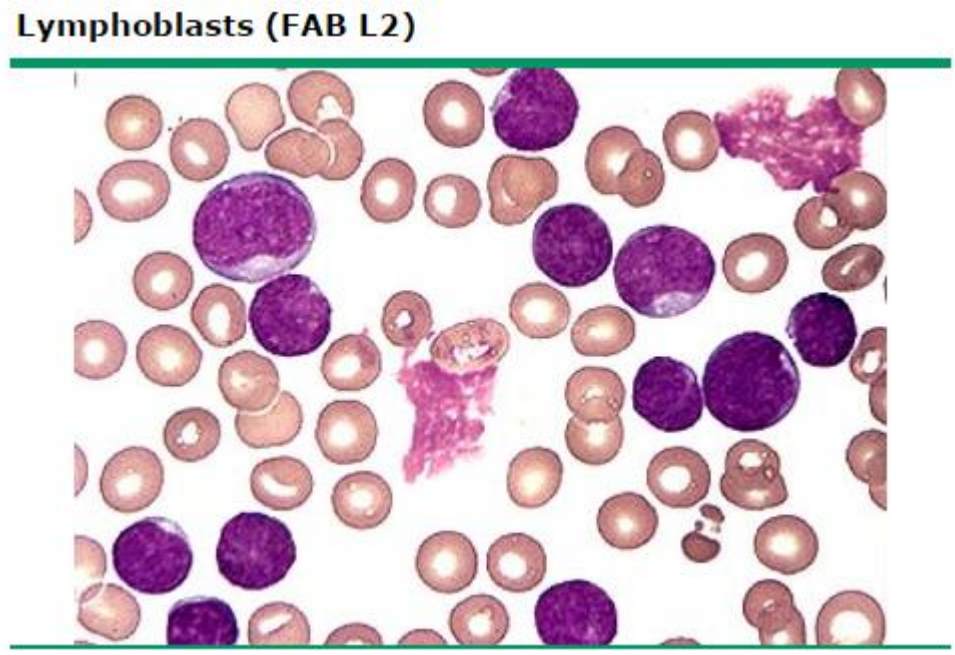


Figure 2.4: L2 morphology of bone marrow aspirate from a patient with ALL.

●L3 lymphoblasts have deep cytoplasmic basophilia with distinct cytoplasmic vacuolation (Figure 2.5). L3 morphology relates with a more guarded prognosis. However, less than 1 percent of cases of pediatric ALL are classified as FAB L3 (Miller et al., 1981 and Lilleyman et al., 1992).

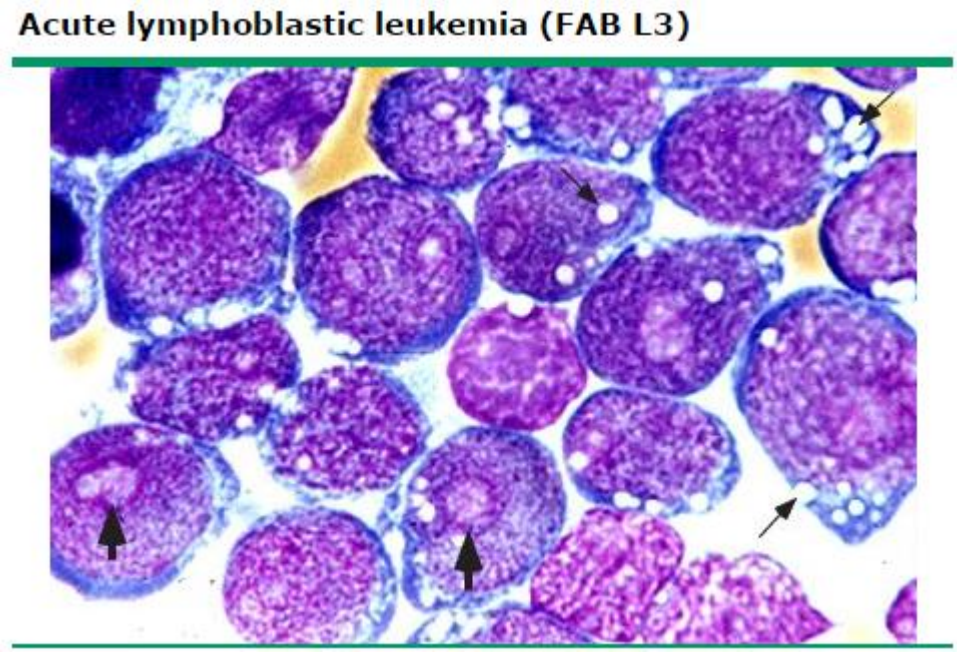


Figure 2.5: L3 morphology of bone marrow aspirate from a patient with ALL.

2.7. Immunophenotyping of ALL

FAB classification of ALL has been modified by the World Health Organization (WHO) depending on immunophenotyping using an extensive panel of monoclonal antibodies to cell surface "cluster of differentiation" (CD) markers (Chiaretti et al., 2014).

Immunophenotyping by means of multi channel flow cytometry has become the standard procedure for ALL diagnosis and sub-classification. It was also developed as useful tool for the detection and monitoring of minimal residual disease. The consensus by European Group for the Immunological characterization of leukemia (EGIL) is that a threshold of 20% should be used to define a positive reaction of blast cells to a given monoclonal antibody, except for myeloperoxidase (MPO), CD3, CD79a and nTdT, which are considered positive at the 10% level of expression, and 30% or more for myeloid markers such as CD33, and CD13 (Chiaretti et al., 2014).

The cases were classified into four main ALL immunological subtypes: precursor B-cell ALL that constitutes about 80% of cases, mature B-cell ALL that constitutes about 2-5% of cases, precursor T-cell ALL, and mature T-cell ALL that constitute about 15-17% of cases (Zipf et al., 2000). A case was considered precursor B-cell ALL, if the cells expressed cytoplasmic CD79a and Immunoglobulin (Ig), and surface CD19, CD10, Human Leukocyte Antigen-D related (HLA-DR), CD34 ,CD20, CD22, and nTdT. A case was considered mature B-cell ALL, if the cells have also surface Ig expression and clonal lambda or kappa light chains, and negative for nTdT and CD34 (Bene et al., 1995 and Chiaretti et al., 2014).

A case was considered precursor T-cell ALL, if the cells expressed cytoplasmic CD3, and surface CD7, CD1a, CD2, CD5,CD4, CD8, CD34, and nTdT. However, it was considered mature T-cell ALL, if the cells have also surface CD3, CD4 or CD8, and negative for CD1a, CD34, and nTdT (Bene et al., 1995 and Chiaretti et al., 2014). In T-cell ALL, the expression of CD10 is quite common (25%) and not specific (Chiaretti et al., 2014).

Furthermore, some B-cell and T-cell ALL also express myeloid markers such as CD13 and CD33 in addition to their specific lymphocyte markers (Chiaretti et al., 2014).

2.8. Cytogenetics of ALL

Cytogenetic features play an important role in the risk stratification in pediatric ALL. Abnormalities concluding both chromosome number and chromosome structure such as gene rearrangements and fusions have an effect on patient prognosis (Harrison, 2009).

2.8.1. Numeric abnormalities in ALL:

Numerical abnormalities may influence the whole chromosome set (ploidy changes), or the gain or loss of individual chromosomes (aneuploidy changes). The following chromosomal numeric abnormalities are prognostic indicators in patients with ALL:

- High hyperdiploidy (50 or more chromosomes) is a suitable prognostic feature, and has been uniformly associated with good prognosis in ALL (Rubnitz et al., 2008). Patients with high hyperdiploidy, those with the best outcomes in clinical trials from the United States include those with either double trisomies of chromosomes 4 and 10, or triple trisomies of chromosomes 4, 10 and 17 (Sutcliffe et al., 2005).
- Hypodiploidy (fewer than 46 chromosomes) is a poor prognostic indicator. The probability of a poor outcome increases with a decrease in chromosomal numbers. However, hypodiploid cases lacking only a single chromosome have a similar prognosis to diploid cases, whereas cases which are near-haploid (24 to 28 chromosomes), have the worst outcome (Nachman et al., 2007).
- Although rare cases with near triploidy (68 to 80 chromosomes) or near tetraploidy (>80 chromosomes) generally are associated with very poor outcome (Pui et al., 1990), and a large series has reported suitable outcome in B-cell lineage cases (Raimondi et al., 2006).

2.8.2. Structural abnormalities in ALL:

Structural gene abnormalities include translocations, deletions, insertions, and inversions. The following structural chromosomal abnormalities are prognostic indicators in patients with ALL:

- The t(12;21) translocation produces the TEL-AML1 fusion gene. This structural change is connected with a suitable prognosis (Shurtleff et al., 2005).
- The t(9;22) translocation also known as the Philadelphia chromosome: is one of two structural rearrangements associated with poor prognosis (Jones et al., 2005).
- Another rearrangements involving the MLL gene at 11q23 are connected with a poor prognosis (Pui et al., 2002).

2.9. Prognostic factors and risk stratification currently used to direct therapy in ALL

Many clinical, and biological features have been identified as having prognostic significance in the clinical outcome of patients with ALL. The standard features are age, gender, WBC count at diagnosis, and immunophenotype pattern of the patients, and the most two important prognostic factors are age at diagnosis, and the initial WBC count (Friedmann and Weinstein, 2000 and Hunger and Mullighan, 2015).

Infants less than one year, adolescents greater than nine years, and pediatrics with WBC count above 50,000/µl being at higher risk. Immunphenotyping of the lymphoblast is highly significant prognostic feature, because T-cell and mature B-cell immunophenotypes are associated with a very poor prognosis, and precursor B-cell is associated with a good prognosis (Table 2.1) (Friedmann and Weinstein, 2000 and Hunger and Mullighan, 2015).

Table 2.1: Prognostic factors in pediatric acute lymphoblastic leukemia.

Risk Factor	Favorable Outcome	Unfavorable Outcome
Age	>1 and <10 years	<1 or >10 years
Gender	Female	Male
WBC count at diagnosis	<50,000/ul	>50,000/ul
Immunophenotype	Precursor B-cell	T-cell, or mature B-cell

The prognostic value of gender is argumentative, due to some studies report no difference in prognosis between males and females, but another centers especially from United Kingdom (UK) and Nordic countries have found a significantly higher relapse rate in males over females (ARYA, 2004).

Most of the prognostic factors currently used to determine the intensity of therapy are clinical or biological and can be determined at the time of diagnosis. There is great variation in risk

stratification of ALL used by the major clinical trials groups treating pediatric cancer in the United State. Mature B-cell ALL is treated completely differently from precursor B-cell and T-cell ALL. For the most part, the treatment of precursor B-cell ALL, and sometimes T-cell ALL are based on age and WBC at diagnosis as in Table 2.2 that shows the association between age, WBC count, and prognosis of pediatrics treated on Pediatric Oncology Group (POG) and Children's Cancer Group (CCG) regimens as of 1993 (Friedmann and Weinstein, 2000).

Table 2.2: Outcome of 4-year event free survival for children with precursor B-cell ALL treated by POG and CCG protocols by multiple age and WBC count categories.

	Age (years)			
WBC count/ul:	**1-2.99**	**3-5.99**	**6-9.99**	**≥10**
<10,000				
4-year event free survival %	82.9	84.7	82	69.6
Number of patients treated	490	937	437	406
% precursor B-cell patients	10.7	20.5	9.6	8.9
10,000-49,999				
4-year event free survival %	74.6	74.5	80.2	59.2
Number of patients treated	436	608	205	236
% precursor B-cell patients	9.5	13.3	4.5	5.2
≥50,000				
4-year event free survival %	68.3	73.9	47.5	41.1
Number of patients treated	278	280	122	140
% precursor B-cell patients	6.1	6.1	2.7	3.1

However, irregularity in the definitions of treatment related risk groups have made it difficult to contrast the results of various clinical tests. Due to of this, a workshop was held in 1993 by the Cancer Therapy Evaluation Program of the National Cancer Institute (CTEP/NCI) to try to create a uniform approach to risk stratification in order to increase the efficiency of clinical research. The participating investigators reached an unanimity on the basis of available data that patients with precursor B-cell ALL, aged one to nine years, and with WBC count less than 50,000/μl at diagnosis which represent the most of patients are at lower risk than other patients (Friedmann and Weinstein, 2000 and Schultz et al., 2007).

Table 2.3 shows the four year event free survival for POG and CCG patients at the time of the workshop for standard risk group compared to the higher risk group (Friedmann and Weinstein, 2000).

Table 2.3: Uniform age and WBC criteria for precursor B-cell ALL standard and high risk cohorts adopted at the CTEP/NCI workshop.

Risk	**Definition**	**4-year event free survival %**	**% of precursor B-cell patients**
Standard	WBC count <50,000/ul and age 1-9.99 years	80.3	68
High	WBC count ≥50,000/ul or age ≥10 years	63.9	32

Nevertheless, current stratification systems use these criteria in addition to several other features to stratify patients into treatment groups such as immunnophenotype among different age and WBC count groups. For instance, T-cell ALL patients are generally older than precursor B-cell ALL patients, partially clarifying the high risk situation of older patients. Similarly, the very poor prognostic feature of older patients, tends to present with a higher WBC count (Friedmann and Weinstein, 2000 and Schultz et al., 2007).

Furthermore, extramedullary disease such as in CNS is a factor used to determine the intensity of treatment. CNS leukemia is more common in T-cell ALL than precursor B-cell ALL that occurs in fewer than 5% of pediatrics at diagnosis, and is generally predictive of a poor treatment outcome (Friedmann and Weinstein, 2000 and Hunger and Mullighan, 2015). CNS disease is defined as more than five white blood cells per ml of spinal fluid which are blasts morphologically.

However, patients with T-cell ALL are more likely to have unfavorable factors such as male, age greater than nine years, high WBC count at diagnosis, low CD10 expression, mediastinal mass, and CNS involvement (de Sousaa et al., 2015).

2.10. Treatment of pediatric ALL

Improvements in the 5-year survival rate for pediatric ALL continue to be seen by improving multiple factors including a better understanding of the immune-biology of ALL and disease burden, recognition of shelter sites and integration of pre-symptomatic central nervous system prophylaxis, lineation of prognostic factors with risk adapted treatment and improvements in supportive care. The overall outcomes are fairly similar, and more than 95% will attain remission and close to 85% will survive free of leukemic recurrence at least 5 years from diagnosis (Seibel, 2008).

One of the hallmarks of the treatment of pediatric ALL is the dependence on risk based stratification by determining the clinical and biological features that have been shown to affect prognosis, patients can be classified into groups based on risk of treatment failure. Those with favorable clinical and biological features can be treated with the modest therapy to spared toxicity, whereas more aggressive regimens are restricted for those with more high risk disease to maximize cure. It is therefore fundamental to identify those features shown to consistently affect prognosis and influence treatment as in Table 2.4 (Rubnitz et al., 2000; Seibel, 2008 and Stacy et al., 2015).

Successful treatment of pediatric ALL involves administration of a multidrug regimen that is divided into several phases (induction, consolidation, and maintenance) and includes therapy directed to the CNS. However, most treatment protocols take two to three years to complete. (Rubnitz et al., 2000; Seibel, 2008 and Stacy et al., 2015).

Table 2.4: Clinical risk assignment and suggested therapies in pediatric ALL.

Risk Group	Features	Percent%	Recommended Therapy
Low	Hyperdiploid or trisomies 4,10,17	20	Conventional anti-metabolite-based therapy
	T(12,21)	20	
Standard	WBC <50,000/microL	15	Intensified antimetabolite therapy
	Age 1 to 9.9 years		
High	T cell Phenotype	15	Intensive multi-agent therapy
	Age > 10 years	15	

	WBC > 50,000/micro , t(1;19)	6	
Very High	t(9;22)	3	Consider allogenic hematopoietic cell transplantation in first remission
	t(4;11); age <1year	4	
	Induction failures and slow responders	2	

2.10.1. Induction therapy of ALL:

Induction therapy is the initial phase of treatment, the first block for chemotherapy, lasting for 4 to 6 weeks, and is designed to put the patient in complete remission, and restore normal bone marrow hematopoiesis. More than 90 percent of children and adolescents with ALL come in complete remission at the end of induction therapy regardless of their initial risk grouping (Seibel, 2008 and Stacy et al., 2015).

Early clearance of lymphoblasts from the bone marrow and the presence of minimal residual disease at day 15 and the end of induction therapy at day 33 are the best indicators of clinical outcome. Patients who repay quickly to the induction regimen appear to have a more favorable outcome, whereas those who have a slow response or who lose induction therapy have a more guarded prognosis (Sutton et al., 2009).

The agents used during induction phase include vincristine, corticosteroids, and asparaginase, with most regimens adding an anthracycline such as usually doxorubicin or daunorubicin. However, both anthracyclines have been shown to have similar efficacy and toxicity in randomized trials. Certain groups spare the addition of anthracycline to those with lower risk groups in an effort to decrease toxicity. The corticosteroid used is often prednisone or dexamethasone, with dexamethasone demonstrating improved CNS penetration and decreased risk of relapse, but with increased incidence of toxicities such as avascular necrosis, infection, and reduction in linear growth (Seibel, 2008 and Stacy et al., 2015).

Several different agents for asparagine depletion exist as well, including polyethylene glycol (PEG) asparaginase and Erwinia asparaginase. PEG asparaginase has been modified by covalently attaching polyethylene glycol, which has been demonstrated to result in a longer half

life and decreased immunogenicity in comparison with native *Escherichia coli* L-asparaginase. However, randomized trials have also shown superior efficacy of the pegylated formulation. Nevertheless, Erwinia asparaginase is often given to the patients who have experienced an allergic reaction to PEG asparaginase, and requires a more frequent administration schedule (Stacy et al., 2015).

2.10.2. CNS preventive therapy:

Leukemic involvement of the CNS at the time of diagnosis is an uncommon result, occurring in less than 5 percent of patients (Bleyer, 1988 and Seibel, 2008). Although bone marrow remission could be achieved using systemic chemotherapy, most children eventually developed CNS relapse in the absence of specific therapy directed toward this site. This approach includes both treatment of patients with clinical CNS disease at diagnosis and prophylaxis for patients with subclinical disease (Stacy et al., 2015).

There are several methods of achieving the goal of eradication of disease from the CNS, including direct intrathecal administration of chemotherapy, systemic administration of chemotherapy able to penetrate the blood-brain barrier, and cranial radiation. All treatment plans include intrathecal administration of chemotherapy beginning during remission induction.
Some protocols include intrathecal treatment throughout therapy, whereas others do not include it in maintenance (Seibel, 2008 and Stacy et al., 2015).

There are several options for intrathecal chemotherapy include intrathecal methotrexate or a combination of intrathecal methotrexate, cytarabine, and hydrocortisone as triple intrathecal therapy. Studies have shown no significant difference in overall or event free survival between them, although some evidence points to decreased frequency of CNS relapse with the use of triple intrathecal therapy. Systemically administered chemotherapy with CNS effects includes dexamethasone, high dose methotrexate, 6-mercaptopurine, cytarabine or cyclophosphamide, and L-asparaginase (Seibel, 2008 and Stacy et al., 2015).

2.10.3. Consolidation therapy of ALL:

Consolidation or intensification therapy is the second phase of ALL treatment and is initiated soon after realization of complete remission. Continuing treatment is required to eradicate the submicroscopic residual disease because small numbers of leukemic lymphoblasts referred to as MRD still in the bone marrow although histologic proof of complete remission after induction therapy. In such cases, relapse occurs quickly if therapy is not continued (Stacy et al., 2015).

The goal of post-induction chemotherapy is to prevent leukemic regrowth, reduce residual tumor burden, and block the evolution of drug resistance in the remaining leukemic cells (Stacy et al., 2015).

Intensification of therapeutic regimens last approximately between 6 to 9 months, and has been regulated depend on the patient's risk of poor outcome. A reduction of intensification therapy for patients with good prognosis while giving more intensive treatment for those in the high risk group with the goal of improving survival (Möricke et al., 2008, and Stacy et al., 2015).

This phase of chemotherapy involves combinations of different chemotherapeutic agents to maximize synergy and minimize drug resistance, usually including agents not used in the initial remission induction such as mercaptopurine, thioguanine, methotrexate, cyclophosphamide, etoposide, and cytarabine (Stacy et al., 2015).

2.10.4. Maintenance therapy of ALL:

Maintenance or continuation chemotherapy phase is the third, final, and longest stage of treatment in pediatric ALL. A much less intensive regimen than the prior chemotherapy, and the prolonged maintenance phase has been demonstrated to lower the risk of relapse once remission has been established after fulfillment of the consolidation or intensification phase of therapy. It often lasts at least 2 years, and the goal is to kill any remaining leukemia cells that may regrow and cause a relapse (Seibel, 2008 and Stacy et al., 2015).

The cornerstone of maintenance therapy is antimetabolite therapy with methotrexate and mercaptopurine, and both drugs are available in oral formulations. Some regimens also include monthly vincristine and steroids, although the evidence for additional benefit is unclear (Seibel, 2008 and Stacy et al., 2015).

2.11. Difficulties in defining complete remission in ALL

The primary goal of induction therapy for ALL is achievement of an initial complete remission defined as the eradication of all leukemia cells (less than 5% of blasts) from the bone marrow and blood detectable by microscopic review and the restoration of normal hematopoiesis (more than 25% cellularity and normal peripheral blood counts) (Cavé et al., 1998, and Stacy et al., 2015). Complete remission has historically been defined depend on morphologic criteria, and it has been proposed that an assessment of MRD might define a more tough (complete MRD response) that may be better able to predict prognosis (Cavé et al., 1998, and Stacy et al., 2015).

This approach is supported by several observations that explain the difficulty in ascertaining whether a patient with ALL in morphologic complete remission is likely to remain disease free (Cavé et al., 1998):

1:Hematogones: Morphologic evaluation may be unable to differentiate between ALL blast cells and lymphoid precursors (hematogones) or activated mature lymphocytes. This uniqueness is particularly difficult in samples of bone marrow recovering from chemotherapy or transplantation, where hematogones may account for 10% of the lymphoid cells.

2:Sampling error: A single bone marrow specimen represents only a very small percentage of the total bone marrow cellular population and thereby presents a potential for sampling error. However, several documented cases exist in which a bone marrow aspiration was normal at one site and showed leukemia at another.

3:Detection limits: The detection of ALL blasts by morphologic review or conventional cytogenetics is limited by operator error and the number of metaphases evaluated.

2.12. Minimal Residual Disease in ALL

In the past, treatment response has been monitored by morphological examination of bone marrow aspirates, and due to this process has a limited sensitivity and specificity, it can result in failure to detect residual leukemic cells, potentially leading to under treatment and an increase risk of relapse. On the Contrary, misclassification of normal cells such as ALL blasts may result in over treatment and an increase in the risk of treatment related morbidity (Gaipa et al., 2013).

Over the past 3 decades, remarkable advances have been achieved in the treatment of ALL in children. Although current treatment strategies result in long term remission for approximately 80% of children with ALL, the remaining 20% ultimately relapse, and cure rate after relapse is about 25% to 40%. Furthermore, some subgroups of children who now receive intensive therapy are likely to be over-treated and may well be cured using less intensive regimens, resulting in reduced toxicity and fewer long term side effects (Conter et al., 2009).

In addition to risk factors associated with the patient such as sex, and age at diagnosis and risk factors associated with the disease such as white blood cell count at diagnosis, and immunophenotyping, measurement of in vivo treatment effectiveness has confirmed to be the most important in predicting patient clinical outcome and risk of relapse. Many techniques have been developed to refine morphology in assessing response to treatment, including immunological or molecular markers, fluorescent in situ hybridization, in vitro drug response, and colony assays. This technologic advancement drove to introducing the concept of Minimal Residual Disease, which has challenged the conventional definition of remission (Conter et al., 2009).

Minimal Residual Disease is the presence of leukemic cells below the threshold of detection by conventional morphologic methods, and is an important component of patient evaluation and powerful predictor of clinical outcome over the course of sequential therapy in pediatric ALL (Karawajew et al., 2015). Studies in children with ALL have demonstrated the strong correlation between MRD and risks of relapse, as well as the prognostic significance of MRD measurements during and after initial induction therapy (Bruggemann and Campana, 2010).

The most important time for MRD assessment are upon completion of initial induction, and additional time points may be useful depending on the regimen of treatment used. However, the minimal limit of assay sensitivity to confirm MRD negativity should be <0.01%, and there are multicolor flow cytometry and PCR methods can detect leukemic cells at a sensitivity threshold of <0.01% bone marrow mononuclear cells (Bruggemann and Campana, 2010).

MRD positivity is defined by the presence of 0.01% or more ALL cells; the risk of relapse is generally proportional to the level of MRD, particularly when measured during or at the end of remission induction therapy. However, MRD is typically an independent strong prognostic factor that can identify poor responders among patients with acute lymphoblastic leukemia (Campana, 2010).

The large majority of patients with newly diagnosed ALL attain a morphologic and cytogenetic complete remission after induction chemotherapy. While most children experience prolonged relapse free survival, some children will relapse and die of leukemia. Relapse is result from residual leukemic cells that remain following the achievement of complete remission, but are below the limits of detection using conventional morphologic assessment. These subclinical levels of residual leukemia are MRD and can be evaluated using more sensitive assays (Coustan-Smith et al., 2000).

Complete remission was defined as the lack of physical signs of leukemia or detectable leukemia cells on blood smears, a bone marrow with active hematopoiesis and less than 5% leukemia blast cells, and normal cerebrospinal fluid (Conter et al., 2009).

Groups collaborating in the International Berlin-Frankfurt-Munster Study Group (I-BFM-SG) have pioneered the evaluation of MRD at days 33 and 78 of treatment as a time point 1 and 2 respectively to stratify patients into low, intermediate or high risk groups (Conter et al., 2009).

In this risk group classification, time point 1 appeared to be particularly useful for recognizing low, and intermediate risk patients and time point 2 for the recognition of high risk patients. MRD based risk group classification is used for treatment stratification in pediatric ALL protocols of the BFM–Associazione Italiana di Ematologia ed Oncologia Pediatrica (AIEOP) to identify the different response groups and differences in overall treatment response within T-ALL and precursor B-ALL (Willemse et al., 2002). However, negativity of MRD at time point 1 (day 33) was the most favorable prognostic factor (Schrappe et al., 2011).

2.13. Methods for detecting minimal residual disease

An assortment of techniques have been studied for the detection of residual disease, including cytogenetics, cell culture systems, FISH, Southern blotting, Multicolor flow cytometry, and Polymerase Chain Reaction (Campana, 2003 and Bacher et al., 2008).

MRD assays for ALL should allow to the detection of one leukemic cell among 10,000 normal cells or more. They should also reliably discriminate leukemic and normal cells, and allow a

timely output of the results. Finally, they should be robust enough to support consistent MRD estimates when applied in different laboratories. Currently, the most reliable methods to study MRD in ALL are flow cytometric analysis of leukemia associated immunophenotypes, and polymerase chain reaction amplification of antigen receptor gene rearrangements (Gaipa et al., 2013).

2.13.1. Multicolor Flow Cytometry:

Multicolor flow cytometry uses a laser to determine specific immunophenotypic features of up to thousands of cells per second. MRD can be identified using flow cytometry depend on the irregular expression of antigens (Al-Mawali et al., 2009).

Two examples explain the power of multicolor flow cytometry technique to determine MRD:

1: In B-cell lineage ALL, leukemic blasts nearly always co-express CD10, CD19, CD20, CD22, CD34, nTdT, and CD58 in addition to some T-cell antigens including CD5 and CD7, and myeloid antigens including CD13 and CD33 (Campanaa and Coustan-Smitha, 2004 and Karawajew et al., 2015).
2: In T-cell lineage ALL, leukemic blasts nearly always co-express terminal transferase, CD2, cytoplasmic CD3 (cCD3), CD5, nTdT, CD34, and CD7 (Krampera et al., 2003, and Campanaa and Coustan-Smith, 2004).

Sequential MRD monitoring using multi-parameter flow cytometry technique has been shown to be a valuable predictor of relapse in pediatric ALL (Coustan-Smith et al., 1998 and Karawajew et al., 2015). As an example, in one study, the cumulative rate of relapse for those negative for MRD by flow cytometry was 10 percent, whereas it was 23, 43, and 72 percent for those with MRD of <0.1, 0.1 to <1.0, and ≥ 1.0 percent, respectively (Coustan-Smith et al., 2000).

2.13.1.1. Advantages of flow cytometry technique:

Advantages of flow cytometry technique for the detection of MRD include (Brüggemann et al., 2010):

1:Wide applicability: Immunophenotyping with flow cytometry technique can be applied to 80-95 percent of patients with ALL.
2:Speed: Reporting of results on the same day.
3:Quantitative: Results from flow cytometry technique are quantitative rather than qualitative, although not yet standardized.
4:Gives additional information on the benign and malignant cells present in the sample, which may allow for drug targeting.
5:Bone marrow and whole blood may be used as the sample source.

2.13.1.2. Limitations of flow cytometry technique:

Limitations of flow cytometry technique for MRD assessment include (Li et al., 2003 and Campanaa and Coustan-Smith, 2004):

1:Hematogones: Low frequency normal hematopoietic progenitor cells (hematogones), similar to leukemic blasts, may express the same cytoplasmic or surface-bound marker profile, displaying distinction between normal and malignant cells difficult in this setting.
2:Immunophenotypic shifts: There is also the potential for a change in immunophenotypic expression of the leukemic cells during disease progression, resulting in a false negative result.
3:Low cellularity of bone marrow samples during and after induction therapy.

2.13.2. Polymerase Chain Reaction:

PCR can amplify a DNA or complementary DNA (cDNA) sequence unique to the leukemic clone resulting in the detection of one malignant cell among 10^4 to 10^5 normal cells. There are two types of targets used to detect MRD in ALL patients (Brüggemann et al., 2006):

1: Immunoglobulin (Ig) or T-cell receptor (TCR) gene rearrangements.
2: Leukemia-specific chromosomal rearrangements.

2.13.2.1. The advantages of PCR technique for MRD monitoring:

Advantages of PCR technique for MRD monitoring include (Brüggemann et al., 2006 and 2010):

1:Extraordinary sensitivity: While the sensitivity for each junctional region target varies, the sensitivity of PCR is typically 0.5 to 1.0 log higher than that obtained with flow cytometry technique.
2:Wide applicability: PCR testing is viable to most cases of ALL given the high frequency of Ig and TCR gene rearrangements in ALL.
3:Speed: In just a few hours, PCR can amplify a single DNA molecule a million fold.
4:Sample stability: The DNA samples tested with PCR are very stable during transport.
5:Minimal tissue requirements.
6:Standardized method.

2.13.2.2. Limitations of PCR technique for MRD monitoring:

The disadvantages of PCR for MRD monitoring enclose a variety of technical and biological issues including (Konrad et al., 2003 and Chen et al., 2006):

1:Contamination of the reaction product, requiring tough quality control.
2:Poor reproducibility when small numbers of transcripts are present.
3:Evolution of the leukemic clone, sub-clone formation, and or the presence of oligo-clonal populations that can cause both false-negative and false-positive results.
4:Need for diagnostic sample: Because the junctional region rearrangements are solitary to the leukemia clone, a sample from the time of diagnosis must be obtainable to determine the suitable primers.
5:Restricted to experienced molecular hematology laboratories.

2.13.3. Response defined by using MRD:

In addition to standardization of the techniques used to assess and quantify MRD, it is important that clinicians and researchers use common terminology to report individual patient findings and allow for the comparison of trial outcomes. As such, a panel of representatives of the major European study groups on children and adult ALL has suggested the following definitions (Brüggemann et al., 2010):

1:Complete MRD response: No MRD is detected with assessment that responds with a set of minimal technical requirements for the method used.
2:MRD persistence: Presence of a continuously quantifiable MRD positivity measurable at least two time points with at least one relevant treatment element in between.
3:MRD reappearance: Conversion from MRD negativity to quantifiable MRD positivity, ideally with confirmation from a second sample prior to a change in treatment.

2.14. The Flow Cytometry Technique

In 1934: Moldavan described a photoelectric technique for counting cells flowing through a capillary tube. The real beginning of modern flow cytometry started in 1965 when Fulwyler built a cell sorter using the Coulter principle to size cells and electrostatic charging of droplets to sort them. After few years by combining the measurement of volume, light scatter and fluorescence into a single instrument, Paul Mullaney introduced multi-parameter flow cytometry. (BD Biosciences., 2000).

However, the ability to measure side scatter was added by extensive experiments carried out by Gary Salzman. By the mid 1970s, flow cytometers were entering the marketplace, and Leonard Herzenberg came up with the term, Fluorescence Activated Cell Sorter (FACS) (BD Biosciences., 2000).

Immunophenotyping using flow cytometry technique has become the method of choice in identifying and sorting cells within complex populations. Applications of this technology have occurred in both basic research and clinical laboratories. Immunophenotyping is useful in our understanding the origin and nature of leukemia, and the regulation of hematopoietic cell differentiation and maturation (BD Biosciences., 2000).

Fluidics, optics and electronics are the three main systems that make up a flow cytometer. The basic operation of a flow cytometer is that a tube containing the prepared cells under investigation is placed in the flow cytometer. The sample is drawn up from the sample vessel and pumped into the flow chamber (flow cell) through tubing. Cells flow through the flow chamber one at a time very quickly and are presented to one or more light sources (Lasers). The laser beam hits the cells as they pass through the flow chamber. The way the light bounces off each cell gives information about the cells physical characteristics. Light scatter and or fluorescence if

fluorescent molecules are present on the particle are captured depending on the type of fluorochromes, spectrally filtered and directed to appropriate photodetectors for conversion to electrical signals (BD Biosciences., 2000).

Light scatter is collected at two angles: Forward Scatter (FSC) and side scatter (SSC). Forward scatter measures scattered light in the direction of the laser path and measures the size of the cell. Side scatter measures scattered light at 90 degrees to the laser path and measures the granularity of the cell.

The electronics in the cytometer amplify and process the resulting data. They convert analogue data to digital data which is stored in the computer. This data can be analyzed to provide information about subpopulations within the sample (Figure 2.6) (BD Biosciences., 2000).

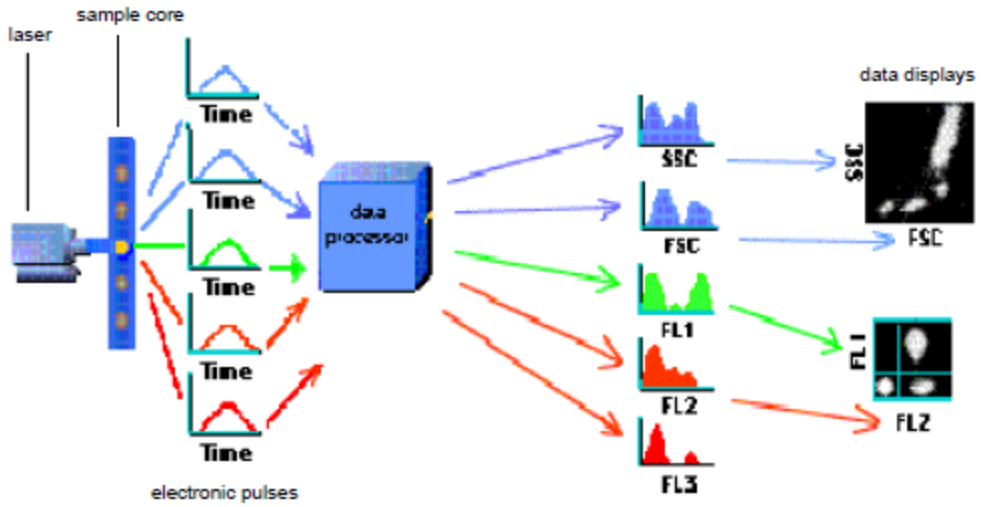


Figure 2.6: Scattered and emitted light signals are converted to electronic pulses that can be processed by the computer.

Chapter three

Methodology

3.1. Study design

This study is an incident study, was conducted in January 2015 based on clinical selection of pediatric ALL due to high incidence of ALL cases in comparison with other leukemia among children. However, the diagnosis of pediatric ALL was based on signs and symptoms, and clinical laboratory results. The immunophenotyping analysis was done by flow cytometry technique that used in routine clinical laboratories for the diagnosis, prognosis and monitoring of ALL.

Pediatric ALL cases were selected from those admitted to Huda Al-Masri pediatric cancer department at Beit-Jala hospital in Bethlehem, this hospital is one of the largest centers of pediatric oncology and hematology centers in Palestine.

3.2. Target population

The target population of this study were Palestinian children with ALL, age between 0-14 years, diagnosed and treated at Huda Al-Masri pediatric cancer department at Beit-Jala hospital in Bethlehem, and west bank area residents through the study period.

3.3. Sampling

According to the previously mentioned aims of this incident study, we had selected pediatric ALL cases from January 2015 to December 2015. A total of 15 consecutive children (7 females, 8 males) < than14 years with newly diagnosed ALL were diagnosed by pediatric oncologists at Huda Al-Masri pediatric cancer department at Beit-Jala hospital.

3.4. Inclusion Criteria

Pediatric ALL patients residents of west bank area of Palestine, < than14 years old, and admitted to Huda Al-Masri pediatric cancer department at Beit-Jala hospital from January 2015 to December 2015.

3.5. Exclusion Criteria

Pediatric ALL patients not a resident of west bank area of Palestine, older than 14 years old, any patient without flow cytometric analysis or transferred outward to another pediatric oncology center.

3.6. Definition of study area

This study was conducted in a single cancer center in southern west bank area of Palestine, which includes patients from west bank area according to the appendix 1, except in Jerusalem governorate.

3.7. Data collection tools

Multiple sources of data were used during this study to achieve a comprehensive idea about the immunophenotyping of pediatric ALL.

3.7.1. Medical files: Data of pediatric ALL patients in their medical files were reviewed with ethical consideration of patient privacy to obtain some clinical features of the given patient at Huda Al-Masri pediatric cancer department at Beit-Jala hospital in Bethlehem.

3.7.2. Flow Cytometer: All cases either retrospective or incident were tested by College of American Pathologists (CAP) accreditation pathology department at King Hussein Cancer Center (KHCC) in Amman-Jordan on bone marrow aspirate samples collected in Ethylenediaminetetraacetic acid (EDTA) tubes according to a two step strategy using panels of monoclonal antibodies based on European Group for Immunological Characterization of Leukemia (EGIL). For the study purpose, the immunophenotyping of the incident target cases at 2015 was performed at Huda Al-Masri pediatric cancer department at Beit-Jala hospital in Bethlehem on bone marrow aspirate samples collected in Ethylenediaminetetraacetic acid (EDTA) tubes according to a two step strategy, and for validation purpose, the achieved results were compared with the KHCC results of the same cases and no significant difference was noted.

At Huda Al-Masri pediatric cancer department at Beit-Jala hospital in Bethlehem, the reactivity of fluorescent conjugated monoclonal antibodies directed against lymphoid and myeloid associated antigens was evaluated on the surface of leukemic cells. The intracytoplasmic Ig, CD3, CD79a and MPO antigens as well as nTdT staining were evaluated by fluorescent conjugated monoclonal antibodies after fixation and permeabilization of leukemic cell. Stained cells were analyzed by flow cytometry technique on a multicolor BD FACS-Callibur flow cytometer (Becton Dickinson Calibrate).

Minimal Residual Disease results were evaluated by examining the most common antigens or CD markers through reactivity of fluorescent conjugated monoclonal antibodies directed against lymphoid associated antigens for each ALL type on the surface of bone marrow cells after the induction point 1 at day 33 of treatment by flow cytometry technique on a multicolor BD FACS-Callibur flow cytometer.

Immunological Staining: A volume of 100 ul of each sample of bone marrow aspirate was mixed with 3 ml of 1X Red Blood Cell lysing buffer solution-eBioscience in each falcon tube, followed by vortex by Turbo mixer –LW Scientific for few seconds, then incubation in the refrigerator without light for 20 to 30 minutes. Each falcon tube was centrifuged by Eppendorf centrifuge-5702 at speed 3000 round per minute for 3 minutes.

The supernatant was removed and put it again on vortex for few seconds. A volume of 3 ml of 0.01M Phosphate buffer saline-eBioscience was added in each falcon tube for washing, then the centrifugation was repeated for 3 minutes at speed 3000 round per minute, and also the supernatant was removed from each falcon tube.

A volume of 10 ul of each antibody (5ul/0.5ug, eBioscience), was added in each falcon tube as in Table 3.1, followed by vortex for few seconds, and then centrifugation for 15 minutes in the refrigerator without light. Then again, a volume of 3 ml of phosphate buffer saline was added followed by centrifugation for 3 minutes at speed 3000 round per minute, and the supernatant was removed from each falcon tube. Finally, 0.5 ml of phosphate buffer saline was added to each falcon tube and the samples were ready for reading.

For intracellular staining: before adding 0.5 ml of phosphate buffer saline for each falcon tube, 100 ul of 1C Dako solution-eBioscience for fixation was added followed by vortex for few

seconds, and incubation for 10 minutes in the refrigerator without light. A volume of 3 ml of phosphate buffer saline was added in each falcon tube followed by centrifugation for 3 minutes at speed 3000 round per minute, and removing the supernatant.

Then, a volume of 100 ul of 10X permeabilization buffer-eBioscience was added in each falcon tube followed by vortex for few seconds, and then immediately a volume of 5 ul of each antibody was added in falcon tubes followed by vortex for few seconds and incubation for 15 minutes in the refrigerator without light.

Moreover, a volume of 3 ml phosphate buffer saline was added in each falcon tube followed by centrifugation for 3 minutes at speed 3000 round per minute and removing the supernatant. Finally, a volume of 0.5 ml phosphate buffer saline was added to each falcon tube to become ready for reading.

However**,** the antibodies used in this study were panels of mouse monoclonal antibodies in Acute Leukemia Immunophenotyping kit (Becton Dickinson) (Table 3.1). There are Anti CD10, Anti CD19, Anti CD20, Anti CD1a, Anti CD2, Anti CD8, Anti CD4, Anti CD5, Anti CD3, Anti CD22, Anti CD7, Anti CD33, Anti CD38, Anti CD117, Anti HLA-DR, Anti CD13, Anti CD34, Anti CD61, Anti CD14, Anti CD11b, Anti cytoplasmic CD79a (cCD79a), Anti nTdT, Anti cMPO, Anti Light chains (Kappa or Lambda), Anti surface Ig, Anti cCD3, and Anti CD45.

Table 3.1: Multicolor parameters of antibodies to CD markers in acute lymphoblastic leukemia profile.

Tube Number	First Color (FITC)	Second Color (PE)	Third Color (Per CP)	Fourth Color (APC)
1 (control)	____	____	CD45	____

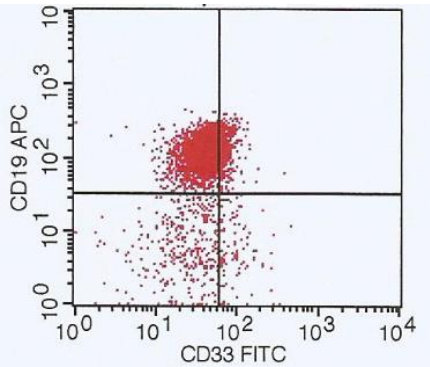
2	CD10	CD19	CD45	CD20
3	CD1a	CD2	CD45	CD3
4	CD4	CD5	CD45	CD7
5	cCD3	cCD79a	CD45	cMPO
6	CD22	CD34	CD45	CD38
7	CD13	CD14	CD45	CD33
8	CD61	CD71	CD45	CD117
9	CD8	CD11b	CD45	HLA-DR
10	nTdT	sIg	CD45	Light chains

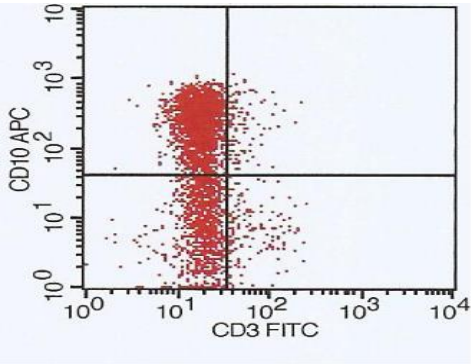
Leukemic samples were considered positive for a particular antigen if 20% or more leukemic cells reacted with a particular antibody for lymphoid markers (CD1a, CD2, CD3, cCD3, CD4, CD5, CD7, CD8, CD10, CD19, CD20, CD22, CD34, CD38, CD45, cCD79a, HLA-DR, nTdT, sIg, Kappa or Lambda light chains) and 30% or more for myeloid markers (CD11b, CD13, CD14, CD33, CD34, CD45, CD61, CD71, CD117, cMPO) (Chiaretti et al., 2014).

In the following 2 representative examples in Figures 3.1 and 3.2, the results of flow cytometry analysis for acute lymphoblastic leukemia patients with different type of ALL depending on immunophenotyping are illustrated.

Positive CD19 and negative CD33

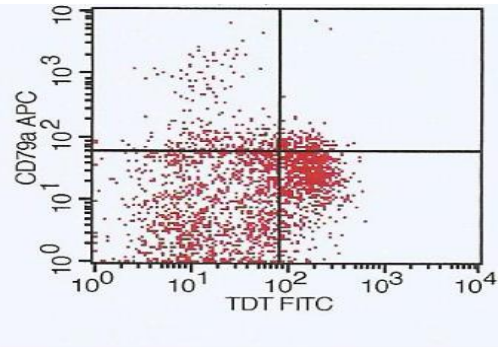
Positive CD10 and negative CD3

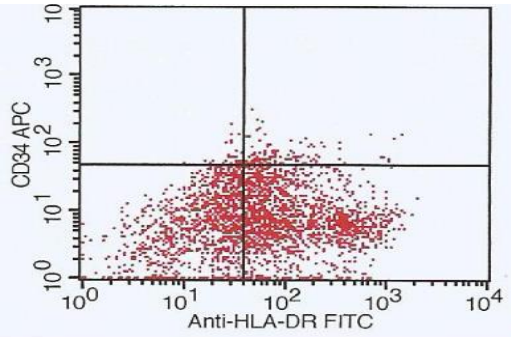




Positive TdT and CD79a

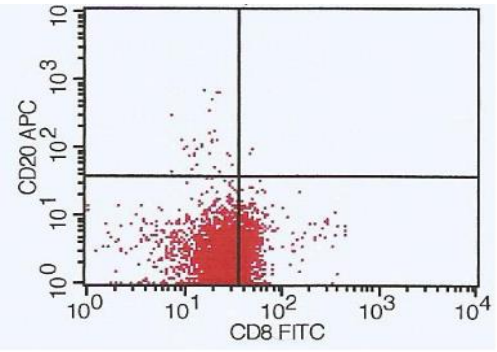
Positive HLA-DR and negative CD34





Negative CD20 and CD33

Positive CD22 and negative CD33



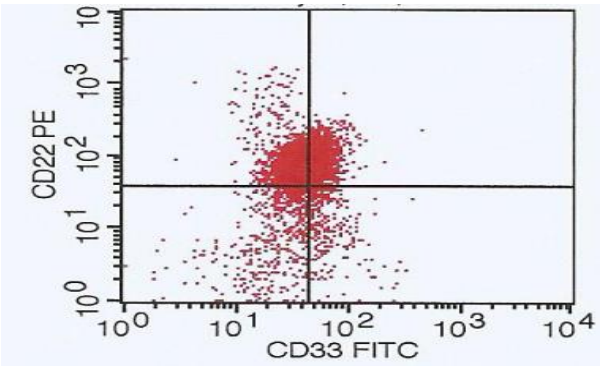
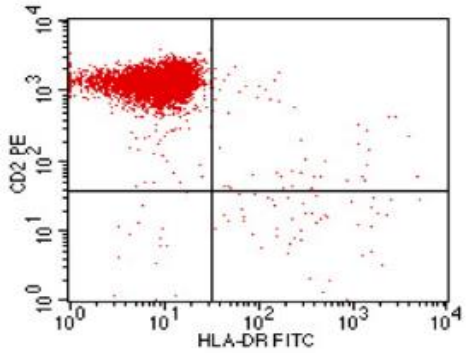


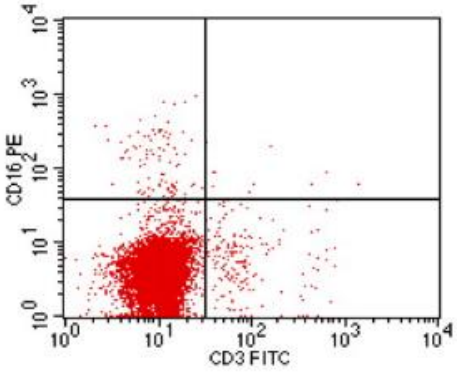
Figure 3.1: Results of flow cytometry analysis for acute lymphoblastic leukemia patient with precursor B-cell immunophenotype.

Positive CD2 and negative HLA-DR

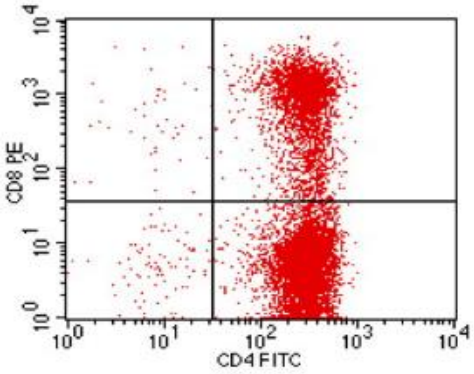
Negative CD3 and CD16

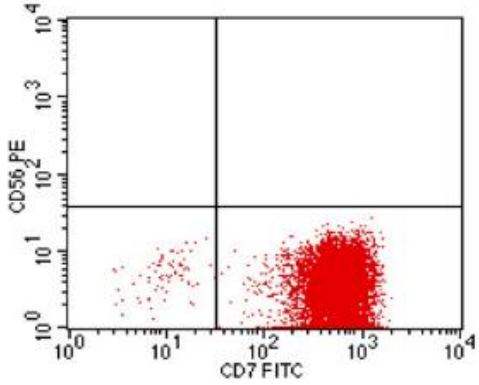


Positive CD4 and CD8



Positive CD7 and negative CD56





Positive CD5 and negative CD20

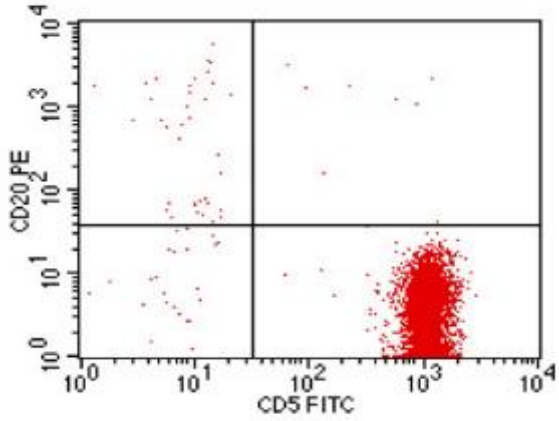


Figure 3.2: Results of flow cytometry analysis for acute lymphoblastic leukemia patient with T-cell immunophenotype.

3.8. Data analysis programs

Statistical analysis of the results was performed using Statistical Package for the Social Sciences (SPSS) version 23.0 (IBM, Corporation) and Microsoft Excel version 2016, showing the immunophenotype of pediatric ALL patients in Palestine, and clinical features of them. Categorical variables and results that presented as frequencies and percentages with p-values were analyzed by using Pearson Chi Square, and Fisher's Exact test. The continuous variables as mean and median were presented and analyzed using Independent Sample T-Test.

3.9. Ethical Consideration

Ethical approval for this study was approved by AL-Quds University (AQU) local Helsinki ethical committee for medical research. This committee follows the moral aspect and high privacy in dealing with the data contained in this study, where it is encoded these data and tabulated without mentioning to the participants. A written consent was taken from all patients or their families. This study also was approved, and permission for the research was given by Palestinian Ministry Of Health (MOH) and administration of Huda Al-Masri pediatric cancer department at Beit-Jala hospital in Bethlehem, Palestine.

Chapter four

Results

4.1. Age distribution of the different ALL subtypes

The age distribution of the precursor B-cell ALL subtype in Figure 4.1, shows that precursor B-cell ALL patients were younger with peak incidence between 2 and 5 years.

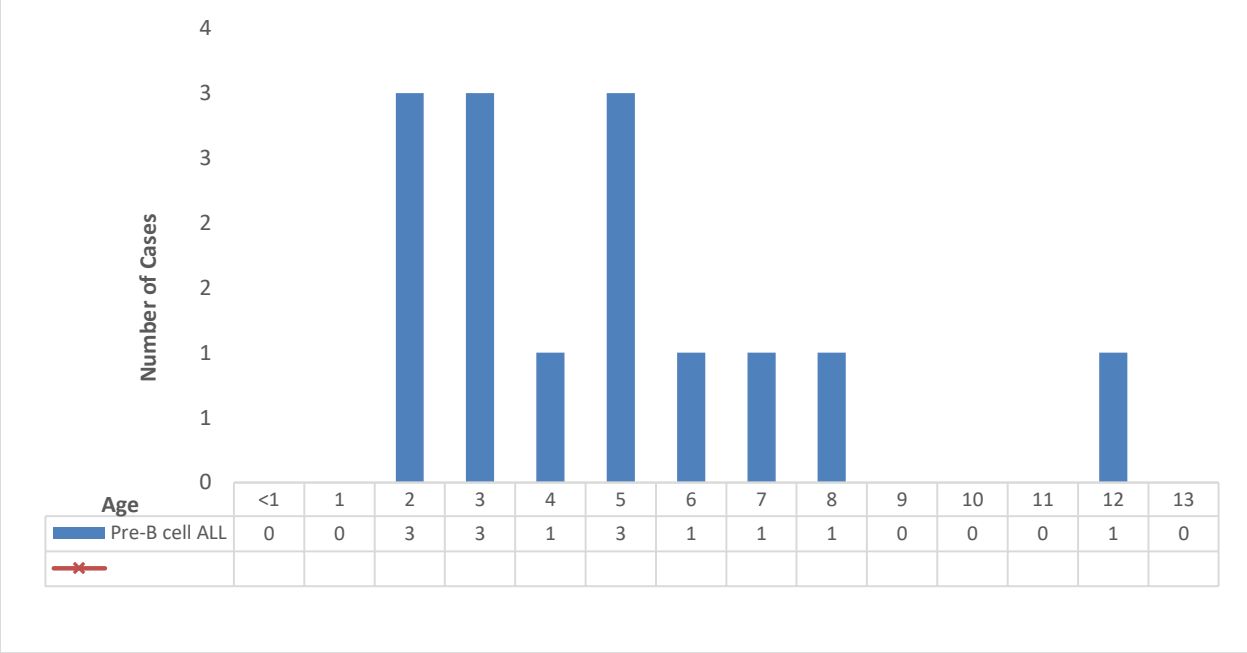


Figure 4.1: Age distribution of 14 children with precursor B-cell acute lymphoblastic leukemia.

4.2. Geographical distribution of ALL subtypes

Geographical distribution of Palestinian pediatric ALL in a single cancer center experience as in Table 4.1, shows that about half of the cases (46.7%) were from Hebron governorate, includes 50% of precursor B-cell ALL cases and 0% of T-cell ALL cases. We also found that 13.3% of ALL cases occurred in Bethlehem governorate with 7.1% of precursor B-cell ALL cases and 100% of T-cell ALL cases, because there was just one case of T-cell ALL. For normalization of the geographical distribution results, the number of inhabitants in Hebron governorate is about 3 times more than the number of inhabitants in Bethlehem governorate, and the number of cases in Hebron governorate was about 3 times more than number of cases in Bethlehem governorate as

in Table 4.1. For this reason, the percentage of cases that diagnosed in Hebron governorate was similar to the percentage of cases that diagnosed in Bethlehem governorate.

In Ramallah governorate, there was 20% of ALL cases with 21.4% of precursor B-cell ALL and 0% of T-cell ALL. Each of Nablus, Jenin and Tulkarm governorates contain 6.7% of ALL cases with 7.1% of precursor B-cell ALL cases and 0% of T-cell ALL cases (Table 4.1). The number of ALL cases in Ramallah, Nablus, Jenin, and Tulkarm don't reflect the total number of cases in these governorates.

However, the results of geographical distribution of pediatric ALL cases were not statistically significant between ALL subtypes in each governorate.

Table 4.1: Geographical distribution of pediatric ALL subtypes at a single cancer center experience in southern west bank area of Palestine.

	All Patients (n=15	**T-cell ALL (n=1)**	**Precursor B-cell ALL (n=14)**	**Number of Inhabitants**	**P-value**
Governorates					>0.05
Hebron	7 (46.7%)	0 (0%)	7 (50%)	**706,508**	
Bethlehem	2 (13.3%)	1 (100%)	1 (7.1%)	**216,114**	
Nablus	1 (6.7%)	0 (0%)	1 (7.1%)	380,961	
Ramallah	3 (20%)	0 (0%)	3 (21.4%)	348,110	
Jenin	1 (6.7%)	0 (0%)	1 (7.1%)	311,231	
Tulkarm	1 (6.7%)	0 (0%)	1 (7.1%)	182,053	

4.3. Distribution of ALL cases according to immunophenotypes

The study group comprised 15 acute lymphoblastic leukemia cases (<14 years). There were 7 females and 8 males with a male/female ratio of 1.14:1. Immunophenotyping analysis showed

that the precursor B-cell phenotype was encountered in 14 (93.3%) cases, mature B-cell phenotype in Zero (0%) cases, and T-cell phenotype in 1 (6.7%) case (Table 4.2).

4.3.1. ALL characteristics depending on the distribution of immunological subtypes:

The patients characteristics are summarized in Table 4.2. The immunophenotypic distribution of ALL subtypes is not clearly influenced by clinical features. Compared with precursor B-cell ALL, Age at diagnosis (P >.05), Gender (P >.05), higher or lower leukocyte count at diagnosis (P >.05), and Positive CNS disease (P >.05), Mediastinal mass (P >.05), and the same relation with T-cell ALL subtype.

4.3.2. Negative MRD at the end of induction point 1 (day 33):

We examined negative MRD at the end of induction point 1 at day 33 after treatment when the reactivity of fluorescent conjugated monoclonal antibodies directed against lymphoid associated antigens for each ALL type on the surface of bone marrow cells is less than 0.01% of total cells expressing the most common CD markers for each ALL type, to test if complete remission will occurs in most of cases as in developed countries. We found that 13 of pediatric ALL cases (86.7%) were subjected to complete remission after the end of induction point 1 at day 33 with the only T-cell ALL case and 12 cases that constituted 85.7% of precursor B-cell ALL cases.

Two cases (14.3%) from precursor B-cell ALL cases were with positive MRD because more than 0.01% of total cells expressing the most common CD markers, one case with 0.85 and the second case with 0.5 of total cells were blasts expressing the most common CD markers for precursor B-cell type (CD19, CD10, CD34). However, the results of negative or positive MRD were not statistically significant (Table 4.2).

4.3.3. CNS leukemia at diagnosis of ALL

We examined the presence of extramedullary disease such as in central nervous system because it is a factor used to determine the intensity of treatment. We found that just 1 case (7.1%) of precursor B-cell ALL cases had positive CNS disease.

Table 4.2: Characteristics of acute lymphoblastic leukemia patients.

Features	**All Patients (n=15)**	**T-cell ALL (n=1) 6.7%**	**Precursor B-cell ALL (n=14)**	**P-value**

			93.3%	
Age of Diagnosis (Year)				>0.05
Mean	5.1	------	5.3	
Median	5	------	4.5	
Range	2 - 12	------	2 - 12	
< 1	0 (0%)	0 (0%)	0 (0%)	
1 - 9	14 (93.3%)	1 (100%)	13 (92.9%)	
10+	1 (6.7%)	0 (0%)	1 (7.1%)	
Gender				>0.05
Male	8 (53.3%)	0 (0%)	8 (57.1%)	
Female	7 (46.7%)	1 (100%)	6 (42.9%)	
M/F ratio	1.14 : 1	0	1.33 : 1	
Leukocyte Count (K/ml) at Diagnosis				>0.05
Mean	35	------	37.3	
Median	18.7	------	25.4	
Range	1.4 - 105	------	1.4 - 105	
<50	9 (60%)	1 (100%)	8 (57.1%)	
≥50	6 (40%)	0 (0%)	6 (42.9%)	
Positive CNS Disease	1 (6.7%)	0 (0%)	1 (7.1%)	>0.05
Positive Mediastinal mass	1 (6.7%)	1 (100%)	0 (0%)	>0.05
MRD Results				>0.05
Negative MRD <0.01 of cells at end of induction point 1 (day33)	13 (86.7%)	1 (100%)	12 (85.7%)	
Positive MRD >0.01% of cells at end of induction point 1 (day33)	2 (13.3)	0 (0%)	2 (14.3%)	

4.4. Imunophenotype of ALL cases

ALL cell lineage divided into two subtypes: B-cell lineage ALL and T-cell lineage ALL. There were 15 children (7 females and 8 males) with ages ranging from 2 to 12 years and median age of 5 years (Table 4.2).

4.4.1. Precursor B-Cell immunophenotype:

Fourteen children with precursor B-cell phenotype were studied. There were 6 females and 8 males (male/female ratio of 1.33:1) with ages ranging from 2 to 12 years and median age of 4.5 years (Table 4.2).

Based on the reactivity of precursor B-cell ALL cells with various anti-precursor B-cell monoclonal antibodies (Table 4.3), all tested precursor B-Cell ALL cases expressed CD19. Many Cases had cCD79a, CD10, nTdT, HLA-DR in 85.7%, 92.9%, 85.7%, 92.9% respectively, and cells in more than 78% of cases expressed CD34. According to CD38, CD58 and CD20 were also found in 71.4%, 71.4% and 35.7% of cases respectively. Furthermore, myeloid antigens CD13 and/or CD33 occurred just in (14.2%) of cases, and CD22 was also found in few cases (7.1%).

4.4.2. T-Cell immunophenotype:

One child with T-cell ALL type was studied. Based on the reactivity of T-cell ALL cells with various anti-T-cell monoclonal antibodies (Table 4.3), the T-cell case cells expressed the most common CD markers for T-cell ALL type, and they were surface CD7, CD10, CD5, CD34, nTdT, and cCD3 antigens.

4.4.3. Lineage heterogeneity between ALL immunophenotypes:

Lineage heterogeneity was found only in precursor B-cell ALL cases having one or two myeloid associated antigens (CD13,CD33) expressed on their blast cells. Lineage heterogeneity for both antigens was found in 7.1% of precursor B-cell ALL cases (n=1). However, CD13 antigen was found in 7.1% of cases (n=1), and CD33 antigen was found in 14.2% of cases (n=2) (Table 4.3).

Table 4.3: Immunophenotyping profiles of 15 acute lymphoblastic leukemia patients.

Markers	**T-cell ALL (n= 1)**	**Precursor B-cell ALL (n= 14)**
CD1a+	0/1 (0%)	__
sCD3+	0/1 (0%)	0/14 (0%)
cCD3+	1/1 (100%)	0/14 (0%)
CD4+	0/1 (0%)	__

CD5+	1/1 (100%)	__
CD7+	1/1 (100%)	0/14 (0%)
CD8+	0/1 (0%)	__
CD10+	1/1 (100%)	13/14 (92.9%)
cCD79a+	0/1 (0%)	12/14 (85.7%)
CD19+	0/1 (0%)	14/14 (100%)
CD20+	0/1 (0%)	5/14 (35.7%)
CD22+	0/1 (0%)	1/14 (7.1%)
nTdT	1/1 (100%)	12/14 (85.7%)
CD34+	1/1 (100%)	11/14 (78.6%)
HLA-DR+	0/1 (0%)	13/14 (92.9%)
CD38+	0/1 (0%)	10/14 (71.4%)
CD58+	0/1 (0%)	10/14 (71.4%)
CD13+	0/1 (0%)	1/14 (7.1%)
CD33+	0/1 (0%)	2/14 (14.2%)
CD13+, CD33+	0/1 (0%)	1/14 (7.1%)
CD13-, CD33+	0/1 (0%)	2/14 (14.2%)
CD13+, CD33-	0/1 (0%)	0/14 (0%)
CD13-, CD33-	0/1 (0%)	12/14 (85.7%)
CD13+, and/or CD33+	0/1 (0%)	2/14 (14.2%)

4.5. CD10 Expression

The correlation of CD10 positivity with age, leukocyte count at diagnosis, and gender was analyzed (Table 4.4). CD10 expression was found in 13 cases (92.9%) of precursor B-ALL cells and 1 cases (100%) of the T-ALL cells.

CD10 expression in precursor B-ALL was not significantly frequent in patients with age range of 1-9 years, and not significant frequent with initial low leukocyte count. In contrast, children with

precursor B-ALL CD10- phenotypes were not significantly more frequently > 10 or < than 1 year, and not statistically significant with initial high leukocyte count.

CD10 positivity was less common in T-cell lineage and was not statistically significant associated with initial higher leukocyte count, and more frequently from 1 to > 10 years. Furthermore, children with T-cell ALL CD10- phenotypes were not statistically significant associated with more frequently between 1-9 years, and initial low leukocyte count.

Table 4.4: Biological features according to CD10 expression in pediatric ALL.

		Precursor B-cell ALL (n=14)			T-cell ALL (n=1)		
Feature	Total of Patients	CD10 -	CD10 +	P-Value	CD10 -	CD10 +	P-Value
Gender				0.201			1
F	7 (53.3%)	0 (0%)	6 (100%)		0 (0%)	1 (100%)	
M	8 (46.7%)	1 (12.5%)	7 (87.5)		0 (0%)	0 (0%)	
Leukocyte Count (K/ml)				0.212			0.123
<50	9 (60%)	1 (12.5%)	7 (87.5%)		0 (0%)	1 (100%)	
≥50	6 (40%)	0 (0%)	6 (100%)		0 (0%)	0 (0%)	
Age (Y)				0.201			1
< 1	0 (0%)	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
1 - 9	14 (93.3%)	1 (7.7%)	12 (92.3%)		0 (0%)	1 (100%)	
>10	1 (6.7%)	0 (0%)	1 (100%)		0 (0%)	0 (0%)	

Chapter five

Discussion

The present work is the first and a unique study takes place in Palestine that discussing the immunophenotyping of pediatric ALL. It's studying small series of acute lymphoblastic leukemia cases, providing an idea about the immunophenotype, some clinical features, CD10

expression, age distribution, and geographic distribution of ALL subtypes in a single cancer center in southern west bank. We reported the results of 15 patients.

This study is a first step towards a better understanding of ALL, as the most common pediatric malignancy in developed countries and in Palestine as well.

5.1. Age and gender distribution of the ALL subtypes

The peak age range of 2 to 5 years for precursor B-cell phenotype was similar to the pediatric ALL reported in developed countries (Kebriaei et al., 2003 and de Sousaa et al., 2015), and with male to female ratio (1.14:1) that may be similar to that reported in developed countries (Bachir et al., 2009), but not statistically significant.

5.2. Geographical distribution of ALL

According to the geographical distribution of pediatric ALL in Palestine that is largely unknown. The present study gives us a small idea about the geographical distribution of pediatric ALL in different governorates in a single cancer center experience in the southern west bank area of Palestine. We found that about a half of the cases (46.7%) were from Hebron governorate, although the percentage of cases in Hebron governorate was similar to the percentage of cases in Bethlehem governorate when we did the normalization for the results according to the number of inhabitants in both Hebron and Bethlehem governorates, so this high number of cases in Hebron governorate due to the huge number of population there, and the results were not statistically significant. However, the cases from Ramallah, Nablus, Jenin, and Tulkarm governorates are not reflected the total number of patients because they are treated in other cancer centers.

5.3. Immunophenotypic distribution of ALL subtypes

In this study, the advantage is to select the best strategy for acute lymphoblastic leukemia diagnosis and management. Immunophenotyping was used in order to analyze 15 children with acute lymphoblastic leukemia. The relative frequencies of immunological subtypes in this study (93.3% for precursor B-cell phenotype and 6.7% for T-cell phenotype) are similar to that reported by the developed countries of Europe and USA (Neelkamal et al., 2013, and Hunger and Mullighan, 2015).

5.3.1. ALL characteristics depending on the distribution of immunological subtypes:

The clinical features of pediatric ALL in Palestine are largely unknown. This study gives us an idea about some clinical features of pediatric ALL in Palestine that help in determining the preferred protocol for the management of ALL. However, the immunophenotypic distribution of ALL subtypes was not clearly influenced by clinical features. May be due to we had a not huge sample size.

MRD examination is a key tool towards the development of personalized treatment for children with ALL and flow cytometry technique has significantly contributed to expand the applicability of this valuable test (Gaipa et al., 2013).

Negative MRD at the end of induction point 1 at day 33 to determine the complete remission was 86.7% of pediatric ALL cases, so this high percentage means that the response to treatment was very good as in developed countries (Schrappe et al., 2011).

Although CNS leukemia is more common in T-cell ALL than precursor B-cell ALL, and it occurs in fewer than 5% of children at diagnosis (Friedmann and Weinstein, 2000 and Hunger and Mullighan, 2015), we found that just one case with positive CNS disease was from precursor B-cell ALL.

5.4. Cell Lineage of ALL cases

Data concerning the antigen expression for each class of ALL showed to some extent as has been reported in pediatric ALL in various developing countries:

Every case of B cell lineage ALL expressed just CD19 as reported in previous studies, and not all cases expressed cCD79a and CD22 as in developed countries, due to most of cases (85.7%) expressed cCD79a and few cases (7.1%) expressed CD22 (Campana and Coustan-Smith, 2002 and Kaleem et al., 2003).

Furthermore, HLA-DR, CD20, CD34 and nTdT were the most sensitive antigens for the precursor B-cell ALL as previous reported studies (Bachir et al., 2009, and Kaleem et al., 2003).

As previous reported by the developed countries, cCD3, CD5, and CD7 were the most sensitive antigens for the T-cell lineage ALL (Campana and Coustan-Smith, 2002 and Kaleem et al., 2003).

5.5. Lineage Heterogeneity

Aberrant expression of myeloid antigens on acute lymphoblastic leukemia cells is a well-documented phenomenon and has no prognostic or therapeutic implications (Putti et al., 1998), but can be used for monitoring MRD (Campana and Pui, 1995). However, the myeloid associated antigens CD13/CD33 used in the current study are the most commonly antigens expressed in pediatric ALL cases.

In this study, myeloid antigens expression occurred only in precursor B-cell ALL cases in 14.2% (n=2), that differ from developed countries due to myeloid antigens occurred in both ALL subtypes, although this frequency of myeloid antigens in precursor B-cell ALL between 6 to 35% as in developed countries (Putti et al., 1998 and Pui et al., 1991).

5.6. CD10 Expression

CD10 expression was detected in 13 cases (92.9%) of the 14 precursor B-cell ALL cases and in 1 T-cell ALL case (100%), due to just this case tested for this antigen. Cases of precursor B-cell ALL having the CD10 had low leukocyte count at diagnosis, but not statistically significant, and also not statistically significant that the majority of cases (92.3%) were between 1-9 years of age. CD10 positive T-cell ALL case was characterized by lower leukocyte count at diagnosis, and between 1-9 years of age, but without statistically significant.

Studies of the prognostic significance of the CD10 expression in ALL have showed that CD10 expression in pediatric B lineage ALL is associated with several favorable presenting features but is not an independent prognostic factor (Pui and Evans, 1998 and Hann et al., 1998). In T-cell lineage, the expression of CD10 was independently associated with favorable clinical outcome (Pui et al., 1990 and 1993).

However, for the larger subgroup of patients with T-lieange ALL, CD10 expression has no independent prognostic significance (Consolini et al., 1998).

5.7. Conclusion

This study is a unique study providing a clear idea about the immunophenotyping of pediatric ALL cases at a single cancer center experience in southern west bank area in Palestine, and this immunophenotypic distribution was similar to the general immunophenotypic distribution pattern in developed countries. The age distribution showed a peak incidence between 2 and 5 years among the precursor B-cell ALL subtype as in developed countries. Geographical distribution of Palestinian pediatric ALL showed that about half of the cases (46.7%) from Hebron governorate, but with the same percentage of cases diagnosed in Bethlehem governorate depending on the number of inhabitants.

The response to treatment for ALL patients was very good as in developed countries, due to the Negative MRD at the end of induction point 1 at day 33 was 86.7% of ALL patients.

This effort aims to confirm the excellent significance of the immunophenotyping method for diagnosis and classification of pediatric ALL at a single cancer center in southern west bank in Palestine, to determine the prognosis and then the preferred protocol for the treatment of pediatric ALL to obtain better quality of life for children with cancer that is similar to the successful efforts of the developed countries.

5.8. Study limitation

1:The major limitation of this study was that the sample size is limited due to the late establishment of the pediatric oncology center and the results just for small number of cases for the study period (one year), thus the T-cell phenotypes weren’t huge which affects the generalization of the results.

2:This study did not include all centers in the west bank, only one of the largest pediatric centers for leukemia patients in Palestine, due to lack of access to other cancer centers in Palestine and limited resources.

Despite of these limitations, this study demonstrates the urgent need of investigators cooperation in the west bank of Palestine to create a functional cooperative group.

5.9. Recommendations

This study could carry important recommendations for pediatric oncologists and pediatric patients as well. It highlights the importance and the prognostic value of flow cytometry

technique as a routine procedure for all pediatric ALL cases in the process of ALL diagnosis, prognosis, and management.

This study also could be directed to policy makers in Palestinian MOH to make the flow cytometry technique a routine procedure for pediatric ALL diagnosis and management protocol, and could be a cornerstone for further researchers about pediatric ALL in Palestine.

References

Neelkamal, Radha Rathee. (2013): Acute Lymphoblastic Leukemia: Characterization and its Prognostic Values. International Journal of Pharma Research & Review, Nov 2013; 2(11):27-36.

de Sousaa D, Ferreiraa F, Félix F, et al. (2015): Acute lymphoblastic leukemia in children and adolescents: prognostic factors and analysis of survival. rev bras hematol hemoter; 37(4):223–229.

Hunger SP, Mullighan CG. (2015): Acute Lymphoblastic Leukemia in Children, N ENG J MED 373;16.

Goldberg JM, Silverman LB, Levy DE, et al., (2003): Childhood T-cell acute lymphoblastic leukemia: the Dana-Farber Cancer Institute acute lymphoblastic leukemia consortiu experience. J Clin Oncol 21 (19); 3616-22.

Willemse M, Seriu T, Hettinger K, et al. (2002): Detection of minimal residual disease identifies differences in treatment response between T-ALL and precursor B-ALL. Blood;99(12):4386-93.

Gaipa G, Basso G, Biondi A, et al. (2013): Detection of Minimal Residual Disease in Pediatric Acute Lymphoblastic Leukemia. Cytometry part B;48B:359-369.

Conter V, Bartram C, Maria V, et al. (2009): Molecular response to treatment redefines all prognostic factors in children and adolescents with B-cell precursor acute lymphoblastic leukemia: results in 3184 patients of the AIEOP-BFMALL 2000 study. Blood,115,16.

Badrinath P, Day NE et al., (1997): Seasonality in the diagnosis of acute lymphocytic leukaemia. Br J Cancer,75(11):1711–1713.

Karimi M, Yarmohammadi H. (2003): Seasonal variations in the onset of childhood leukemia/lymphoma: April 1996 to March 2000, Shiraz, Iran. Hematol Oncol,21(2):51-5.

Ward E, De Santis C, Robbins A, et al., (2014): Childhood and adolescent cancer statistics, 2014. CA Cancer J Clin; 64:83.

AL-Mulla N,Chandra P,Khattab M, et al., (2014): Chilhood Acute Lymphoblastic Leukemia in the Middle East sand Neighboring Countries: A Prospective Multi-Institutional International Collaborative Study (CALLME1) by the Middle East Childhood Cancer Alliance (MECCA). Pediatr Blood Cancer; 61:1403_1410.

Palestinian Health Information Center. (2015): National Focal Person for Health Information & Statistics, National Cancer and Death Registry, Ministry of Health. Nablus-Palestine.

National Cancer Institute, (2016): Childhood Acute Lymphoblastic Leukemia Treatment (PDQ(R)), Last Modified: 01/04/2016 from http://www.cancer.gov/ cancertopics/ pdq/treatment/childALL/patient.

Ribera JM, Oriol A. (2009): Acute lymphoblastic leukemia in adolescents and young adults. Hematol Oncol Clin North Am, 23(5):1033-42.

Hunger SP, Lu X, Devidas M, et al. (2012): Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: a report from the children's oncology group. J Clin Oncol, 10. 30(14):1663-9.

Howlader N, Noone AM, Krapcho M, et al., (2013): SEER Cancer Statistics Review, 1975-2010. Bethesda, MD, National Cancer Institute, Section 28.

Steliarova-Foucher E, Stiller C, Kaatsch P, et al., (2004): Geographical patterns and time trends of cancer incidence and survival among children and adolescents in Europe since the 1970s (the ACCISproject): an epidemiological study. Lancet; 364:2097.

Shah A, Coleman MP. (2007): Increasing incidence of childhood leukaemia: a controversy re-examined. Br J Cancer; 97:1009.

Svendsen AL, Feychting M, Klaeboe L, et al., (2007): Time trends in the incidence of acute lymphoblastic leukemia among children 1976-2002: a population-based Nordic study. J Pediatr; 151:548.

Margolin JF, Steuber CP, et al., (2001): Acute Lymphoblastic Leukemia. In:Principles and Practice of Pediatric Oncology, 4th ed, Pizzo PA, Poplack DG (Eds), Lippincott-Raven, Philadelphia; p.489.

Rogalsky RJ, Black GB, Reed MH. (1986): Orthopaedic manifestations of leukemia in children. J Bone Joint Surg Am; 68:494.

Jonsson OG, Sartain P, Ducore JM, Buchanan GR. (1990): Bone pain as an initial symptom of childhood acute lymphoblastic leukemia: association with nearly normal hematologic indexes. J Pediatr;117:233.

Sinigaglia R, Gigante C, Bisinella G, et al., (2008): Musculoskeletal manifestations in pediatric acute leukemia. J Pediatr Orthop; 28:20.

Bleyer WA. (1988): Central nervous system leukemia. Pediatr Clin North Am; 35:789-814.

Chiaretti S, Vitale A, Cazzaniga G, et al., (2013): Clinico-biological features of 5202 patients with acute lymphoblastic leukemia enrolled in the Italian AIEOP and GIMEMA

protocols and stratified in agecohorts. Haematologica; 98:1702.

Brunning RD, Flandrin G, Borowitz M, et al., (2001): Precursor B lymphoblastic leukaemia/lymphoblastic lymphoma (Precursor B-cell acute lymphoblastic leukaemia). In: World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues, Jaffe ES, Harris NL, Stein H, Vardiman JW (Eds), IARC Press, Lyon; p.111.

Simone JV, Verzosa MS, Rudy JA. (1975): Initial features and prognosis in 363 children with acute lymphocytic leukemia. Cancer; 36:2099.

Bennett JM, Catovsky D, Daniel MT, et al., (1976): Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. Br J Haematol; 33:451.

Miller DR, Leikin S, Albo V, et al., (1981): Prognostic importance of morphology (FAB classification) in childhood acute lymphoblastic leukaemia (ALL). Br J Haematol; 48:199.

Lilleyman JS, Hann IM, Stevens RF, et al., (1992): Cytomorphology of childhood lymphoblastic leukaemia: a prospective study of 2000 patients. United Kingdom Medical Research Council's Working Party on Childhood Leukaemia. Br J Haematol; 81:52.

Chiaretti S, Zini G, et al., (2014): Diagnosis and Subclassification of Acute Lymphoblastic Leukemia, Mediterr J Hematol Infect Dis, 6(1):e2014073.

Zipf, TF, Berg, SL, Roberts, WM, et al., (2000): Childhood Leukemias. In: Clinical Oncology (2nd ed), Abeloff, MD, Armitage, JO, Lichter, AS, et al (Eds), Churchill Livingstone, New York; p.2402.

Bene MC, Castoldi G, Knapp W, et al. (1995): Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia;9(10):1783-1786.

Harrison CJ. (2009): Cytogenetics of paediatric and adolescent acute lymphoblastic leukaemia. Br J Haematol;144:147.

Rubnitz JE, Wichlan D, Devidas M, et al., (2008): Prospective analysis of TEL gene

rearrangements in childhood acute lymphoblastic leukemia: a Children's Oncology Group study. J Clin Oncol; 26:2186.

Sutcliffe MJ, Shuster JJ, Sather HN, et al., (2005): High concordance from independent studies by the Children's Cancer Group (CCG) and Pediatric Oncology Group (POG) associating favorable prognosis with combined trisomies 4, 10, and 17 in children with NCI Standard-Risk B-precursor Acute Lymphoblastic Leukemia: a Children's Oncology Group (COG) initiative. Leukemia; 19:734.

Nachman JB, Heerema NA, Sather H, et al., (2007): Outcome of treatment in children with hypodiploid acute lymphoblastic leukemia. Blood;110:1112.

Pui CH, Carroll AJ, Head D, et al., (1990): Near-triploid and near-tetraploid acute lymphoblastic leukemia of childhood. Blood; 76:590.

Raimondi SC, Zhou Y, Shurtleff SA, et al., (2006): Near-triploidy and near-tetraploidy in childhood acute lymphoblastic leukemia: association with B-lineage blast cells carrying the ETV6-RUNX1 fusion, T-lineage immunophenotype, and favorable outcome. Cancer Genet Cytogenet;169:50.

Shurtleff SA, Buijs A, Behm FG, et al., (1995): TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with an excellent prognosis. Leukemia; 9:1985.

Jones LK, Saha V. (2005): Philadelphia positive acute lymphoblastic leukaemia of childhood.Br J Haematol;130:489.

Pui CH, Gaynon PS, Boyett JM, et al., (2002): Outcome of treatment in childhood acute lymphoblastic leukaemia with rearrangements of the 11q23 chromosomal region. Lancet; 359:1909.

Friedmann A, Weinstein H. (2000): The Role of Prognostic Features in the Treatment of Childhood Acute Lymphoblastic Leukemia. Oncologist;5(4):321-8.

ARYA L.S. (2004): Acute Lymphoblastic Leukemia:Current Therapy. Indian Journal of Medical and Paediatric Oncology; 25,5.

Schultz KR, Pullen DJ, Sather HN, et al., (2007): Risk- and response-based classification of childhood B-precursor acute lymphoblastic leukemia: a combined analysis of prognostic markers from the Pediatric Oncology Group (POG) and Children's Cancer Group (CCG). Blood; 109:926.

Seibel N.L. (2008): Treatment of Acute Lymphoblastic Leukemia in Children and Adolescents: Peaks and Pitfalls, Hematology Am Soc Hematol Educ Program; 2008:374-380.

Rubnitz, JE, Look, AT. (2000): Pathobiology of Acute Lymphoblastic Leukemia. Basic Principles and Practice . Elsevier Science, New York;1052.

Stacy L. Cooper, Patrick A, et al., (2015): Treatment of Pediatric Acute Lymphoblastic Leukemia, Pediatr Clin North Am; 62(1): 61–73.

Sutton R, Venn NC, Tolisano J, et al., (2009): Clinical significance of minimal residual disease at day 15 and at the end of therapy in childhood acute lymphoblastic leukaemia. Br J Haematol; 146:292.

Möricke A, Reiter A, Zimmermann M, et al., (2008): Risk-adjusted therapy of acute lymphoblastic leukemia can decrease treatment burden and improve survival: treatment results of 2169 unselected pediatric and adolescent patients enrolled in the trial ALL-BFM 95.Blood; 111:4477.

Cavé H, van der Werff ten Bosch J, Suciu S, et al., (1998): Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia. European Organization for Research and Treatment of Cancer--Childhood Leukemia Cooperative Group. N Engl J Med; 339:591.

Karawajew, L., Dworzak, M., Ratei, R., et al., (2015): Minimal residual disease analysis by eight-color flow cytometry in relapsed childhood acute lymphoblastic leukemia. Haematologica,100(7), 935–944.

Bruggemann M, Schrauder A, Raff T, et al. (2010): Standardized MRD quantification in European ALL trials: proceedings of the Second International Symposium on MRD assessment in Kiel, Germany,18-20 September 2008. Leukemia;24:521-535.

Campana D. (2010): Minimal residual disease in acute lymphoblastic leukemia. Hematology

Am Soc Hematol Educ Program;2010:7-12.

Coustan-Smith E, Sancho J, Hancock ML, et al., (2000): Clinical importance of minimal residual disease in childhood acute lymphoblastic leukemia. Blood; 96:2691.

Schrappe M, Valsecchi M, Bartram C, et al. (2011): Late MRD response determines relapse risk overall and in subsets of childhood T-cell ALL: results of the AIEOP-BFM-ALL 2000 study. Blood,118,8.

Campana D. (2003): Determination of minimal residual disease in leukaemia patients. Br J Haematol; 121:823.

Bacher U, Zander AR, Haferlach T, et al., (2008): Minimal residual disease diagnostics in myeloid malignancies in the post transplant period. Bone Marrow Transplant; 42:145.

Al-Mawali A, Gillis D, et al., (2009): The role of multiparameter flow cytometry for detection of minimal residual disease in acute myeloid leukemia. Am J Clin Pathol; 131:16.

Campanaa D, Coustan-Smith E, (2004): Minimal Residual Disease Studies by Flow Cytometry in Acute Leukemia. Acta Haematol, 112:8–15.

Krampera M, Vitale A, Vincenzi C, et al., (2003): Outcome prediction by immunophenotypic minimal residual disease detection in adult T-cell acute lymphoblastic leukaemia. Br J Haematol; 120:74.

Coustan-Smith E, Behm FG, Sanchez J, et al., (1998): Immunological detection of minimal residual disease in children with acute lymphoblastic leukaemia. Lancet; 351:550.

Coustan-Smith E, Sancho J, Hancock ML, et al., (2000): Clinical importance of minimal residual disease in childhood acute lymphoblastic leukemia. Blood; 96:2691.

Brüggemann M, Schrauder A, Raff T, et al., (2010): Standardized MRD quantification in European ALL trials: proceedings of the Second International Symposium on MR assessment in Kiel, Germany, 18-20 September 2008. Leukemia; 24:521.

Li A, Zhou J, Zuckerman D, et al., (2003): Sequence analysis of clonal immunoglobulin and T cell receptor gene rearrangements in children with acute lymphoblastic leukemia at diagnosis and at relapse: implications for pathogenesis and for the clinical utility of PCR-based methods

of minimal residual disease detection. Blood; 102:4520.

Brüggemann M, Raff T, Flohr T, et al., (2006): Clinical significance of minimal residual disease quantification in adult patients with standard-risk acute lymphoblastic leukemia. Blood;107:1116.

Konrad M, Metzler M, Panzer S, et al., (2003): Late relapses evolve from slow-responding subclones in t(12;21)-positive acute lymphoblastic leukemia: evidence for the persistence of a preleukemic clone. Blood;101:3635.

Chen IM, Chakerian A, Devidas M, et al., (2006): Resolution of ambiguous low-level positive quantitative polymerase chain reaction results in TEL-AML1 positive ALL using a post-PCR fluorescent oligoligation method. Br J Haematol; 135:358.

BD Biosciences. (2000): Introduction to Flow Cytometry: A Learning Guide. San Jose, CA; 11-11032-01.

Kebriaei P, Anastasi J, et al., (2003): "Acute lymphoblastic leukemia: diagnosis and classification," Best Practice and Research: Clinical Haematology, vol. 15, no. 4, pp. 597–521.

Bachir F, Bennani S, Lahjouji A, et al., (2009): Characterization of Acute Lymphoblastic Leukemia Subtypes in Moroccan Children. International J Pediatr; 2009:1_7.

Campana D and Coustan-Smith E, (2002): "Advances in the immunological monitoring of childhood acute lymphoblastic leukaemia," Best Practice and Research: Clinical Haematology, vol. 15, no. 1, pp. 1–19.

Kaleem Z, Crawford E, Pathan M. H, et al., (2003): "Flow cytometric analysis of acute leukemias: diagnostic utility and critical analysis of data," Archives of Pathology and Laboratory Medicine,vol. 127, no. 1, pp. 42–48.

Putti M. C, Rondelli R, Cocito M. G, et al., (1998): "Expression of myeloid markers lacks prognostic impact in children treated for acute lymphoblastic leukemia: Italian experience in AIEOP-ALL 88- 91 studies," Blood, vol. 92, no. 3, pp. 795–801.

Campana D and Pui C. H. (1995): "Detection of minimal residual disease in acute leukemia: methodologic advances and clinical significance," Blood, vol. 85, no. 6, pp. 1416–1434.

Pui C. H, Raimondi S. C, Head D. R, et al., (1991): "Characterization of childhood acute leukemia with multiple myeloid and lymphoid markers at diagnosis and at relapse," Blood, vol. 78, no. 5, pp. 1327–1337.

Pui C. H and Evans W. E. (1998): "Acute lymphoblastic leukemia,"The New England Journal of Medicine, vol. 339, no. 9, pp. 605–615.

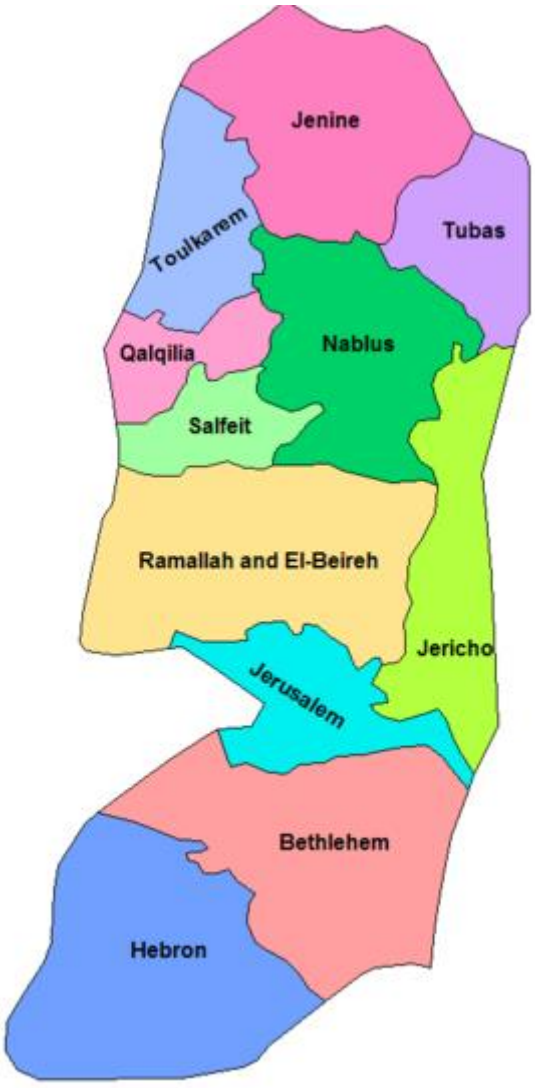
Hann M, Richards S. M, Eden O. B, et al., (1998): "Analysis of the immunophenotype of children treated on the Medical Research Council United Kingdom Acute Lymphoblastic Leukaemia trial XI (MRC UKALLXI)," Leukemia, vol. 12, no. 8, pp. 1249–1255.

Pui C. H, Behm F. G, Singh B, et al., (1990): "Heterogeneity of presenting features and their relation to treatment outcome in 120 children with T-cell acute lymphoblastic leukemia," Blood, vol. 75, no. 1, pp. 174–179.

Pui C. H, Behm G. F, et al., (1993): "Clinical and biologic relevance of immunologic marker studies in childhood acute lymphoblastic leukemia," Blood, vol. 82, no. 2, pp. 343–362.

Consolini R, Legitimo A, Rondelli R, et al., (1998): "Clinical relevance of CD10 expression in childhood ALL. The Italian Association for Pediatric Hematology and Oncology (AIEOP)," Haematologica, vol. 83, no. 11, pp. 967–973.

Appendix 1: Definition of the study area.



Appendix 2: Definition of the Estimated Population in West Bank by Region, 2015.

Region	**Population**
West Bank	**2,862,485**
Jenin	311,231
Tubas	64,719
Tulkarm	182,053
Nablus	380,961
Qalqilia	110,800
Salfit	70,727
Ramallah & Al-Bireh	348,110
Jericho & Al Aghwar	52,154
Jerusalem	419,108
Bethlehem	**216,114**
Hebron	**706,508**

Appendix 3: An Arabic official letter from Al-Quds University to Beit-Jala Hospital to conduct the study.

Al-Quds University
Faculty of Medicine
Abu-Dies, Jerusalem

جامعة القدس
كلية الطب
أبوديس – القدس

2015\11\15

حضرة الدكتور \ زيـــاد شقير المحترم

مدير مستشفى بيت جالا الحكومي

تحية طيبة و بعد:

الموضوع: المساعدة في بحث لطالب ماجستير

أرجو من حضرتكم التكرم بمساعدة الطالب : أسامة نبيل غطاس سلامة - أحد باحثي درجة الماجستير في برنامج الكيمياء الحيوية و الأحياء الجزيئية بكلية الطب - جامعة القدس في بحثه بعنوان:

"Immuno-phenotyping of Palestinian Pediatric ALL cases in West Bank using flow cytometry".

وذلك من خلال الايعاز للزميل الدكتور رئيس قسم أورام الأطفال بالسماح له بالاطلاع على ملفات المرضى المسجلين مع مراعاة توصيات لجنة هلسنكي المحلية للأخلاق الطبية للبحث و الحفاظ على خصوصية المرضى.

و تفضلوا بقبول فائق الاحترام

المشرف الأكاديمي:

الدكتور\ غسان موسى بعلوشة

أستاذ مساعد علم الأمراض

رئيس دائرة علم الأمراض و الأنسجة- كلية الطب

P.O Box 20002
Tel 02-2799203, Fax 02-2796110

ص. ب 20002
هاتف 022799203 فاكس 022796110

Appendix 4: An Arabic official letter to conduct the study in Beit-Jala Hospital.

15. DEC. 2015 14:29 HOSPITALSAFEET NO. 405 P. 1/1

State of Palestine
Ministry of Health
General Hospital Directorate
Nablus
Fax : 092385956 Tel 2374840
P.O :14

دولـــة فلســـطين
وزارة الصـــحة
الإدارة العامة للمستشفيات
نابلس
فاكس : 092385956 تلفون 092374840
ص.ب : 14

التاريخ: ٢٠١٥/١٢/١٥

الاخ مدير مستشفى بيت جالا المحترم، ،

تحية فلسطينية وبعد ،،

الموضوع : بحث لطالب الماجستير / أسامه نبيل سلامه

اشارة الى كتابكم رقم خ/431/2/1028 بتاريخ 2015/11/25 ، لا مانع لدينا من تسهيل مهمة الطالب المذكور اعلاه ، على عمل بحث في قسم اورام الاطفال ، على ان يلتزم بقوانين وانظمة وزارة الصحه الفلسطينيه .

مع الاحترام ،،

ق.أ. مدير عام الإدارة العامة للمستشفيات
د. عبد الرحيم شويكه

النمط الظاهري المناعي لحالات سرطان الدم الليمفاوي الحاد لدى الأطفال الفلسطينيين من خلال تجربة مركز سرطان واحد في جنوب الضفة الغربية باستخدام جهاز التدفق الخلوي

إعداد: اسامة نبيل غطاس سلامة

إشراف: الدكتور غسان بعلوشة

الملخص

المقدمة : سرطان الدم الليمفاوي الحاد هو أكثر انواع السرطان شيوعا لدى الأطفال, يشكل حوالي 75 الى 80% من حالات مرض السرطان الحاد. تشكل نسبة الاصابة عالميا حوالي 3 الى 4 لكل 100.000 من السكان عند عمر أقل من 15 سنة. و تعتبر الأبحاث المنشورة في هذا الموضوع محدودة جدا في فلسطين. و من هنا قدمت هذه الدراسة نتائج واضحة و مهمة حول النمط الظاهري المناعي لمرضى سرطان الدم الليمفاوي الحاد عند الأطفال في أهم مركز سرطان للأطفال في جنوب الضفة الغربية في فلسطين.

الاهداف : تهدف هذه الدراسة الى تحديد أنماط سرطان الدم الليمفاوي الحاد عند الأطفال الفلسطينيين, مع توضيح أهمية المظاهر السريرية مثل عدد خلايا الدم البيضاء بالنسبة للعمر و النمط الظاهري المناعي, و تحديد الاستجابة للعلاج بعد المرحلة التعريفية باستخدام الحد الأدنى من خلايا المرض المتبقية عند النقطة الزمنية الأولى بعد 33 يوم من العلاج.

الأدوات والطرق: لقد استخدمت دراسة مستحدثة, و أجريت هذه الدراسة من يناير 2015 حتى ديسمبر 2015 على مرضى أطفال سرطان الدم الليمفاوي الحاد في مركز هدى المصري لسرطان الأطفال في مستشفى بيت جالا الذي يقع في المنطقة الجنوبية من الضفة الغربية. و تتكون عينات البحث لدينا من حالات شُخصت حديثا بمجموع 15 طفل على التوالي (7 اناث, 8 ذكور) تقل أعمارهم عن 14 عاما بسرطان الدم الليمفاوي الحاد في مركز هدى المصري لأورام الأطفال في مستشفى بيت جالا 2015. هذا المستشفى هو واحد من أكبر مراكز الأطفال لحالات سرطان الدم في فلسطين.

لقد تم استعراض البيانات لمرضى أطفال سرطان الدم الليمفاوي الحاد في ملفاتهم الطبية مع أخذ الاعتبارات الأخلاقية لخصوصية المرضى للحصول على بعض المظاهر السريرية. لقد تم تنفيذ فحص النمط الظاهري المناعي لكل المرضى السابقة أو الحديثة على عينات نخاع العظم التي تم جمعها بإستخدام أنابيب EDTA وفقا لإستيراجية إستخدام الأجسام المضادة بخطوتين بناءً على المجموعة الأُوروبية لتوصيف النمط الظاهري المناعي لسرطان الدم من خلال تقنية التدفق الخلوي في مركز الحسين للسرطان في عمان, الأردن الحاصل على إعتماد من كلية أطباء الباثولوجي الأمريكية. و لغرض الدراسة, لقد فحصنا النمط الظاهري للحالات الجديدة في 2015 في مركز هدى المصري لسرطان الأطفال في مستشفى بيت جالا على عينات نخاع العظم التي تم جمعها بإستخدام أنابيب EDTA وفقا لإستيراجية إستخدام الأجسام المضادة بخطوتين باستخدام جهاز التدفق الخلوي. و لقد تم توثيق النتائج من خلال فحصها مرة أخرى في مركز الحسين للسرطان بعمان و لم يسجل فرق يذكر بينهما.

لقد تم تقييم نتائج الحد الأدنى من خلايا المرض المتبقية عن طريق فحص المواد المضادة الشائعة خلال فعالية الأجسام المضادة المشعة مباشرة مع هذه الأجسام المضادة على سطح خلايا سرطان الدم لكل نوع من سرطان الدم الليمفاوي الحاد عند النقطة الزمنية الأولى بعد 33 يوم من العلاج باستخدام تقنية التدفق الخلوي.

النتائج: لقد تم إجراء التحليل الإحصائي للنتائج لمجموع عينات البحث و هي 15 حالة سرطان دم ليمفاوي حاد تم تشخيصها حديثا بعمر أقل من 14 سنة. كان هناك 7 حالات من الإناث و 8 حالات من الذكور بنسبة 1.14:1 من الذكور للإناث. أظهر التوزيع العمري لمختلف أنواع سرطان الدم الليمفاوي الحاد بأن مرضى نوع الخلية بي غير الناضجة من سرطان الدم الليمفاوي الحاد هم أصغر سناَ مع أعلى نسبة إرتفاع بين 2 و 5 سنوات. و قد أظهر التوزيع الجغرافي لحالات سرطان الدم الليمفاوي الحاد عند الأطفال حدثت

تقريبا في نصف الحالات و بنسبة 46.7% في محافظة الخليل و لكن بنسبة متساوية للحالات التي شخصت في محافظة بيت لحم اعتمادا على التعداد السكاني للمحافظتين.

و أظهر تحليل النمط الظاهري المناعي أن هناك 14 حالة (93.3%) من نوع خلية بي غير الناضجة من سرطان الدم الليمفاوي الحاد, عدم وجود حالات من نوع الخلية بي الناضجة من سرطان الدم الليمفاوي الحاد, و وجود حالة واحدة (6.7%) من نوع الخلية تي من سرطان الدم الليمفاوي الحاد.

لقد وجدنا أن نسب التجانس فقط موجودة في نوع الخلية بي غير الناضجة من سرطان الدم الليمفاوي الحاد بوجود واحدة أو إثنتان من المضادات النخاعية (CD13,CD33) على خلاياها المنفجرة. فكانت نسبة التجانس لكلا المضادين النخاعين بنسبة 7.1% في حالات نوع الخلية بي غير الناضجة من سرطان الدم الليمفاوي الحاد.

لقد فحصنا عدم وجود الحد الأدنى من خلايا المرض المتبقية في نهاية المرحلة التعريفية من العلاج. فوجدنا انه 86.7% من المرضى تعرضوا لاختفاء كامل للمرض بعد المرحلة التعريفية من العلاج أي بعد 33 يوم بحيث يشمل الحالة الوحيدة من سرطان الدم الليمفاوي الحاد من نوع تي, و 85.7% من حالات سرطان الدم الليمفاوي الحاد من نوع خلية بي غير الناضجة لكن بدون دلالة احصائية.

الملخص: أن هذه الدراسة المميزة تقدم فكرة واضحة عن النمط الظاهر المناعي لحالات سرطان الدم الليمفاوي الحاد عن الأطفال من خلال تجربة مركز سرطان واحد يقع في جنوب الضفة الغربية في فلسطين, و هذا التوزيع كان شبيه لنمط التوزيع بشكل عام كما في الدول المتقدمة. أظهر التوزيع العمري أن حدوث الذروة كان

ما بين 2 و 5 سنوات لحالات سرطان الدم الليمفاوي الحاد من نوع الخلية بي غير الناضجة كما في البلدان المتقدمة. و قد أظهر التوزيع الجغرافي لحالات سرطان الدم الليمفاوي الحاد عند الأطفال حدثت تقريبا في نصف الحالات و بنسبة 46.7% في محافظة الخليل و لكن بنسبة متساوية للحالات التي شخصت في محافظة بيت لحم اعتمادا على التعداد السكاني للمحافظتين.

الأستجابة للعلاج لمرضى سرطان الدم الليمفاوي الحاد كان جيد جدأ كما في الدول المتقدمة بسبب أن عدم وجود الحد الأدنى من خلايا المرض المتبقية عند النقطة التعريفية أي بعد 33 يوم من العلاج كان بنسبة 86.7% من مرضى سرطان الدم الليمفاوي الحاد.