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THE EVALUATION OF MULTIPLE DRUG RESISTANCE (MDR-1) PROTEIN AS A PROGNOSTIC FACTOR IN THE PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA (B-ALL)

PEDİYATİK B FENOTİPLİ AKUT LENFOBLASTİK LÖSEMİ (B-ALL) HASTALIĞINDA PROGNOSTİK FAKTÖR OLARAK MULTİPLE DRUG RESİSTANCE (MDR-1) PROTEİNİN DEĞERLENDİRİLMESİ

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THESIS APPROVAL / TEZ ONAYI

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İstanbul Üniversitesi Sağlık Bilimleri Enstitüsü İmmünoloji Anabilim Dalı, İmmünoloji Programı'nda Yüksek Lisans öğrencisi Amani AL KENDİ tarafından Doç. Dr. Suzan ÇINAR'ın danışmanlığında hazırlanan "Pediyatik B Fenotipli Akut Lenfoblastik Lösemi (B-ALL) Hastalığında Prognostik Faktör Olarak Multiple Drug Resistance (MDR-1) Proteinin Değerlendirilmesi" başlıklı tez aşağıdaki jüri üyeleri tarafından 26.04.2019 tarihinde yapılan Tez Savunma Sınavında başarılı bulunmuş ve Yüksek Lisans Tezi olarak kabul edilmiştir.

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Bu tez çalışmasının kendi çalışmam olduğunu, tezin planlanmasından yazımına kadar bütün safhalarda etik dışı davranışımın olmadığını, bu tezdeki bütün bilgileri akademik ve etik kurallar içinde elde ettiğimi, bu tez çalışmasıyla elde edilmeyen bütün bilgi ve yorumlara kaynak gösterdiğimi ve bu kaynakları da kaynaklar listesine aldığımı, yine bu tezin çalışılması ve yazımı sırasında patent ve telif haklarını ihlal edici bir davranışımın olmadığı beyan ederim.

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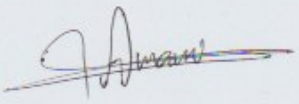

DEDICATION / ITHAF

This thesis is dedicated to my parents; my father, Mohammed and my mother, Norya, who love and support me throughout my life. Thank you both for encouraging me to go for as much education as I could. Thank you for giving me strength to reach for the stars and chase my dreams. I am truly thankful for having you in my life.

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LIST OF ABBREVIATIONS / KISALTMALAR LISTESI

ABC	:	ATP-binding cassette
ABCB1	:	Adenosine triphosphate-Binding Cassette subfamily B transporter-1
ALL	:	Acute Lymphoblastic Leukemia
AML	:	Acute myeloid leukemia
APC	:	Allophycocyanin
ATP	:	Adenosine Tri-phosphate
B-ALL	:	B-cell acute lymphoblastic leukemia
BCP	:	B-cell precursors
BCRP	:	Breast cancer resistance protein
BFM	:	Berlin-Frankfurt-Münster
BM	:	Bone marrow
BMNC	:	Bone marrow mononuclear cells
CD	:	Cluster of differentiation
CLPs	:	Common lymphoid progenitors
CNS	:	Central nervous system
CV	:	Coefficient of variation
DI	:	DNA Index
DNA	:	Deoxyribonucleic acid
EGIL	:	European Group for Immunological Classification
FAB	:	French, American, and British
FACS	:	Fluorescence activated cell sorting
FCS	:	Flow cytometer simulator
FHR	:	Flow High Risk
FISH	:	Fluorescent *in situ* hybridization
FITC	:	Fluorescein isothiocyanate
FLR	:	Flow Low Risk
FMR	:	Flow Medium Risk
FSC	:	Forward Scatter
G0	:	Gap 0
G1	:	Gap 1
GSH	:	Glutathione
GSSG	:	Oxidized glutathione
HR	:	High Risk
HSC	:	Hematopoietic stem cells
IGH	:	Immunoglobulin heavy chain
LRP	:	Lung resistance protein
MDR1	:	Multiple Drug Resistance 1
MRD	:	Minimal Residual Disease
MRP-1	:	Multidrug Resistance-Associated Protein 1

MSD	:	Membrane spanning domains
NBD	:	Nucleotide binding domains
NCI	:	National Cancer Institute
NK	:	Natural-killer
NOS	:	Not otherwise specified
PBS	:	Phosphate buffer salt
PCR	:	Polymerase Chain Reaction
PerCP	:	Peridinin- chlorophyll protein
Pgp	:	P-glycoprotein
Ph	:	Philadelphia chromosome
PH	:	Potential of Hydrogen
PI	:	Propidium iodide
QRT-PCR	:	Quantitative reverse transcriptase-PCR
RBC	:	Red Blood Cell
RQ-PCR	:	Real time quantitative polymerase chain reaction
RT-PCR	:	Reverse-Transcriptase-PCR
SR	:	Standard Risk
SSC	:	Side Scatter
TCR	:	T-cell receptor
TdT	:	Terminal deoxynucleotidyl transferase
UK	:	United Kingdom
US	:	United States
WBC	:	White Blood Cell
WHO	:	World Health Organization

SUMMARY /ABSTRACT

Amani Alkendi (2019). Assessment of Multiple drug resistance associated protein 1 (MDP-1) as a prognostic factor in pediatric B-cell phenotype acute lymphoblastic leukemia (B-ALL). Istanbul University, Aziz Sancar Institute of Experimental Medicine, Department of Immunology, Master Thesis Istanbul.

Acute lymphoblastic leukemia (ALL) is the most prevalent type of cancer in children. After chemotherapy, minimal residual disease (MRD) is still the most important indicator of clinical results and relapse. Multidrug resistance is the main obstacle to successful treatment. Multidrug resistance associated protein 1(MRP-1) may play a key role in throwing the chemical drug out of cells which lead to therapy resistance.

The goal of this study is to detect protein MRP-1 on cells from the bone marrow of children with B-cell ALL, and to determinate the value of MRP-1 as a prognostic factor in comparison with other factors such as DNA index (DI) and MRD.

Bone marrow samples were obtained from children who are diagnosed as B-ALL (n=20). The expressions of MRP-1 and DI were determined using flow cytometry at diagnosis. On the 15^{th} day of treatment, MRD in the bone marrow was detected also by flow cytometry.

There was no statistically significant difference of MRP-1 expression between risk groups like other prognostic factor. Protein level of MRP-1 expression measured seemed to have no prognostic importance.

Utilization of MRP-1 as predictive factor may not provide information on the B-ALL prognosis. Our results can help to better understand the nature of MRP-1 in B-ALL patients.

Key Words: Leukemia; B-cell, MRP-1, MRD, Flow cytometry, Multiple drug resistance.

The present work was supported by the Research Fund of Istanbul University. Project No. TYL-2018-28342

ÖZET

Amani Alkendi (2019). Pediyatik B fenotipli Akut lenfoblastik lösemi (B-ALL) hastalığında prognostik faktör olarak Multiple Drug Resistance (MDR-1) proteinin değerlendirilmesi. Istanbul Üniversitesi, Aziz Sancar Deneysel Tıp Araştırma Enstitüsü, İmmünoloji Anabilim Dalı, Yüksek Lisans Tezi, İstanbul.

Akut lenfoblastik lösemi (ALL) çocuklarda görülen en yaygın kanser tipidir. Kemoterapiden sonra Minimal Kalıt Hastalık (MRD) klinik bulgular ve relapsın hala en önemli indikatörüdür. Çoklu ilaç direnci başarılı bir tedavinin önündeki belli başlı engellerden biridir. Çoklu ilaç direnci ile ilişkili protein-1(MRP-1) kimyasal ilacın hücre dışına atılmasında anahtar rol oynayarak tedaviye karşı direnç gelişmesine neden olur.

Çalışmanın hedefi B hücre ALL'li çocukların kemik iliği hücrelerinde MRP-1 proteinini saptamak ve DNA indeks (DI) ve MRD gibi diğer faktörler gibi MRP-1'in prognostik faktör değerini belirlemektir.

Kemik iliği örnekleri B-ALL tanısı alan çocuklardan (n=20) elde edilmiştir. Tanı sırasında MRP-1 ve DI düzeyleri akan hücre ölçer ile tayin edilmiştir. Tedavinin 15.gününde kemik iliğindeki MRD de akan hücre ölçer ile saptanmıştır.

Risk grupları arasında MRP-1 düzeyleri bakımından diğer prognostik faktörler gibi anlamlı fark gözlenmemiştir. Ölçülen MRP-1 protein düzeylerinin prognostik öneminin olmadığı görünmektedir.

MRP-1'i kestirici faktör olarak kullanmak B-ALL prognozu hakkında yeterince bilgi vermeyebilir. Sonuçlarımız B-ALL hastalarında MRP-1'in doğasını daha iyi anlaşılmasına yardım edebilir.

Key Words: Lösemi; B-hücreli, MRP-1, MRD, Akan Hücre Ölçer (Flow cytometry), Çoklu ilaç direnci.

Bu çalışma, İstanbul Üniversitesi Bilimsel Araştırma Projeleri Birimi tarafından desteklenmiştir. Proje No: TYL-2018-28342

1. INTRODUCTION AND PURPOSE / GİRİŞ VE AMAÇ

Leukemia consists of two Greek words, leukos which means white and emia means blood and characterizes as a blood cancer with high levels of malignant white blood cells. Leukemia is by far the most prevalent pediatric cancer and tends to cause 29% of all deaths related to cancer in kids from 1 and 14 years. Leukemia also is a group of cancer types of blood cells that generally start in the bone marrow. It is basically composed of abnormal growth of white blood cells (WBC). These aberrant WBCs are indeed immature cells and are namely blasts or even leukemia cells. These cells cannot perform their physiological functions any longer, also they constitute rapidly proliferating blast cells, which produces a phenotype of disease by means of invasion of healthy tissues and organs. The major cause of disease-based infant mortality is childhood cancer (Pizzo and Poplack 2015) .

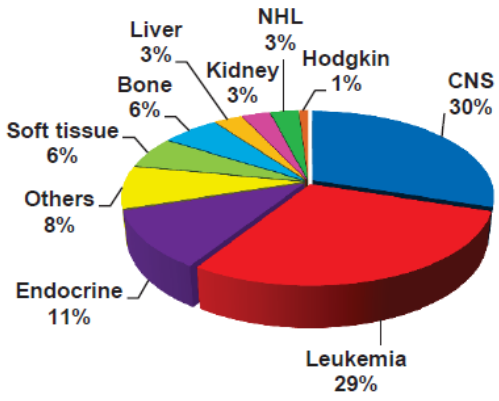


Figure 1-1: Deaths related to cancer in children from 1 to 14 years old (Pizzo and Poplack 2015). CNS, central nervous system; NHL, non-Hodgkin lymphoma.

Acute lymphoblastic leukemia is a malignant disease most prevalent in childhood with the greatest therapeutic progress in the last three decades. This progress was achieved by recognition of certain prognostic risk factors which enable the treatment to be adjusted accordingly. Many major risk factors that are used in diagnosis and treatment stratification includes age, WBC count, DNA index, and treatment response. According to National Cancer Institute (NCI) stratification, prognosis is associated with age and / or initial WBC count of patient (Smith, et al. 1996). In Addition, the DNA Index (DI) is an independent prognostic factor and gives valuable information for diagnostic and treatment stratification (Williams, et al. 1982) (Trueworthy, et al. 1992). As well, minimal residual disease (MRD) is considered the most important indicator of clinical results and relapse in childhood. Research has

demonstrated that high level of MRD is related to an unfavorable prognosis regardless of other risk markers commonly used for risk stratification (Conter, et al. 2010).

Chemotherapy is the main way of treating leukemia. Drug resistance in patients diagnosed with leukemia is often considered as the principal clinical obstacle to an efficient chemotherapy. Nowadays several resistance mechanisms were identified, and the most important and common mechanism for chemotherapy failure is multidrug resistance (MDR) (Du and Chen 2017). Many MDR drug efflux pumps belong to the superfamily of ATP-binding cassette (ABC) transporters. Multidrug-resistance-associated protein 1 (MRP-1) also belongs to the ABC transporter family. Findings indicate that MRP-1 plays a key role in elimination and sequestration of chemotherapy drug, which results in reduced levels in concentrations at their target locations (El-Sharnouby, et al. 2010). However, the precise clinical value of these MDR proteins in childhood ALL is not clear and the data available are contradictory.

1.1. AIM OF THE STUDY

The purpose of this study is to assess the importance of MRP-1 as predictor for responsiveness to therapy of childhood B-ALL. It is questionable whether the detection of MRP-1 at diagnosis is an independent prognostic factor for B-ALL. Depending on certain actual independent predictive factors like DI at diagnosis and the MRD risk evaluation on the 15^{th} day of treatment, the correlation of MRP-1 expression with these factors was investigated.

2. GENERAL INFORMATION / GENEL BILGILER

2.1. HEMATOPOIESIS

The process of blood cell formation is termed as haematopoiesis and usually occurs in the bone marrow. Initially, all blood cells originate from pluripotent stem cells and undergo a number of development phases before the formation of distinguished cells of each type. Then they get in the bloodstream. Every blood cell type comes from a hematopoietic stem cell (HSC) that is self-renewed. A massive proliferation happens during the differentiation into all different lineages, including myeloid (macrophages, granulocytes, and polymorphonuclear cells), erythroid-megakaryocytic (erythrocytes and platelets) and lymphoid lines (B-cells, T-cells, natural killer cells and dendritic cells) (Figure 2-1) (Katsura 2002).

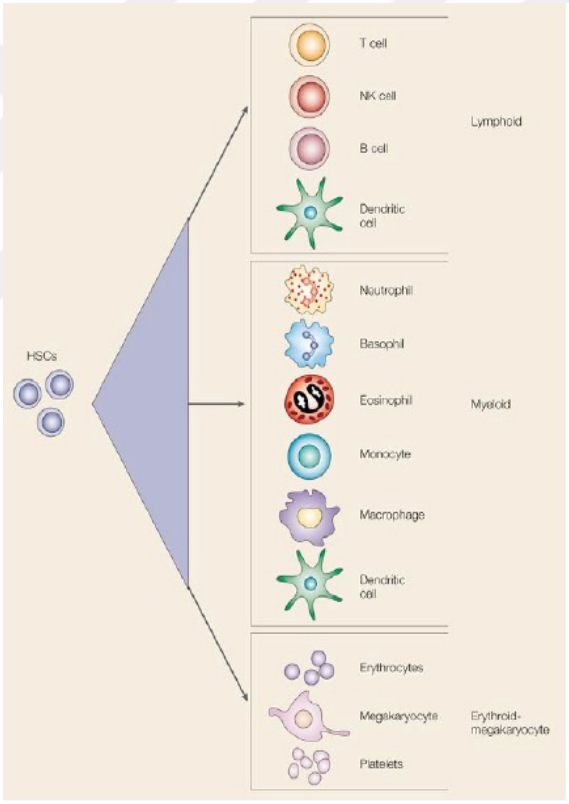


Figure 2-1: Categories of hematopoietic cells (Katsura 2002). HSCs: Hematopoietic stem cells

The process of hematopoiesis begins in the initial weeks of embryogenesis and HSCs immigrate to the liver and spleen. At the end of the fetal phase, bone marrow is the main place of hematopoiesis. In early childhood, the whole bone marrow can generate hematopoietic cells. In time, the hematopoiesis is increasingly limited to the central skeleton and proximal ends of femur and humerus (Boisset and Robin 2012) (Ciriza, et al. 2013).

2.2. LEUKOCYTES

Leukocytes are a type of white blood cells that represents approximately 1% of the blood volume. Also have nuclei contrary to erythrocytes. The nucleus contains chromatin substance which is deoxyribonucleic acid (DNA) and acts as the carrier of genetic information. Blood naturally contains mature leucocytes and could be divided into two main groups. The first one is polymorphonuclear leukocytes (granulocytes) and the other one is mononuclear leukocytes (agranulocytes) (Table 2-1) (Scott and Fong 2004).

Table 2-1: Leukocyte Types (Scott and Fong 2004).

Major Types	Specific Types	Percentage of the WBC's
Granulocytes	Neutrophils Eosinophils Basophils	50%-70% Less than 5% Fewer than 1%
Agranulocytes	Lymphocytes Monocytes	25-35% 4-10%

2.3. LYMPHOCYTES

Lymphocytes are a subgroup of white blood cells present both in blood and bone marrow. Lymphocytes are split into B or T lymphocytes, and natural killer cells. B-lymphocytes are differentiated within the bone marrow while T-lymphocytes are matured in the thymus gland. Both kinds of lymphocytes are crucial for certain immune reactions. Specialist WBC play an important role in the immune defense mechanism, i.e. the further division of lymphocytes into; B-cells which produce antibodies, and T-cells that mediate cellular defense mechanisms.

The main biological function of B-cells is to identify and remove foreign antigens. The capability to recognize millions of specific antigens is based on the huge variety of antigen receptor molecules. This variety is created through specific rearrangement mechanisms which take place during maturation of B-cells, whereby the different *Ig* gene segments are combined. As a consequence, a single B-cell expresses identical receptors with completely identical antigen specificity coded with the distinct rearrangement (Tonegawa 1983).

2.3.1. B-cell Development

The bone marrow is the only place of B lymphopoiesis from mid-gestation. HSCs generate common lymphoid progenitors (CLPs), that differentiate into B-cells, T-cells, and NK

cells (Tonegawa 1983). The development of lymphocytes takes place in the bone marrow and begins with the hematopoetic pluripotent stem cell. Signals from BM stromal cells are sent to this stem cell for lymphoid differentiation and differentiation of B-lineage. At specific developmental stages, a variety of different surface and intracellular markers are expressed such as the cluster of differentiation (CD). These markers and the different stages of immunoglobulin (*Ig)* gene rearrangements help to define distinctly different stages of B-cell maturation (Davi, et al. 1997).

2.3.2. Hematological Malignancies (Blood Cancer)

Cancer is a generic term for a group of malignant diseases with uncontrolled and invasive cells and metastases. In most organs or tissues such as blood, bone, skin, breast, lymph node, colon or nerve tissue, it may usually spread. Haematological malignancies represent a significant proportion of human cancers in various types. Blood, bone marrow and lymph node cancers are a heterogeneous group of blood cancers and are called hematological malignancies (Jemal, et al. 2009). These malignancies can be derived from any of the two main lineages of the blood cells: myeloid or lymphoid lineages. Myeloproliferative disorders, myelodysplastic disorders, and myelogenic leukemia usually originate from the myeloid lineage, while lymphoma, lymphocytic leukemia, and myeloma originate from the lymphoid lineage (Ge and Wang 2010). Table 2-2 lists a few examples of blood diseases together with their basic pathologies.

Table 2-2: Blood disease different types and their pathology (Beeker 2000).

Disorders	Pathology	Disease
Erythrocyte	Increased RBC Decreased RBC	Polycythemia Anemia
Leukocyte	Increased WBC (nonmalignant)	Eosinophilia Infectious Mononucleosis Sepsis
	Decreased WBC (nonmalignant)	Leukopenia
	Malignant disorders of WBC	Leukemia Lymphomas
Hemostatic	Quantitative Platelet Disorder	Primary Thrombocythemia Allergic Purpura
	Coagulation Disorder	Hemophilia
	Vascular Disorder	Purpura Simplex

RBC; Red blood cell, WBC; White blood cell

2.4. LEUKEMIA

Leukemia, that usually develops from blood, bone marrow, and lymphatic system cells, is also definitely known as fluid cancer because of not generating solid masses or tumors. The aberrant white blood cells start overhelming the bone marrow, providing no room for red blood cells and platelets and, as a natural consequence, a reduction in red blood cells can lead to anemia while a declination of platelet count decreases the blood's coagulation ability. Moreover, the abnormal nature of WBC makes them not able to fight infections. Exhaustion, frequent and severe infections, bruising and bleeding are the typical symptoms of leukemia. Leukemia includes a variety subsets based on the clinical and pathological characteristics. The classes are acute and chronic leukemia. Leukemia diseases are further divided into lymphoblastic or myeloid leukemia, depending on the involved blood cell type.

The most frequent form of childhood leukemia is acute form. Acute leukemia is characterized by fast and uncontrolled proliferation of immature cells. In such conditions, the bone marrow cannot generate healthy blood cells, because of the rapid development of the malignant cells and their accumulation. Also, leukemic cells move into the bloodstream and spread to other body tissues.

Chronic leukemia occurs mostly in the elderly, although it can be seen in all ages. An excessive increase in abnormal mature white blood cells characterizes chronic leukemia. It takes usually long to make progress, the production of cells is much higher than normal, this leads to many abnormal white blood cells (Miss.PatilTejashri and Raskar 2015).

Finally, several subtypes according to the specific precursor are included in the leukemia classification. A schematic diagram summary of the present classification of acute leukemia by the World Health Organization (WHO) can be found in Tables 2-3 (Foon and Todd 1986).

Table 2-3: World Health Organization's Classification of Acute Leukemia (Foon and Todd 1986)

Acute myeloid leukemia
AML with recurrent cytogenetic abnormalities
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML NOS
Morphologic and immunophenotypic subtypes
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm
Acute leukemia of ambiguous lineage
Acute undifferentiated leukemia
Mixed phenotype acute leukemia with t(9;22)(q34;q11.2); BRC-ABL1
Mixed phenotype acute leukemia with t(v;11q23); MLL rearranged
Mixed phenotype acute leukemia, NOS (sub classify by phenotypes)
B-lymphoblastic leukemia / lymphoma
B-lymphoblastic leukemia / lymphoma with recurrent genetic abnormalities
B-lymphoblastic leukemia / lymphoma NOS
T-lymphoblastic leukemia / lymphoma

AML, Acute Myeloid Leukemia; MLL, Mixed-lineage leukemia; NOS, Not otherwise specified.

2.4.1. Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is a malignant disease due to genetic mutations of lymphocyte precursor cells or blasts, as in hematology language. Overproduction of immature lymphocytes in bone marrow prevents normal hematopoiesis. Untreated ALL can lead to death because of huge number of blasts occupying the bone marrow and diffusing to vital organs through the peripheral blood. The clinical and biological characteristics of ALL differ sufficiently from their myeloid counterpart and certainly with its diagnostic and treatment protocols. In addition due to advances in molecular biology, the classification of ALL has become important for prognostic evaluation and proper chemical planing (Mohapatra 2013).

Acute lymphoblastic leukemia is present in two main types as B and T cell ALL. B-cell ALL come from the uncontrolled growth of abnormal immature B lymphocytes while T-ALL comes from abnormal immature T-lymphocytes. Both forms are characterized by the accumulation of blast cells, leading to suppression of hematopoietic differentiation in BM (Cobaleda and Sanchez-Garcia 2009).

2.4.1.1. B-cell acute lymphoblastic leukemia

B-ALL is a blood cell cancer which is characterized mostly by the unregulated, rapid cell growth of immature B cell progenitors that cannot properly mature in lymphocytic B-cells and is also associated with the highest childhood mortality due to cancer. Its timely and precise diagnosis is therefore essential for a stable and successful treatment (Zhou, et al. 2012).

B-ALL comes from an uncontrolled development of anomalous B-lymphocytes immature also called precursor B-lymphoblasts. B-ALL is usually classified according to the supposed origin of cell and the development stage of B-cells, from immature B-cells such progenitor (pro-B-cells), precursor (pre-B-cells), and pre-pro-B cells, to the more mature B-cells, such as plasma cells (Swerdlow, et al. 2008)

2.4.2. Epidemiology

Leukemia is the most frequent cause of cancer-related death in men younger than 40 years. While leukemia was associated with approximately half of new cancer-related deaths among females. Leukemia is considered as the main cause of death among women under 20 which is a matter of concern. The most frequent form is acute lymphocytic leukemia among the children younger than 14 year (Du and Chen 2017). B-cell precursor (BCP) constitutes about 80% of pediatric ALL cases, while about 15% is T-cell and 5% is mature B-cell leukemia (Kato and Manabe 2018).

In United States, diagnose of 5,960 new ALL cases were expected in 2018. Over 88% of ALL children are B-cell ALL. The incidence of ALL commonly increases in ages between 1-4 years and then decreases until about 45 years of age. The average age at diagnosis is 15 years (Raetz and Gojo 2018). In US, the number of new cases of leukemia was 50,149 for both sexes and all ages, and of the total cancer mortality of 616,714 cases, about 23,882 (4.29%) died from leukemia in 2018 (WHO 2018). The number of new cases of leukemia in Yemen was 763 for both sexes and all ages, 370 of them were male and 393 were female. Cancer mortality in Yemen was 9,085, and about 782 (9.49%) died from leukemia in 2018 (WHO 2018). In Turkey, cancer mortality was 116,710 and about 4,681 (4.31%) died from leukemia, also there were 6,029 new cases of leukemia in 2018 (WHO 2018). Australia, New Zealand, Northern America, and Western Europe have the highest incidence of leukemia for both sexes, while the lowest incidence was in western Africa. In general, the rates in male were higher than in female (Miranda-Filho, et al. 2018).

2.4.3. Diagnosis and Classification

Various diagnostic methods were used to characterize and classify B-ALL, including clinical features, morphological properties, immunophenotyping and cytogenetic investigations (Swerdlow, et al. 2008). Findings of biopsy from bone marrow aspiration analysis are the basis of ALL diagnosis. Initially, ALL diagnosis is based on the existence of over 25 to 30% blast cells in the bone marrow. Leukemias have traditionally been classified by the morphological system which is based on the blast cells morphology. Immunophenotyping currently provides a more precise and valuable classification system for acute leukemias (Bene, et al. 1995). Cytogenetic and molecular genetic methods provide valuable and important specific information which are often relevant to patient prediction and options of treatment (Pui and Evans 1998).

2.4.3.1. Morphology

Classification of ALL is based mainly on the microscopy of leukemic cells, which are stained by Wright-Giemsa and known as the French, American, and British (FAB). FAB is classified by three morphological ALL groups L1, L2, and L3. The most commonly, blast cell sizes are small to medium in ALL with insufficient cytoplasm, intense chromatin, and pale or non-existent nucleoli FAB L1 subtype. Less commonly, ALL cells generally tend to become more large, with moderate amounts of weak basophilic cytoplasm, thin scattered chromatin, and notable nucleoli, particularly regarding FAB L2. ALL may rarely have as a subtype FAB L3, composed of large blast cell, many intensely basophilic and vacuolated cytoplasms, roughly clumped chromatin and varied nucleoli that are prominent (Zafar 1985).

2.4.3.2. Immunophenotype

The detection of blast cell antigens using flow cytometry actually allows distinguishing the origin and the stage of maturation of leukemia. Immunological markers that are expressed on leukemic cells are used for ALL classification. The first diagnostic method applied sheep rosette formation for identification of the T-lineage, a polyclonal antiserum with the common acute lymphoblastic antigen leukemia (CALLA) to identify the B-lineage. The development of polyclonal antibodies versus lineage-associated markers (cluster of differentiation or CD markers) permits the identification of different and specific leukemic cell maturation stage. ALL was therefore classified by different study groups into many B lineage and T lineage subcategories (Bene, et al. 1995).

Depending on the European Group for Immunological Classification of Leukaemias (EGIL), ALL is subdivided into two main subsets, B-ALL, T-ALL (Pui and Evans 1998). Unlike the morphological characteristics, immunophenotype divides ALL disease into two wide categories: precursor B-cell ALL (B-ALL) and precursor T-cell ALL (T-ALL). According to the *Ig* gene expression, B-ALL can be re-classified into three subgroups. The first, early pre-B (or pro-B) ALL, lacking Ig. Second, pre-B-ALL with an expression of cytoplasmic μ chains and without expression or restriction of Ig light chains. Third, transitional phase pre-B-ALL with cytoplasmic and low surface expression of μ chains, but without or low expression of Ig light chains (Table 2-4) (Béné, et al. 2011).

Table 2-4: EGIL classification of acute lymphoblastic leukemia (Béné, et al. 2011).

B-lineage ALL	B-lineage markers (CD19,CD22, CD79…)	CD10	Intracytoplasmic *mu* chains (IgM)	Surface immunoglobulins or light chains
B-I (pro-B)	Positive	Negative	Negative	Negative
B-II (common)	Positive	Positive	Negative	Negative
B-III (pre-B)	Positive	Positive/Negative	Positive	Negative
B-IV (matureB)	Positive	Positive/Negative	Positive / Negative	Positive

2.4.3.3. Molecular (genetic) method

In the majority of ALL conditions, cytogenetic or molecular changes happen. By the methods currently available, the chromosomal or molecular anomalies could be identified in 60-75% of ALL cases in childhood, that can also be classified according to these results (Pui and Evans 1998). In few last decades chromosomal analysis has been in use for diagnostic classification, stratification of risk and progression monitoring. ALL can be generally classified by leukemic cell (ploidy) by modal chromosome number. Many different groups of ploidy have a prognostic and therapeutic significance: diploid (46 chromosomes), pseudodiploid (46 chromosomes with numerical or structural abnormalities), hyperdiploid (47 to 50 chromosomes, hyperdiploid (with 51 to 65 chromosomes) and triploid / tetraploid with over 65 chromosomes. In contrast, the ALL group with characteristically poor prognosis appears to be

defined as hypodiploid (< 45 chromosomes), which is recognized as a high-risk feature in most strategies for risk classification (Trueworthy, et al. 1992). Additionally chromosomal abnormalities were detected by cytogenetic / fluorescent *in situ* hybridization (FISH) analysis with success (Williams, et al. 1982). B-ALL was included in the WHO classification as distinct disease entity with cytogenetic abnormalities as following (Swerdlow, et al. 2008).

- Numerical chromosomal abnormalities
  - Hyperdiploidy
  - Hypodiploidy
- Structural chromosomal abnormalities
  - t(9;22)(q34;q11.2); BCR-ABL1
  - t(v;11q23); MLL- rearranged
  - t(12;21)(p13;q22); TEL-AML1 (ETV6-RUNX1)
  - t(5;14)(q31;q32); IL3-IGH
  - t(1;19)(q23;p13.3); E2A-PBX1 (TCF3-PBX1)

2.4.4. Prognostic factors

ALL prognostic factors are used to predict the potential to remission and to help differential treatment. Systems of prognostic have been developed and become covering features of ALL including diagnostic and clinical whether at diagnosis or after induction therapy. There are various factors which may determine the outcome of ALL patients. These include demographic factors such as age and sex, disease factors such as the percentage of WBC or cytogenetics and therapeutic factors such as drug regimen, duration, and intensity of treatment. Table 2-5 lists the most important risk factors affecting prognosis (Rabin and Poplack 2011).

Table 2-5: Prognostic factors in childhood ALL (Rabin and Poplack 2011)

Risk factors	Favorable	Unfavorable
Age	1-10 years	<1 or > 10 years
Gender	Female	Male
Race	Caucasian, Asian	Black, Hispanic
White blood cells	< 50,000 /ul	> 50,000 /ul
Immunophenotype	B-precursor	T-cell, mature B-cell
Cytogenetic and genomic features	Hyperdiploidy ETV6-RUNX1/t (12;21)	BCR-ABL1/t (9;22) MLL rearrangement Hypodiploidy iAmp(21) CRLF2 overexpression
End- induction bone marrow MRD	< 0.01 %	> 0.01 %

ALL, Acute Lymphoblastic leukemia; MRD, minimal residual disease

Various factors exist that could serve to predict patient outcomes, and one of them is patients characteristic. A uniform age and WBC counting selection criteria for pediatric ALL patients have been published in 1996 by National Cancer Institute (NCI). A consensus conference defined that age between 1 to 10 and initial WBC count of < 50,000 per mm^3 fits for the standard risk , and age ≥10 years or initial WBC count of ≥50,000 per mm^3 or both fits for the high risk ALL subgroups (Smith, et al. 1996). There is a particularly special subset of patients with poorer results, children under 1 year of age.

Some cytogenetic abnormalities, especially in B-ALL, have proven to have prognostic significance. For instance, hyperdiploidity has been associated with a better or favorable prognosis, which characterized as 51–65 chromosomes or index of DNA that are higher than or equal to 1.16, in about 30% of B-ALL childhood cases, noted. The ALL cases with a poor or unfavorable prognosis are defined as a hypodiploidy < 45 chromosomes, which is a high-risk characteristic in most risk classification strategies (Trueworthy, et al. 1992). Likewise, the ETV6-RUNX1 (TEL-AML1) fusion detected in approximately 20–25% of B-ALL cases in childhood has been linked to a good or favorable outcome (Dastugue, et al. 2013). Rearrangements of MLL gene that exists in up to 5% of ALL cases of childhood aged between 1-18 and about 80% of ALL childhood are linked to poor or unfavorable prognosis (Moorman,

et al. 2010). The presence of chromosome [t(9;22)(q34;q11.2)] is another cytogenetic aberration which influences prognosis and treatment (Schrappe, et al. 1998).

In the diagnostic assessment, immunophenotyping was traditionally used to determine the cell lineage of leukemia. The children's ALL is classified in precursor B-cells or T-cells subgroups according to the cell surface and cytoplasmic markers expression of the lineage. Historically, T-ALL have been associated with poor prognosis. However, T-ALL results have been improved with contemporary treatment regimes, which now resemble B-ALL results. A highly significant prognostic characteristic was the lymphoblast immunophenotype most frequently B-precorsor, subsequently the T-cell and finally mature B-cell (Mitchell, et al. 2010).

In children prognosis, the time needed to eliminate the sizable leukemia population to undetectable grade is really the most powerful predictor of ALL. A polymerase chain reaction amplification is used to measure submicroscopic particular levels of MRD (1 leukemia cell of 10^4 to 10^5 normal cells) or rearrangements of Immunoglobulin *Ig* and T-cell receptor *TCR* genes could be used, also flow cytometric detection of cell-surface antigen could be used for the same purpose (Conter, et al. 2010).

2.4.5. Treatment

ALL is indeed the most common cancer malignancy. Treatment stratification dependent on the biological characteristics of leukemia is a major contributor for improvement of pediatric ALL outcomes. Treatment was modified on the basis of biological features (aneuploidy and translocation) and the response to therapy.

The first stage of treatment is induction, with the aim of returning normal production of blood cells. Steroids, vincristine, and asparaginase are usually being used as induction therapies, with or without anthracycline. Over 95% of cases reached complete remission after 4-6 weeks of this strategy. Thanks to enhanced support therapy, the death rate decreased to 2-3% in induction therapies, but severe negative events are occurring and some children still are suffering from those negative events, such as infection (Kato and Manabe 2018).

Consolidation therapy starts to completely eradicate residual cells following induction therapy. Different cytotoxic agent combinations and methotrexate are very important to prevent relapses and can avoid the need for cranial irradiation.

Maintenance therapy is usually given for a period of 1 to 2 years after consolidation therapy. The standard combination is daily 6-mercaptopurine and weekly methotrexate. Intensified therapy with vincristine and steroid are used in some maintenance therapies. The duration, intensification or reduction of therapy is dependent on response and relaps risk of patient (Kato and Manabe 2018).

2.5. MINIMAL RESIDUAL DISEASE

MRD simply refers to the existence of a small number of blast cells, undetectable mostly by the traditional methods of morphology, after treatment in the bone marrow or other areas. The morphologic remission of the bone marrow means that less than 5% of blasts occur in the bone marrow, the detection limit for blast cells is 1-5% by cytomorphologic methods (Campana 2003). The most powerful predictor in childhood with ALL is the detection of MRD in bone marrow, and its evaluation is included in the most of the treatment plans. Numerous studies continue to show the prognostic value of MRD in newly diagnosed ALL patients, and predictive value for relapsing as well.

2.5.1. Methods of Minimal Residual Disease detection

In the method of MRD detection, a number of properties are needed. The most important properties are high sensitivity and specificity, the ability to produce rapid results and easy standards. Different techniques for MRD detection are applicable which differs in the specificities of the markers used and in the detection levels. The sensitivity of methods should reach at least 10^{-3}, 1 malignant cell of 1000 normal cells, ideally $l0^{-4}$ to 10^{-6}. Each method has advantages, disadvantages and limitations. Cell morphology, clonogenic tests and traditional cytogenetics are limited due to their low sensitivity. Today, the highly sensitive methods used in MRD detection are quantitative polymerase chain reaction (RQ-PCR), quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) and flow cytometry (Takamatsu 2017) .

Bone marrow cells are used for standard detection of MRD. In addition to bone marrow, MRD also can be identified in other body tissues such as peripheral blood, cerebrospinal fluid or autograft of peripheral stem cells. The presence and the level of MRD are considerably lower in B-ALL peripheral blood compared to the bone marrow. Peripheral blood therefore can not be used for MRD monitoring in patients with B-ALL, however, MRD of peripheral blood can be used to recognize B-ALL patients with very high risk of relapse (Short and Jabbour 2017).

Flow cytometry is a way to study and measure multiple intrinsic and excited optical signals (light-scatter and fluorescence) from cells or particles in the moving flow of fluids

(Figure 2-2).The fundamental principle of flow cytometry builds upon to use a fluidic system in order to deliver cells that are randomly suspended to a specific point intersecting with the lighting laser beam. The cells pass through the light of laser beam one after another and reflect lighting forward scatter (FSC) or at the right angle side scatter (SSC) according to their features. FSC gives information about the size of the cell, whereas SSC informs about the cell structure especially granularity. If cells are marked by fluorescent labeled monoclonal antibodies, the laser beam of a specific wavelength excites the fluorochromes and emits light from other specific wavelengths. The most frequently used fluorochromes are fluorescein isothiocyanide (FITC), R-phycoerythrin (PE, RPE), peridinin- chlorophyll protein (PerCP), allophycocyanin (APC), propidium iodide (PI), AlexaFluor and fluorochrome conjugates like phycoerythrin-cyanines and others. Some of these are used directly for the labeling of cellular components such as nucleic acids labeled with PI, but most of these are used to label other probe covalently such as monoclonal antibodies against cellular antigens. The dispersion and fluorescent light produced by cells are collected by a system of photodetectors that simply convert the photon pulses into electrical signals and further electronic and computer processing results in the graphical display and statistical analysis of the sample. The combination of FSC, SSC and multiple fluorochrome enables multiple parameter measurements on a single cell simultaneously such as 6 parameters in case of 4-colour flow cytometry (Ormerod 2000) (Owens, et al. 2000).

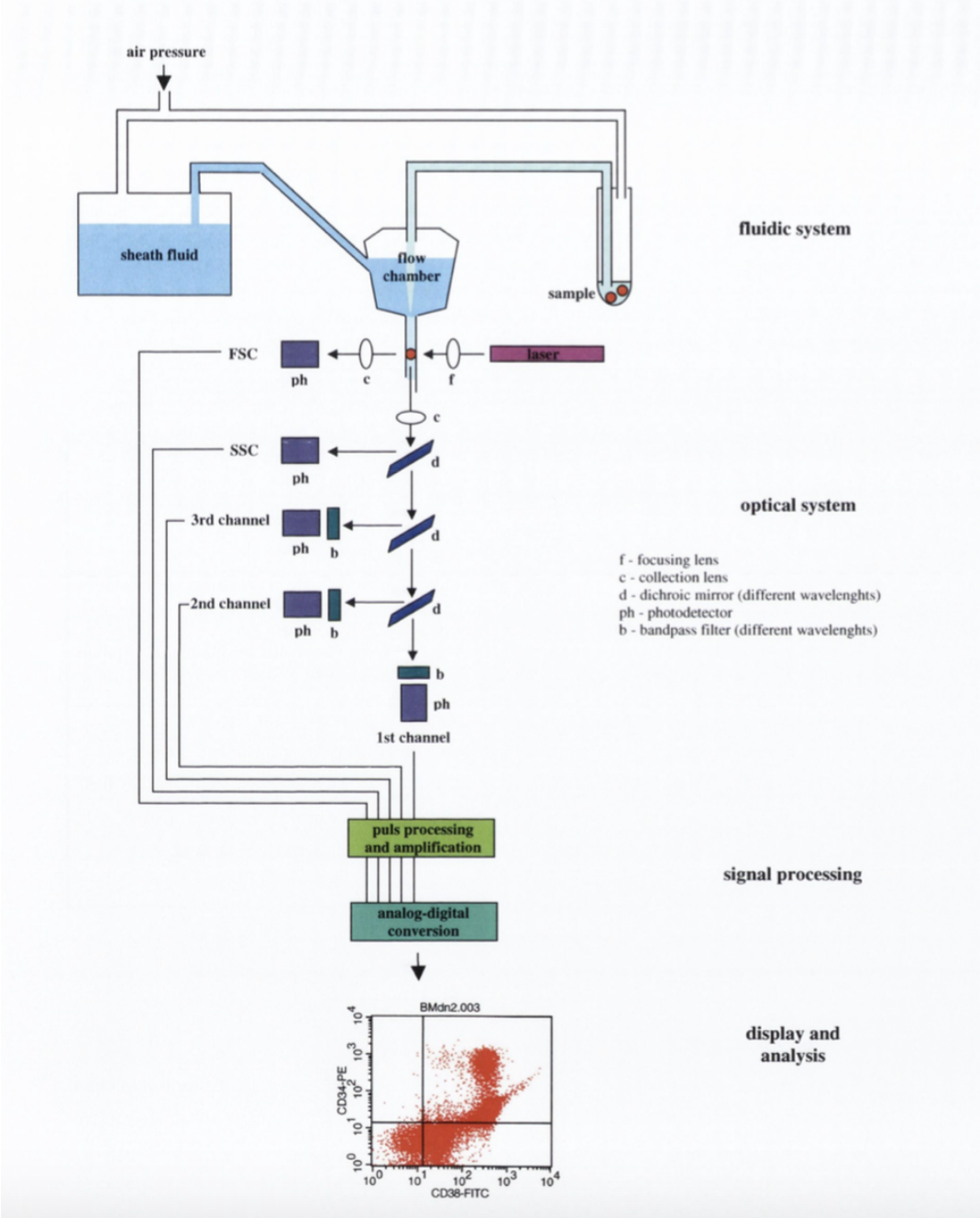


Figure 2-2: Flow cytometer scheme (Owens, et al. 2000).

A larger panel of antibodies is needed to deal with MRD of B-lymphocytes in ALL cases. Expression simultaneous of CD19, CD10, and CD34 or TdT is used to detect immature B cells. Different quantitation of CD38, CD45, and CD22 markers are useful for distinguishing normal immature B-cells and leukemic cells. With the combination of these markers, it can be investigated up to 85 percent of B-ALL in 10^{-4} sensitivities (Chatterjee, et al. 2016).

The detection of MRD has highly predictive and independent prognostic factor for the ALL relapse risk at the end of induction therapy. Cases that have a high level of MRD are associated with an unfavorable prognosis regardless of other risk markers commonly used for risk stratification. MRD levels (10^{-3} or more) have been classified as high-risk, MRD levels (10^{-4} or less) as standard risk, and MRD as moderate risk levels between them (Conter, et al. 2010). MRD detection methods have many pros and cons. Table 2-5 summarizes the features of the two MRD detection methods most commonly used based on van Dongen et al., Scrideli and Tone, Pui et al., Schrappe et al (Rocha, et al. 2016).

Table 2-4: Characteristics of methods mostly used in MRD detection (Rocha, et al. 2016).

Characteristics	**Detection of aberrant immunophenotypes by flow cytometry**	**Analysis of clonal rearrangement of Ig and TCR genes by RQ- PCR**
Analytical sensitivity	$10^{-3} – 10^{-4}$	$10^{-4} – 10^{-5}$
Applicability	>90% of patients	>90% of patients
Advantages	- Rapid turnaround time of results - Allows early MRD analyses - Ability to distinguish between viable and apoptotic cells - Relatively less expensive	- Standardized methods - High sensitivity
Disadvantages	- False positive results due to phenotypic similarities between leukemic lymphoblasts and regenerating B- lymphocyte precursors - False negative results due to phenotypic changes in residual leukemic cells throughout treatment - Limited standardization	- High cost - Technical complexity - Difficulty in providing fast results - Difficulty to rapidly design clone-specific primers for early MRD analyses - False negative results due to oligoclonality or clonal evolution - Inability to distinguish between viable and apoptotic cells

Ig, immunoglobulin; TCR, T-cels receptor; RQ-PCR, real time quantitative polymerase chain reaction; MRD, minimal residual disease. *Based on (Van Dongen, et al. 2015) , (Scrideli and Tone 2015), (Pui, et al. 2009) , (Schrappe, et al. 2000).

2.5.2. Importance of Minimal Residual Disease detection

The evaluation of initial responses at the end of induction therapy and continuous remission monitoring are two main objectives for the MRD detection in acute leukemia. At the end of induction therapy, MRD assessment can impact the treatment strategy. The MRD evaluation provides better stratification and individual approach, that may improve the results of therapy at the end of induction. Intensive therapy can help to improve the cure rate for patients at high risk while reducing therapy should prevent overtreatment.

In children with ALL, researches have demonstrated that MRD existence and level are the strongest predictive and important/independent prognostic factor for outcome and relapse at the end of the induction therapy. The presence of MRD ($\geq 10^{-4}$) is linked to a high risk of relapse, and high levels of MRD ($\geq 10^{-2}$) are linked to especially unfavorable prognosis. Week 4 and week 12, maybe also day 15 seem to be the main time points (Dworzak 2001). During remission in ALL, the continuous monitoring has to date limited clinical value and importance. A continuous drop in MRD level is usually linked to positive prognosis, while continuously high levels of MRD is linked to poor prognosis and signal for a high risk of relapse MRD also showed an important prognostic effect in patients in whom ALL relapsed (Campana and Coustan 2002) (Campana and Coustan-Smith 2004).

2.6. DNA INDEX

The biological characterization of the leukemic clone is performed by using cytology, the immunophenotype, the flow cytometric DNA Index (DI), the karyotype and molecular biology and it is imperative. Historically, leukemia's DNA ploidy has been well known as a prognostic factor for childhood ALL. DI provides information often during diagnosis about the disease diagnosis and treatmen (Rachieru-Sourisseau, et al. 2010). DI is used to obtain a quantitative evaluate of relative DNA content between the tumor cells and the normal diploid controls. It is defined as the ratio of the mean channel position of G0/G1peak of the tumor sample divided by the mean channel position of the G0/G1 peak of the normal diploid controls. The DNA analysis is one of the most important flow cytometry applications with predictive implications. It contains a measurement of the DNA content that assesses the status of ploidity, as well as the cell cycles giving information regarding cell population interest proliferation (M Abu Taleb, et al. 2002).

The content of DNA is the cellular major component most often measured. It can be quantified in order to evaluate DNA ploidity level, the position of the cell within the cell cycle,

and apoptotic cells characterized by their fractional DNA contents may reveal. Cell distribution is based entirely on fundamental differences in DNA content during the main phases of the cell cycle between cells of the pre-replicative phase (G0/1) and those that actually replicated DNA (S phase), compared with post-replicative, more mitotic (G2+M) cells. DNA content is defined as DNA ploidy or DNA index (DI) that is measured by flow cytometry. Normal cells (euploid or non-tumor) G0/1 cell cycle phase's DI is equal to 1.0 (Darzynkiewicz 2011).

One advantage of application of the DI in risk stratification is that it is easy to obtain and almost always successful, unlike cytogenetic analyses not available routinely and not technically possible for all patients (Trueworthy, et al. 1992).

In childhood ALL, DI is an independent prognostic factor. Precision in DI measurement is essential for stratification of diagnosis and treatment. The DI represents the proportion of DNA content of the leukemic cell to normal cells and measured through flow cytometry. DI greater than 1.16 is categorized as hyperdiploid and is linked to a very positive prognosis irrespective of age and WBC. DI > 1.16 as hyperdiploid is prediction of good prognosis while hypodiploidy DI ≤ 1.6 is linked to poor prognosis. Hyperdiploid group accounts for 25 to 30% of ALL children and has more favorable characteristics and higher curative rates than others. However, the importance of DI determination via flow cytometry is limited by the fact that it does not detect chromosomes changing (Rachieru-Sourisseau, et al. 2010).

2.7. MULTIDRUG RESISTANCE (MDR)

After one or more cycles of treatment, cancer cells may become resistant not only to the allready applied chemotherapy but to other drugs if they have different structures and targets. This fenomenon is defined as Multidrug Resistance (MDR) due to the over-expression of certain efflux proteins which are able to traffic chemotherapy from the cells, such as permeability-glycoprotein (P-gp) and multidrug-resistance-associated protein 1 (MRP1), or removed from the nucleus such as lung resistance protein (LRP) (El-Sharnouby, et al. 2010).

Many reports were completely focused on multidrug resistance mechanisms in leukemia. These mechanisms of resistance include increasing drug metabolism because of changed molecular targets, faults in apoptotic mechanisms, efflux pumps overexpression such as P-gp, MRP and BCRP, drug resistance mechanisms mediated by enzymes, the resistance of microenvironmental, and enhanced repair of DNA damage caused by drugs. It is common knowledge that the appearance of MDR made leukemia very difficult to treat effectively. Early, precise and sensitive detection of MDR genes is therefore essential. The MDR efflux pumps

are typically located in epithelial cells, also they are often expressed with other membrane proteins (Du and Chen 2017).

2.7.1. ABC transporters

ATP-binding cassette (ABC) is a transporter superfamily that belongs to the largest and most widely known protein superfamilies. The transportation of a wide variety of substrates such as ions, phospholipids, steroids, is a responsibility of superfamilies members. ABC is present in all cells of every organism and uses ATP binding /hydrolyze energy to carry substrates throughout cell membranes. Two types of structural domains exist for the ABC transport systems: hydrophobic, polytopic membrane-spanning domains (MSD), and hydrophilic, cytosolic nucleotide binding domains (NBD). There are two MSDs and two NBDs for the typical ABC transport system. In the drug intake and efflux, the ABC transporters are major molecules. The ABC human transporters are made up of 48 or 49 members. Amongst these are P-glycoprotein (ABCB1), breast cancer resistance protein (BCRP/MXR/ ABC-P/ABCG2), and multidrug resistance-associated proteins (MRP1/ABCC1 and MRP2/ABCC2) works as a drug efflux pump anticancer and their expression gives the cancer cell MDR-phenotype (Katayama, et al. 2014). The correlation between the reduced accumulation of drug and expression of 170 kDa cell-surface glycoprotein was identified in 1976 by Juliano and Ling, and P-glycoprotein (*ABCB1gene*) was designated (Juliano and Ling 1976).

2.7.2. MDR-Associated Protein1 (MRP-1)

Many studies have found multidrug resistance phenotype in certain tumor cell strains without expression of protein ABCB1, which supports the hypothesis that an additional protein is involved in xenobiotic efflux. In 1992, the amplification of new genes sequence (non-expression of ABCB1) responsible for the MDR phenotype has been identified by Cole and colleagues and was encoded as *abcc* gene (MRP). Following discovery of MRP, five other symmetrical proteins were identified as members of the same family and called ABCC / MRP transporter subfamily (MRP2–MRP6). MRP protein was then called ABCC1/MRP-1 protein. In 1994, is described ABCC1 as a full diaphragm, glycosylated protein with an approximate molecular weight 190 kDa (De Moraes, et al. 2015).

MRP-1 is an ATP-dependent transmembrane protein that is encoded by the gene *abcc1* and on the chromosome 16p13.12. Protein ABCC1 is also a member of the ABC superfamily transporter and carries hydrophobic and anionic substances and organic anions. Protein ABCC1 is constitutively expressed in hematopoietic cells in blood, placenta, brain, and other

organs. The action of the ABCC1 protein occurs in the transportation of physiological substrates like Leukotriene C4 and oxidized glutathione GSSG. Glutathione (GSH) is important factor for predisposition to multidrug resistance. Furthermore, cells are protected by ABCC1, which has a role in expulsion of xenobiotic toxic metabolites produced in order to prevent accumulation. Protein ABCC1 thus plays a significant physiological role in the detoxification of cells either from toxins generated by metabolism or exogenic toxic factors such as chemotherapy drugs. Some famous antineoplastic agents like Vinca alkaloids, anthracyclines, epipodophyllotoxins, methotrexate, daunorubicin, and doxorubicin are substrates of ABCC1 (De Moraes, et al. 2015).

MRP-1 consists of three membrane-spanning structural domains (MSD0, MSD1, MSD2) and of two cytosolic nucleotide binding domains (NBDs) (Figure 2‑3). Each MSD1 and MSD2 have 6 segments of transmembrane (TM) in form helixes, while MSD0 has 5 with about 200 amino acids. The duty of the cytosolic NBD is binding to ATP and by hydrolyzation of ATP, cytosolic NBD supplies energy for the transport of substrates. Some hydrophobic anticancer drugs carried by MRP-1 enter cytoplasm through the plasma membrane by passive diffusion. In addition, MRP-1 possibly prevents sequestration of drugs in intracellular compartment, since it was found in endoplasmic reticulum and endocytic vesciles (Du and Chen 2017).

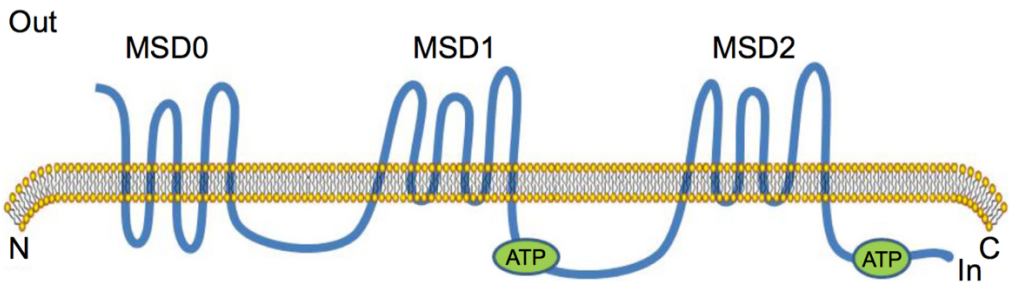


Figure 2-3: Protein ABCC1 schemes (Du and Chen 2017).

There are many detection methods for MRP-1, such as flow cytometric analysis. The RNA expression could also be detected for MRP-1 through Northern blot analysis or by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) (Valera, et al. 2004).

Many research shows that the *abcc1* expression gene is linked to treatment resistance in various types of cancer. Concerning acute leukemias, the role of protein ABCC1 when expressed is controversial. Whereas some studies continue to claim that ABCC1 is associated with multidrug resistance, others show that expression of protein does not seem to affect treatment resistance. There is a potential explanation of the difficulties in identification of the real clinical significance of these proteins in acute leukemias. It is based upon the fact that gene *abcc1* is expressed normally throughout all normal hematopoietic cell strains (De Moraes, et al. 2015).

The predictive value of drug-efflux ATP-binding cassette (ABC) such as MRP-1 in acute leukaemia has been determined by many groups, that conflicting data revealed either for ALL or AML. Overall, in pediatric patients with AML or ALL and adults with ALL, both proteins do not have prognostic value or have very weak prognostic strength. While, in adult AML, p-gp may be related to an unfavorable prognosis, but not MRP1 (Van den Heuvel-Eibrink, et al. 2000). Although the role of some MRPs is not fully understood, the importance and the influence of MRPs on the strategy and outcome of clinical cancer treatment cannot be neglected (Zhang, et al. 2015).

ALL diagnosis is based on lymphoblasts being identified and quantified by flow cytometry. There are important risk factors used for risk stratification and treatment stratification in ALL diagnosis. For example, poor prognosis of ALL in children is associated with age of less than 1 year or more than 10 years, high WBC, DNA index < 1.16 (hypodiploid) and high percentage of MRD. Drug resistance in patients diagnosed with leukemia is often considered as the principal clinical obstacle to efficient chemotherapy. Findings indicate that MRP-1 plays a key role in eliminating and sequestrating of chemotherapy drug, which results in reduced levels in concentrations at their target locations. The purpose of this study is to assess the importance of MRP-1 as predictor for responsiveness to therapy of childhood B-ALL. It is questionable whether the detection of MRP-1 at diagnosis is an independent prognostic factor for B-ALL. Depending on certain actual independent predictive factors like DNA index and the MRD risk evaluation on the 15^{th} day of treatment, the correlation of MRP-1 expression with these factors was investigated. All of the four variables; B-ALL phenotypes, multidrug resistance (MRP-1), DNA index, and MRD at 15^{th} day of therapy were examined by the flow cytometric method. The usage of DI, MRD and MRP-1 in a combined way may give more information can be reached concerning B-ALL prognosis.

3. MATERIAL AND METHOD / GEREÇ VE YÖNTEM

3.1. CASES

Newly diagnosed and untreated pediatric patients with ALL during the period of 2017 – 2018 admitted to laboratory of Istanbul University, Aziz Sancar Institute of Experimental Medicine, Department of Immunology were enrolled in the study, after their parents approved the Informed Consent forms. Approval for the study was obtained from the Local Ethical Committee of Istanbul University, Istanbul Faculty of Medicine (no: 2018/ 28342).

Assays have been performed as the following scheme:

1) At the time of diagnosis:

a. Immunophenotyping of a leukemia diagnosis,

b. MRP1 expression in B-ALL,

c. DNA index of B-ALL patients,

2) Minimal residual disease at 15^{th} day of therapy.

All examinations were performed by flow cytometry.

3.2. SOLUTIONS USED IN EXPERIMENTS

3.2.1. Lysing Solution

The solution was used to make the sample suitable for analysis in flow cytometry to ensure the breakdown of red blood cells after direct immunofluorescent staining with monoclonal antibodies of bone marrow and peripheral blood. FACS Lysing Solution (Becton Dickinson; BD Bioscience, San Jose, USA) with a concentration of 10X containing <15% formaldehyde, <50% diethylene glycol. Solution was diluted in ratio of 1:10 with distilled water and stored at room temperature (RT).

3.2.2. Ficoll

Ficoll (Sigma St. Louis, USA) is a solution containing high molecular weight sucrose polymers and sodium diatrizoate for the isolation of lymphocytes from peripheral blood or bone marrow material. It ensures the isolation of living mononuclear cells (MCs) in a high efficiency and purity. The blood or bone marrow sample to be isolated is mixed with phosphate buffer salt solution in a ratio of 1: 1, spread on ficoll and centrifuged for 20 minutes at 2000 rpm in RT. It must be stored at +4°C until used.

3.2.3. Phosphate Buffer Salt Solution

Phosphate Buffer Salt Solution (PBS; Sigma-Aldrich, USA) is an isotonic phosphate buffered salt solution in which the salts provide a stable physiological pH 7.2 to maintain cell integrity and viability. It is used for preparation of cell suspensions and for washing steps.

3.2.4. Fixation and Permeabilization Kit

Fixation and permeabilization solution (FIX & PERM, Nordic Mubio, Netherlands) used for the analysis of intracellular structures such as nuclear enzymes, oncoproteins, and immunoglobulins etc. provides detection of intracellular antigens in the presence of the appropriate antibody. The fixation and permeabilization kit consists of two reagents. Monoclonal antibodies can pass through pores formed by permeabilization solution and reach intracellular structures; as well as preserving the characteristic morphological characteristics of the cell. In this study fix and perm kit was used for detection of MRP-1 content of bone marrow cells.

3.3. METHODS

3.3.1. Detection of B-cell phenotype of ALL at diagnosis and MRD risk on the 15th day

The phenotype of blast cell obtained from bone marrow was specified by flow cytometry (FACSCalibur, BD Bioscience, San Jose, USA). In assay tube, 100 µl of heparinized bone marrow samples were incubated with anti-CD7, anti-CD10, anti-CD19 anti-CD34, anti-CD45 monoclonal antibodies for 20 minutes in the dark at RT (Table 3-1). Then 2 ml of lysing solution was added to the cells and incubated in dark at RT for 15 minutes. The residual erythrocytes were removed by centrifugation. After that, 2 ml PBS was added and centrifuged 2 times at 2000 rpm for 5 minutes. Following centrifugation, the cell surface molecule expression was determined with flow cytometry after resuspension of cell pellets in 300 µL of FACS buffer.

Table 3-1: Monoclonal antibodies used for diagnosing and phenotyping of ALL

Monoclonal Antibody	Label	Company
CD34	FITC	BD Bioscience, San Jose, USA
CD45	PerCP	BD Bioscience, San Jose, USA
CD7	PE	BD Bioscience, San Jose, USA
CD10	PE	BD Bioscience, San Jose, USA
CD19	APC	BD Bioscience, San Jose, USA

If $CD10^+$ and $CD19^+$ cells were found to be predominant in BM sample, the determined phenotype was B precursor cell ALL. If $CD7^+$ cells are in majority, patients were diagnosed as T precursor cell ALL and are excluded. In the case of B-ALL-diagnosis, bone marrow was labeled with additional anti-CD11a, anti-CD20, anti-CD34, anti-CD58 monoclonal antibodies (BD Bioscience, San Jose, USA) in the appropriate dilutions in order to determine the immunologic subclass of the disease according to the B-cell development stages.

On the 15^{th} day of treatment, the MRD of bone marrow samples were identified with specific monoclonal antibodies (CD10, CD11a, CD19, CD20,CD34, CD45, CD58) according to the phenotypic characteristics and ALL IC-BFM 2009 Standard Operating Procedure, and then the residuel blast cells were determined as percentage by FCM. The rate of leukemic cells in the nucleated $CD19^+$ cell population were determined. The percentages of leukemic blast cells or MRD were classified as flow low- (FLR), medium- (FMR), and high-risk (FHR).

3.3.2. Detection of MRP-1 expression.

MRP-1 expression levels of the blast cells were determined by a flow cytometer. From the bone marrow samples with anticoagluant additive EDTA, 100 µl of cell suspension ($2x10^5$ cells/ml) was put in the test tubes and incubated for 20 minutes with anti-CD19, -CD45, -CD10 monoclonal antibodies in the dark at RT (Table 3-2). Erythrocytes were lysed by incubation for 15 minutes with 2 ml of lysing solution in dark. The cells were washed by centrifugation 2 times at 1800 rpm for 10 minutes by adding 2 ml of PBS. Once the supernatant has been removed, the intracellular MRP-1 molecule was stained. At this stage, 100 µl of fixation medium was added to the 2×10^5 cell suspension and was incubated in the dark for 15 minutes and followed by washing with PBS for 5 minutes at 1800 rpm. Then, 100 µl of permeabilization medium were added along with anti-MRP-1 monoclonal antibodies (FITC, BD Bioscience, San Jose, USA) in appropriate dilutions. After 15 minutes of incubation in the dark, the washing step was done and MRP-1 detected on flow cytometry.

Table 3-2: Monoclonal antibodies used in detection MRP-1

Monoclonal Antibody	label	Company
MRP-1	FITC	BD Bioscience, San Jose, USA
CD45	PerCP	BD Bioscience, San Jose, USA
CD10	PE	BD Bioscience, San Jose, USA
CD19	APC	BD Bioscience, San Jose, USA

3.3.3. DNA content (DNA Index)

From samples of the bone marrow obtained at diagnosis, cell cycle phases were determined according to the DNA content. The effects on the cell cycle were assessed quantitatively using the BD Cycletest Plus DNA Reagent Kit (BD Cycletest^{TM}, San Jose, USA). In this study, BM nucleated mononuclear cells **(**BMNCs**)** were used as a biological material. BMNC were separated by Ficoll density gradient centrifugation of bone marrow samples. The method is briefly as follows. A PBS-diluted bone marrow sample (1:1 ratio) was spread on 4 ml Ficoll with conical bottomed tube. It was centrifuged at RT for 20 minutes at 2000 rpm. After centrifugation, the bone marrow contents are divided into layers according to their density, from top to bottom, plasma, buffy coat, Ficoll and granulocytes/erythrocytes. In this way, the desired BMNC can be collected easily by pipette. BMNC were suspended with 1 ml buffer solution and washed by centrifugation at RT for 5 minutes at 2000 rpm. After centrifugation, the supernatant was discarded and 1 ml of the buffer solution was added. It was centrifuged again at room temperature for 5 min at 2000 rpm. Cells were stored at -20^{o}C at this stage until the experiment was done. After melting, cells were centrifuged and the supernatant was removed, cell suspension was incubated with 200 µl of the trypsin buffer from the kit for 10 minutes at RT. At the end of this period, 200 µl of trypsin inhibitor and RNase buffer were added and incubated for 10 minutes at RT. Then 200 µl of cold propidium iodide staining solution was added. It was kept in the refrigerator for 10 minutes in the dark. Finally, the samples were filtered and data of DNA content were obtained using flow cytometry and CELLQuest software. The histograms were analyzed for the DI by ModFit LT program software (ModFit LT^{TM}, Verity Software House, USA).

3.4. STATISTICAL ANALYSIS

In conjunction with the SPSS program, the data collection and management was performed using the Microsoft Office Excel package. Nonparametric methods were used as Mann Whitney U test.

4. RESULTS / BULGULAR

4.1. Patient characteristics

The clinical and laboratory data of 20 B-ALL cases are shown in (Table 4-1). Patients' ages ranged between 3 and 15 years with a median age of 4.5 years. The gender ratio was balanced (female: male; 10:10). WBC counts ranged from 12.47 to 186.36 at median of 68.39 ($\times 10^9$/L) and RBC counts ranged from 0.14 to 3.97 at median of 3.215 ($\times 10^{12}$/L). Hemoglobin levels ranged from 0.3 to 13 at median of 9.75 (g/dL). Platelet counts ranged from 42 to 876 at median of 339 (10^3/uL). Blast cell percentages ranged from 63.9 to 95.35 at median of 82.16 (%).

Table 4-1: Clinical data of B-ALL patient

Demographic data		
Number of patients	20	
Age (year)	4.5 (3 - 15)	
Sex, male/female	10/10	
Clinical features		**Normal ranges**
WBC (x10^9/L)	68.39 (12.4 -186.36)	5.0 - 17.0
RBC (x10^{12}/L)	3.215 (0.14 - 3.97)	3.9 - 5.3
Hemoglobin (g/dL)	9.75 (0.3 - 13)	115 - 135
Platelet count (x10^3/uL)	339 (42 - 876)	150 - 400
Blast cells (%)	82.16 (63.9-95.35)	0.0- 2.0

4.2. NCI risk groups or WHO stratification

The stratification of the risk group for acute lymphoblastic leukemia in children was classified as Standard Risk (SR), and High Risk (HR) according to the National Cancer Institute (NCI). SR ALL group have both of the following criteria: WBC count is less than 50,000 cells/mm3 (50.0 x 10^9 cells/L) and the child is between 1 and 10 years old (8 of 20); and HR ALL which can have either of the following criteria: WBC count is greater than 50,000 cells/mm3 (50.0 x 10^9 cells/L) or the child is less than 1 or more than 10 years (12 of 20). There was no case with age less than one year which associated with unfavorable prognostic factors (Table 4-2).

Table 4-2: Distribution of ALL patients based on NCI risk stratification

NCI risk groups	N	Female	Male
Standard risk (SR)	8	3	5
High risk (HR)	12	7	5
Total	20	10	10

4.3. DNA index measurement for risk statification

We investigated all B-ALL patients BM samples for DNA index by flow cytometry. DNA content measurement determines ploidy status and also cell cycle analysis provides information about the proliferative activity of the cell population of interest. The patient's samples were run after that using the same setting of the FL2 Area of the control. This helped determination of the diploid population in the patient's sample. DNA Index is defined as the ratio of the mean channel of G0/G1 cells of an aneuploid population divided by normal diploid of G0/G1 mean channel (M Abu Taleb, et al. 2002). DNA histograms consist of X-axis: the content of DNA, and Y-axis: cell number (Figure 4-1). An anomalous DI was measured in 20 patients with DI mean as 1.21 and in 12 patients ranged from 1 to 1.15 as hypodipliod and in 8 patients ranged from 1.17 to 1.83 as hyperdipliod. The results of DNA index analysis among the patients revealed the hyperdiploidy and hypodiploidy as 40%, 60% respectively (Table 4-3).

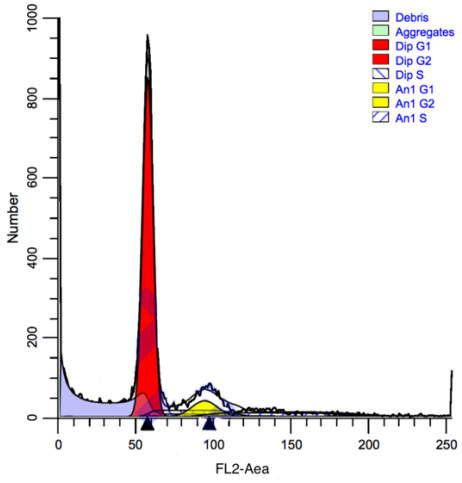


Figure 4-1: The histogram of the cell cycle by MODFIT LT program of representive case. Based on MODFIT analysis this case was found to be aneuploidy (yellow) to the right of first G0/G1 peak (red) with DNA index of 1.64 hyperdiploidy (DI>1.16).

Table 4-3: Distribution of DNA index of B-ALL patients as Hypodiploidy and Hyperdiploidy groups

DNA index	N (%)	Female	Male
Hypodiploidy (<1.16)	12 (60%)	7	5
Hyperdiploidy (>1.16)	8 (40%)	3	5
Total	20	10	10

4.3.1. DNA index and NCI risk groups

HR cases are associated with DI < 1.16 (hypodiploidy) and unfavorable factors such as over ten years of age and WBC count >50 × 10^9 /L which is a poor prognosis. SR cases are associated with DI>1.16 (hyperdiploidy) and favorable factors such as age between 1 and 10 years and WBC count <50 × 10^9 /L which is a good prognosis (Lustosa de Sousa, et al. 2015). So we considered that HR cases associated with DI < 1.16 (hypodiploidy) as a group, and SR cases associated with DI > 1.16 (hyperdiploidy) (Table 4-4).

Table 4-4: High and standard risk groups were associated with hypodiploidy and hyperdiploidy groups respectively

DNA index	NCI Stratification		
	HR	SR	N
Hypodiploidy (<1.16)	12	0	12
Hyperdiploidy (>1.16)	0	8	8
Total	12	8	20

4.4. Blast phenotypes and classification of ALL at diagnosis

The immunophenoptypes of patients with B-ALL were evaluted by flow cytometry. Bone marrow from ALL patients were stained with antibodies against CD7 or CD10, CD19, CD34 and CD45 for discrimination of B-ALL and T-ALL. The low SSC population (at lymphocytes position) obtained from the SSC-CD45 graph with negative or low positive for CD45 (CD45dim) were defined as blasts (Figure 4-2). The blast cell population of the patients diagnosed with B-ALL were confirmed by immunophenotyping profile with expression of CD10, CD19, CD20, CD34 and CD45. Bone marrow samples with cell ratio more than 20% of $CD10^+$ and / or $CD19^+$ positivity were considered as B-ALL; the presence of $CD7^+$ cells indeed were evaluated for T-ALL that were excluded from the study. $CD10^+CD19^+$ blast phenotype was detected in all cases (Figure 4-3).

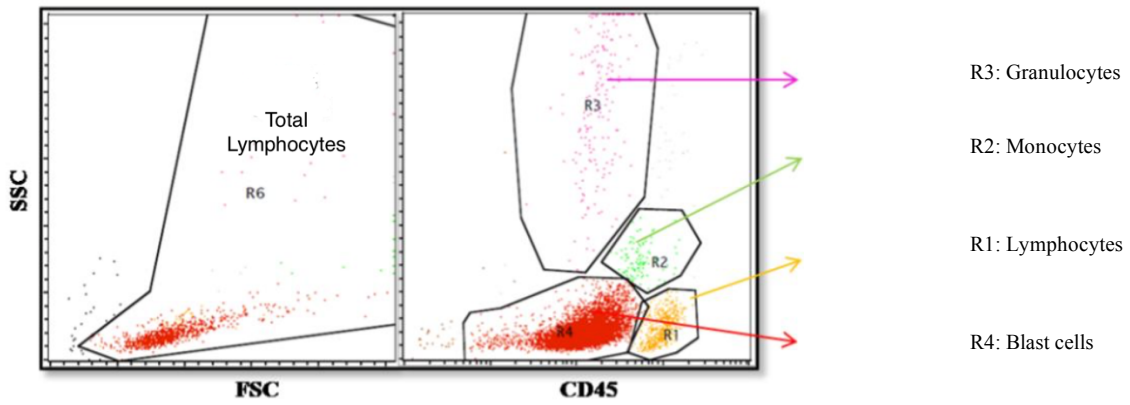


Figure 4-2: Gating strategy for blast cells (R4) SSC-CD45 graph; SSC low CD45dim

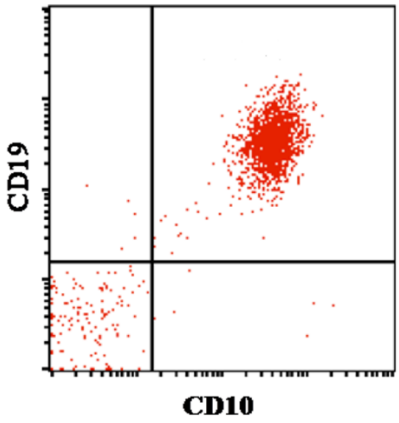


Figure 4-3: $CD10^{+}CD19^{+}$ blast phenotype captured in $SSC^{low}CD45^{dim}$ graph region (R4: blast cells)

4.5. Detection of Minimal Residual Disease on 15th day of therapy according BFM protocol

On the 15th day of therapy the bone marrow samples obtained from B-ALL cases were examined for MRD by flow cytometry. Samples with a normoblast rate less than 2% indicate hemodilution and do not reflect the real MRD percentage and risk. There was only one sample with hemodilution (normoblast 1.6 %, MRD % 6.7534 evaluated as FMR) and excluded. On the MRD day 15 risk analysis revealed two patients with high risk (10.52% FHR), 16 patients with moderate risk (84.21% FMR) and one with low risk (5.26% FLR) (Table 4-5). Majority of patients had response to therapy (90%). About 19 of 20 (95%) cases had MRD levels greater than 0.1% which were labeled as FMR and FHR, among cases considered as positive,

the median of MRD percentage was 1.5732%. There was only one case with negative result that was considerated as FLR (0.0000%).

Table 4-5: BFM MRD day 15 risk groups.

BFM MRD	N (%)	Female	Male
FLR	1 (5.26)	0	1
FMR	16 (84.21)	8	8
FHR	2 (10.52)	2	0
Total	19	10	9

4.5.1. Distribution of NCI and DNA index by level of Minimal Residual Disease

There were 5 SR cases with FMR and there were 11 HR cases with FMR as shown in Table 4-6.

Table 4-6: NCI risk groups and DNA index distribution in BFM MRD risk stratification.

BFM MRD risk Stratification at day 15	NCI risk groups and DNA index at diagnosis		
	Standard risk (hyperdiploidy)	**High risk** (hypodiploidy)	**N**
FLR	0	1	1
FMR	5	11	16
FHR	2	0	2
Total	7	12	19

4.6. MRP-1 expression in health subject

The expression of MRP-1 of peripheral blood cells was analyzed as a positive control. From a healthy volenteer MRP-1 expressions in different types of cells were analyzed by flow cytometry. Lymphocytes, monocytes, myeloid and blasts cells were gated on SSC/CD45 graph (Figure 4-4).

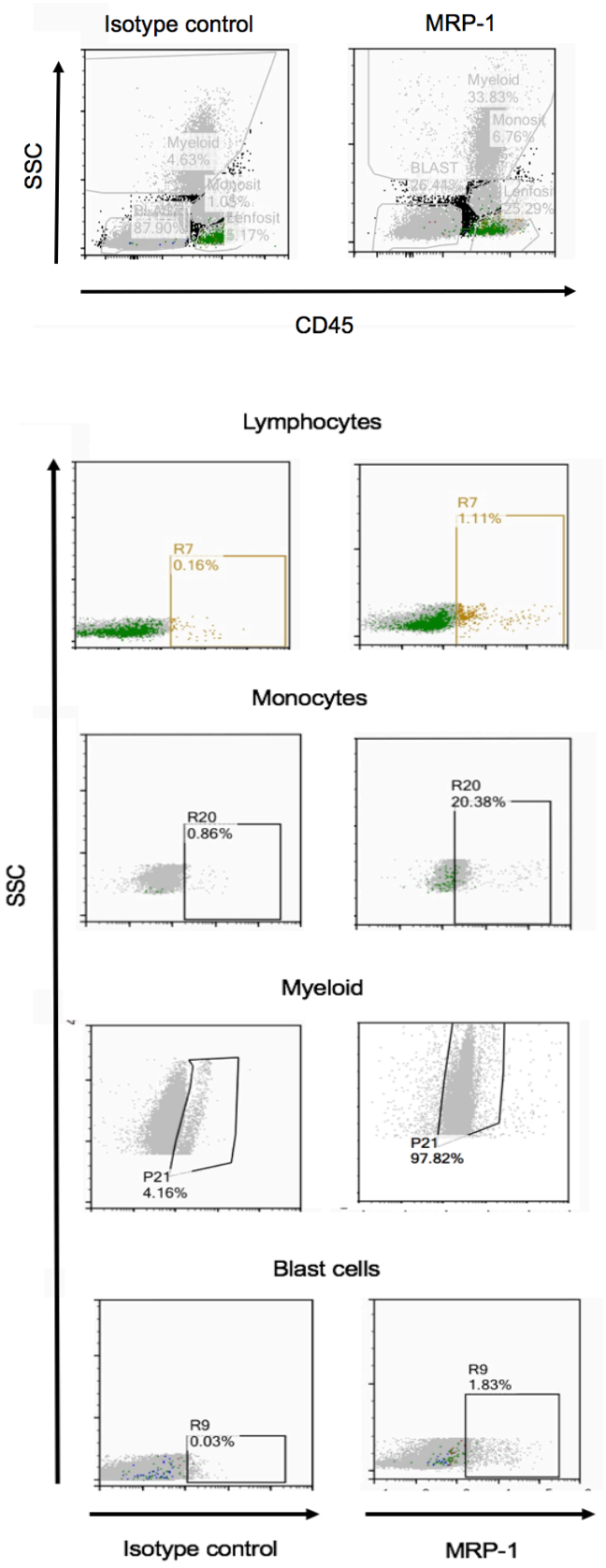


Figure 4-4: Gating strategy for detection of MRP-1 in different peripheral blood derived cells: lymphocytes, monocytes, myeloid, and blasts cells.

4.7. MRP-1 expression in B-ALL patients

The focus of this study is the MRP-1 expression of different cells of the bone marrow obtained from B-ALL children at diagnosis. The expression MRP-1 was investigated in 20 cases of B-ALL patients and determined by flow cytometry by direct immunofluorescence intracellular staining. MRP-1 positive cells were determined by reference to the negative level obtained from isotype control of MRP-1 monoclonal antibody on leukocytes**,** lymphocytes, monocytes, myeloid and blasts cells. Lymphocytes, monocytes and myeloid were gated on SSC/CD45 graph (Figure 4-5). Leukocytes were gated on all events excluded debris (Figure 4-6). Blast cells were gated on CD10/CD45 graph obtained from CD19 positive cells (Figure 4-7).

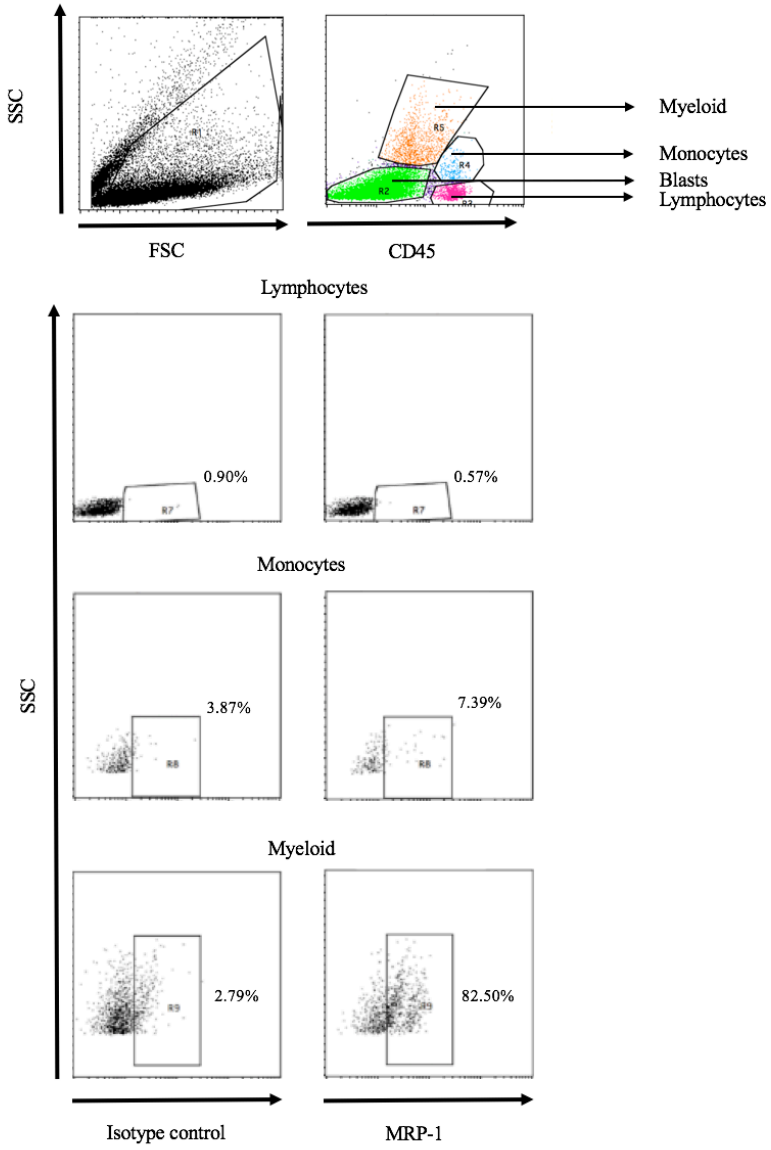
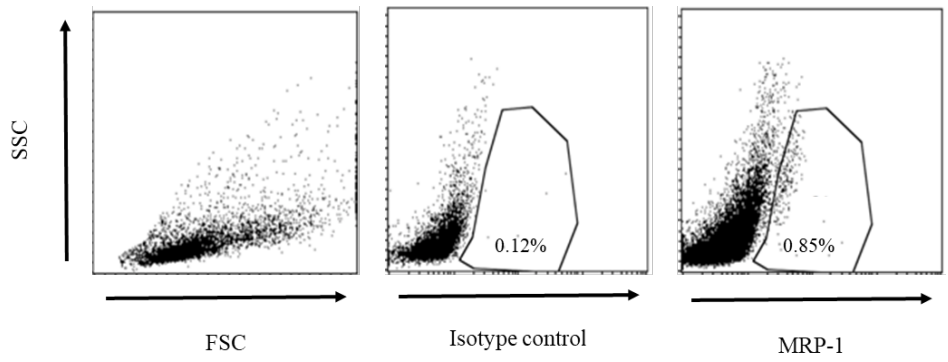


Figure 4-5: Gating strategy for detection of MRP-1 in different bone marrow derived cells: lymphocytes, monocytes, myeloid.



4: Gating strategy for detection of MRP-1 in leukocyte.

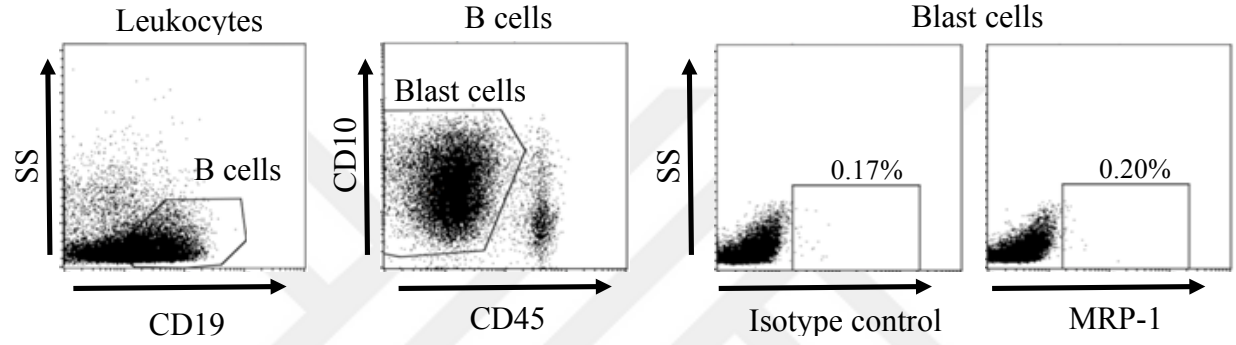


Figure 4-6: Gating strategy for detection of MRP-1 in blast cells.

4.7.1. MRP-1 expression of NCI risk groups and/or DNA index groups

We analyzed MRP-1 regarding to NCI risk and DNA index at diagnosis. Mann Whitney-U test was used to define the difference of BM cells MRP-1 expression between NCI risk groups. There was no statistical difference ($p > 0.05$). MRP-1 expression of bone marrow cells of two NCI risk groups are shown in Table 4-7.

Table 4-7: MRP-1 expression at diagnosis of bone marrow cells obtained from B-ALL patients grouped regarding NCI risk and/or DNA index groups.

Cell populations	**DNA index < 1.16 (High risk) (n=12)**	**DNA index > 1.16 (Standard risk)(n=8)**	
	Median (min-max)	**Median (min-max)**	**p**
Leukocytes	0.21 (0.03-0.98)	0.43 (0.04-0.97)	0.537
Lymphocytes	0.71 (0.10-1.82)	0.44 (0.22-0.73)	0.157
Monocytes	5.61 (1.11-62.56)	7.21 (1.19-15.38)	0.970
Myeloid	36.47 (1.77-89.33)	33.54 (1.51-89.76)	0.970
Blast cells	0.17 (0.09-0.46)	0.23 (0.15-0.28)	0.181

4.7.2. MRP-1 expression within MRD risk groups

To investigate MRP-1 expression of bone marrow cells obtained at diagnosis as a candidate predictive factor for therapy response, we compared with the response of B-ALL patients as MRD day 15 by flow cytometry. Regarding to response B-ALL patients were groupped as FLR, FMR and FHR. Since the frequences of patients who had FLR and FHR MRD risk groups was too small, one and two respectively, they were excluded. The FMR with MRD risk group (n=17) was reclassified into two subgroups depending on their representative NCI risk and/or DNA index at diagnosis. The two subgroups were FMR high risk (hypodiploidy) and FMR standard risk (hyperdiploidy). To compare the difference of MRP-1 expression of BM cell populations in these two subgroups, we used Mann Whitney-U test. As shown in table 4-8, there was no statistical difference.

Table 4-8: MRP-1 expression of bone marrow cells obtained from B-ALL patients with FMR risk at day 15 and NCI and/or DNA index at diagnosis.

Cell populations	**FMR High Risk (Hypodiploidy) (n=11)**	**FMR Standard Risk (Hyperdiploidy) (n=5)**	
	Median (min-max)	**Median (min-max)**	**p**
Leukocytes	0.19 (0.03-0.98)	0.43 (0.04-0.97)	0.404
Lymphocytes	0.78 (0.10-1.82)	0.31 (0.22-0.57)	0.733
Monocytes	6.73 (1.11-62.56)	5.58 (1.19-9.80)	0.884
Myeloid	26.3 (1.77-89.33)	33.54 (2.79-89.76)	0.591
Blast cells	0.17 (0.09-0.46)	0.23 (0.15-0.28)	0.733

Furthermore, we ranked B-ALL patients with FMR response at day 15 MRD depending on their CD34 cell surface marker expression at diagnosis. Two subgroups as FMR $CD34^-$ and FMR $CD34^+$ were compared by their MRP-1 expression and there was no statistical difference ($p > 0.05$; Mann Whitney U test) (Table 4-9).

Table 4-9: MRP-1 expression at diagnosis of bone marrow cells obtained from B-ALL patients grouped regarding CD34 at diagnosis and FMR response at day 15.

Cell populations	**FMR $CD34^-$ (n=8)**	**FMR $CD34^+$ (n=8)**	
	Median (min-max)	**Median (min-max)**	**p**
Leukocytes	0.43 (0.04-0.98)	0.16 (0.03-0.97)	0.163
Lymphocytes	0.68 (0.25-1.82)	0.26 (0.10-1.45)	0.541
Monocytes	4.87 (1.19-16.15)	8.57 (1.11-62.56)	0.815
Myeloid	13.83 (2.79-89.76)	43.56 (1.77-66.03)	0.481
Blast cells	0.19 (0.12-0.45)	0.18 (0.09-0.46)	0.370

5. DISCUSSION / TARTIŞMA

Multi drug resistance is traced directly by the overexpression of many efflux proteins which can traffic chemotherapeutic drugs from the cell, like P-gp and MRP-1. However, the real clinical value of MDR proteins is not completely clear in childhood ALL and existing information are contradictory (Lepelley, et al. 1998) (Zhang, et al. 2015). The aim of this research was to identify that MRP-1 had a relation with other independent prognostic factors like age, baseline white blood cell count (NCI risk stratification), DNA index and MRD (BFM risk stratification) , which may help to be used in prognosis of therapy for patients with B-ALL childhood.

In the earlier study by Lustosa de Sousa et al, they identified that SR cases were associated with hyperdiploidy (DI > 1.16) and favorable factors such as age between 1 and 10 years and WBC count $<50 \times 10^9$ /L which is recognized as a good prognosis (Lustosa de Sousa, et al. 2015). While HR cases were associated with hypodiploidy (DI < 1.16) and unfavorable factors such as age more than 10 years and WBC count $>50 \times 10^9$ /L which is a poor prognosis. The results of this study are matched to this previous study. When we examine the baseline white blood cell count and DNA Index data, all HR cases were simultaneously hypodiploid and SR cases were hyperdiploid.

The number of male children in this study was equal to the number of female children. When gender distribution to NCI risk and DNA index was examined, males showed equal distribution and the majority of female (70%) was in hypodiploid (HR) group. Our finding conflicted to earlier study that have been considered male gender as an independent predictor and related to poor prognosis (Shuster, et al. 1998). We could not prove that the male children had poor prognosis because of the low frequency of cases.

According BFM protocol at 15^{th} day of therapy MRD risk stratification by flow cytometry is usually used for distributing patients into low, medium and high-risk groups. Panzer et al. found the ability to distribute patients into various risk groups and mentioned that early therapy response is a consistent and independent prognostic factor for ALL at childhood (Panzer, et al. 2000). MRD was closely related to the NCI risk group. High-risk NCI patients were much more likely than standard risk patients to be positive for MRD (Borowitz, et al. 2008). Three out of 20 patients had controversial findings; one patient who had MRD negativity as risk FLR was HR at diagnosis and two patients with SR at diagnosis had weak early response to therapy (FHR with MRD > 10%). The controversial finding may be due to

the frequencies of patients at risk groups FLR and FHR was too small. Majority of our patients (85%) had a medium response (FMR). Also, MRD risk stratification was compared with MRP-1 expression in this study.

As regard to MRP-1, Den Boer et al. have reported that the this protein is unlikely to participate in childhood leukemia drug resistance (WHO 2018). Also, they mentioned that WBC count and age in ALL patients was not associated with P-gp, MRP-1 and LRP expressions. In our study, MRP-1 protein expression at diagnosis was examined by flow cytometry and we found no relation with other risk stratification criterias (NCI, DI) which was consistent with previous studies. Another research showed that MRP-1 mRNAs were overexpressed especially in pre-B-ALL children by semi quantitative RT-PCR (Ogretmen, et al. 2000). However Crist et al. have reported that the pre-B immunophenotype was significantly linked to a poorer outcome (Crist, et al. 1989). Plasschaert suggested that high MRP-1 expression was linked to unfavorable outcomes, and that patients with relapse had higher MRP-1 *gene* expression than those with complete remission (Plasschaert, et al. 2005). These findings were in concordance with those of Huh et al, who concluded that ALL patients with high MRP-1 levels could have poorer outcomes because of the correlation between the high levels of MRP-1 mRNA expression and poor 2-year survival (Huh, et al. 2006). Also, there was a study on childhood ALL patients that indicate association of bad prognosis with MRP1 positivity (El-Sharnouby, et al. 2010). Another report from India on ALL patients revealed the increase of MRP-1 mRNA expression at relapse in comparison with presentation or remission (Gurbuxani, et al. 2001). As concerning resistance to chemotherapy, the presence of MRP-1 in ALL adult patients may be one cause of the induction failure (Chauhan, et al. 2012). However, there are many contradictory findings that have not confirmed MRP-1 protein level to be significant for prognosis. For example Kakihara's group have not found any correlation between MRP-1 mRNA expression and risk factors at diagnosis (Kakihara, et al. 1999). Furthermore, a retrospective study of Sauerbrey's group reported no prognostic significance of the *MRP* gene expression (Sauerbrey, et al. 2002). Olson et al, observed the overexpression of MRP-1 during the diagnosis of T- lineage, and revealed no correlation with risk factors, adverse prognosis, and clinical unresponsiveness to therapy in pediatric ALL (Olson, et al. 2005).

MRP-1 protein expression at diagnosis of newly diagnosed B-ALL cases was investigated in our study, relapsed cases were not included. In addition to the above studies, we compared the cell types in the bone marrow such as leukocyte, lymphocyte, monocytes,

myeloid and blast cells according to their fluorescence characteristics and examined the levels of MRP-1 protein expression on those cells. Unlike the studies mentioned above, MRD levels of the patients that were examined by flow cytometry on the 15th day of therapy and MRP-1 levels at diagnosis were compared and no significant difference was found. After 15 days of therapy 85% of our patients had FMR risk. The correlations between increased expression of the *MRP* genes and the MRD were calculated in some study and they have reported that no relation between variables (Valera, et al. 2004) (Fedasenka et al. 2008). Frequencies of patients who had MRD at risk FLR (n=1) and FHR (n=2) was too small to compare the levels, so only patients with FMR response were grouped according to NCI / DI risk at diagnosis and MRP-1 protein levels were compared with MRP-1 expression and no significant difference was found.

In addition, we divided patients who have FMR response into two groups according to the CD34 expression at the time of diagnosis, taking into consideration that $CD34^+$ is associated with pro-B-ALL while $CD34^-$ with pre- and mature B-ALL. Then, MRP-1 expression of bone marrow cells was examined. Although CD34 expression was correlation with other predictively favorable factors such as hyperdiploidy and cytoplasmic Ig, it had a favorable independent impact on treatment outcome (Borowitz, et al. 1990). Also, Drach et al. showed that $CD34^+$ cell lineage expressed high mRNA MRP-1 gene (Drach, et al. 1995). We did not determine the relation between CD34 and other prognostic factors. Regarding MRP-1 expressions in our finding, there was no significant difference.

There are many studies that have not confirmed the correlation between overexpression of MRP-1 and clinical drug resistance in hematologic malignancies (Arlin, et al. 1992) (Büchner 1997). No differences were found in the expression of *MRP-1* gene in relation to *in vitro* cellular drug resistance to predinosone, vincristine, asparaginase, and daunorubicin (Slovak, et al. 1993). Investigations in leukemia have been controversial regarding the clinical importance of the prognostic significance of MRP-1 gene expression (Fujimaki, et al. 2002) (Schneider, et al. 1995). In concordance with these findings our results declared that overexpression of MRP-1 could not be used as a predictor for drug resistance.

In approximately 3 to 5% of all ALL children, the Philadelphia chromosome (Ph 1) is considered as a molecular indicator of high risk for failure of treatment or resistance to treatment (Schrappe, et al. 1998). Because of scarce data about the presence of this factor we couldn’t evaluate the relationship with MRP-1.

In our study, we showed MRP-1 levels with flow cytometric method in patients with B-ALL because it was easier and faster than PCR. The low number of cases made it difficult to compare subgroups. The majority of the FMR group as a response on the 15^{th} day of therapy allowed to create $CD34^-$ and $CD34^+$ groups. It is thought that the cause of conflicting results of MRP-1 in literature is due to the fact that the disease phenotype ($CD34^-$, $CD34^+$) or cell subgroups (leukocyte, lymphocyte, monocytes, myeloid, and blast cells) cannot be evaluated separately. When we combine all of our results, we do not support MRP-1 protein expression as a prognostic factor. Further studies are needed to determine the MRP-1 impacts on ALL patients' outcomes.

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Diagnosis																Day 15		
Patient	Age	Sex	Bone marrow WBC (x10^9/L)	Lymphocytes (%)	Blast (%)	NCI or WHO	CD10 (%)	CD19 (%)	CD20 (%)	CD45 (%)	CD34 (%)	MRP-1 Leucocytes (%)	MRP-1 Small Lymphocyte (%)	MRP-1 Large Lymphocytes (%)	MRP-1 Blast cells (%)	Normoblast (%)	MRD (%)	MRD Risk
1	3	F	69.43	15.72	74.47	HR	98.48	98.57	5.37	49.42	73.6	0.16	0.08	8.47	0.07	3.86	0.9326	FMR
2	3	F	121.60	6.76	89.28	HR	97.53	97.54	94.78	6.64	97.7	0.81	0	2	0.01	2.52	0.1071	FMR
3	6	F	186.36	1.24	95.03	HR	98.45	98.49	1.39	97.74	0.49	0.08	0.06	6.63	0.04	18.44	0.8678	FMR
4	5	M	24.61	15.19	65.97	SR	75.89	85.24	2.83	28.19	0.44	0.36	0.12	4.26	0.1	3.1	1.5732	FMR
5	7	F	32.99	1.00	87.99	SR	5.83	95.05	0.93	100	2.81	0.11	0.02	3.36	0.02	8.2	15.9297	FHR
6	9	F	81.34	12.01	79.39	HR	42.52	96.88	30.83	99.51	42.14	0.29	0.04	4.12	0.01	2.64	4.3599	FMR
7	3	M	74.25	8.83	78.34	HR	97.7	98.11	3.85	65.05	84.82	0.52	0.1	4.74	0.09	58	0	FLR
8	12	F	110.59	2.73	91.44	HR	81.84	99.5	6.00	99.66	94.44	0.19	0.14	11.8	0.11	61.1	6.8486	FMR
9	2	M	67.35	5.05	89.82	HR	98.64	98.66	26.58	3.75	98.53	0.04	0.06	10.85	0.13	9.16	0.1391	FMR
10	5	F	30.31	12.89	75.10	SR	93.26	93.59	38.8	26.16	62.26	0.52	0.19	11.23	0.13	4.8	66.6032	FHR
11	4	M	12.47	20.56	75.24	SR	70.89	98.46	10.51	70.58	74.1	0.97	0.19	6.47	0.22	14.84	0.7524	FMR
12	10	M	115.90	2.08	95.35	HR	98.96	99.21	3.41	80.02	3.51	0.98	0.29	7.1	0.04	19.74	1.4366	FMR
13	15	F	29.25	10.02	84.06	HR	85.88	98.1	4.28	68.8	98.86	0.23	0.07	1.99	0.01	19.5	9.2981	FMR
14	13	M	85.61	5.78	81.31	HR	7.11	97.95	2.01	97.8	98.05	0.07	0.5	4.44	0.41	18.4	7.547	FMR
15	3	M	30.45	12.22	82.98	SR	98.22	98.24	35.59	26.7	29.6	0.88	0.16	7.7	0.14	31.9	0.7942	FMR
16	4	F	26.45	15.27	63.90	SR	89.87	89.98	74.4	41.83	86.26	0.5	0.01	1.2	0	32.1	1.6212	FMR
17	6	M	28.05	6.07	89.22	SR	98.24	98.35	1.99	6.14	95.42	0.04	0.11	6.9	0.11	1.65	6.7534	FMR
18	3	F	132.88	7.39	81.34	HR	94.6	94.73	1.84	2.32	60.86	0.03	0.06	3.75	0.6	4.44	0.7064	FMR
19	3	M	40.36	10.20	75.24	SR	93.23	93.59	3.69	86.32	68.07	0.09	0.19	12.13	0.18	4.68	0.3396	FMR
20	3	M	99.46	6.54	88.61	HR	72.69	99.49	3.02	61.82	92.6	0.59	0.3	5.16	0.22	19	9.2005	FMR

CD, Cluster of differentiation; MRD, Minimal Residual Disease; MRP-1, Multidrug resistance associated Protein1; NCI, National Cancer Institute; SR, Standard Risk; HR, High Risk; FLR, Flow Low Risk; FMR, Flow Medium Risk; FHR, Flow High Risk; WBC, White blood cell; WHO, World Health Organization.

Distribution of DNA index stratification and NCI risk groups of B- ALL patients

Patient	DI index	Diploidy		NCI Risk group
1	1.07	<1.16	Hypodiploidy	HR
2	1.08	<1.16	Hypodiploidy	HR
3	1.09	<1.16	Hypodiploidy	HR
4	1.19	>1.16	Hyperdiploidy	SR
5	1.83	>1.16	Hyperdiploidy	SR
6	1.12	<1.16	Hypodiploidy	HR
7	1.15	<1.16	Hypodiploidy	HR
8	1.15	<1.16	Hypodiploidy	HR
9	1.15	<1.16	Hypodiploidy	HR
10	1.23	>1.16	Hyperdiploidy	SR
11	1.17	>1.16	Hyperdiploidy	SR
12	1.11	<1.16	Hypodiploidy	HR
13	1.14	<1.16	Hypodiploidy	HR
14	1.13	<1.16	Hypodiploidy	HR
15	1.35	>1.16	Hyperdiploidy	SR
16	1.25	>1.16	Hyperdiploidy	SR
17	1.42	>1.16	Hyperdiploidy	SR
18	1	<1.16	Hypodiploidy	HR
19	1.28	>1.16	Hyperdiploidy	SR
20	1.08	<1.16	Hypodiploidy	HR
DI, DNA Index; SR, Standard Risk; HR, High Risk				

FORMLAR

HASTA TAKİP FORMU

ADI VE SOYADI:			
T.C. KİMLİK NO:		**TARİH:**	
YAŞI:		**TELEFON NO:**	
CİNSİYET:			
İLK TANI TARİHİ:			
ANAMNEZİ			
HALEN KULLANDIĞI İLAÇLAR:			
HASTALIĞIN DURUMU:			
Ek bulgular			
Periferik kan sayımı	**WBC** **Lym %** **Nötr %** **Monosit %**		
Kemik iliği hücre sayımı	**WBC** **Lym %** **Nötr %** **Monosit %**		

İMMÜNFENOTİPLEME:
CD19 (%) CD10 (%) CD34 (%) CD20 (%) CD11a (%) CD45 (%) CD58 (%)
CD19 + CD10 +(%) CD19 + CD34 (%)

MRP-1 düzeyi:
(%)

ETHICS COMMITTEE DECISION / ETIK KURUL KARARI

T.C.
İSTANBUL ÜNİVERSİTESİ
İSTANBUL TIP FAKÜLTESİ
KLİNİK ARAŞTIRMALAR ETİK KURULU

Sayı : 1143 **Tarih : 17.10.2017**
Konu: Doç. Dr. Suzan ÇINAR hk.

Sayın Doç. Dr. Suzan ÇINAR
İmmünoloji Anabilim Dalı

İlgi : İmmünoloji Anabilim Dalının 14/09/2017 gün ve 336831 sayılı yazısı

Sorumlu araştırıcılığını üstlendiğiniz ve Amani ALKENDİ' nin yürüteceği 2017/1119 dosya numaralı "Pediyatik B fenotipli Akut lenfoblastik lösemi (B-ALL) hastalığında prognostik faktör olarak Multiple Drug Resistance (MDR-1) proteinin değerlendirilmesi" başlıklı çalışma kurulumuzun 13/10/2017 tarih ve 16 sayılı toplantısında görüşülerek etik yönden uygun bulunmuş olup, tutanaklar ekte sunulmuştur.

Bilgilerinizi rica ederim.

Prof. Dr. A.Yağız ÜRESİN
İstanbul Tıp Fakültesi Klinik Araştırmalar
Etik Kurul Başkanı

Eki: İstanbul Tıp Fakültesi Klinik Araştırmaları Etik Kurulu Karar Formu

PLAGIARISM REPORT / İNTİHAL RAPORU İLK SAYFASI

THE EVALUATION OF “MULTIPLE DRUG RESISTANCE (MDR-1) PROTEIN” AS A PROGNOSTIC FACTOR IN THE PEDIATRIC “ACUTE LYMPHOBLASTIC LEUKEMIA (B-ALL)”

ORIJINALLIK RAPORU

%13	%9	%8	%1
BENZERLIK ENDEKSI	İNTERNET KAYNAKLARI	YAYINLAR	ÖĞRENCI ÖDEVLERI

BIRINCIL KAYNAKLAR

1	ethesis.nitrkl.ac.in İnternet Kaynağı	%1
2	Nevenka Bihari, Milena Mičić, Renato Batel, Rudolf K. Zahn. "Flow cytometric detection of DNA cell cycle alterations in hemocytes of mussels (Mytilus galloprovincialis) off the Adriatic coast, Croatia", Aquatic Toxicology, 2003 Yayın	<%1
3	theses.gla.ac.uk İnternet Kaynağı	<%1
4	repub.eur.nl İnternet Kaynağı	<%1
5	Mihaela Onciu. "Acute Lymphoblastic Leukemia", Hematology/Oncology Clinics of North America, 2009 Yayın	<%1

CV / ÖZGEÇMIŞ

Kişisel Bilgiler

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Email	amanikendi@hotmail.com	**Tel**	05397076662

Eğitim Düzeyi

	Mezun Olduğu Kurumun Adı	**Mez. Yılı**
Doktora		
Yük.Lis.		
Lisans	Sana'a University	2011
Lise	19 Lisesi	2005

İş Deneyimi (Sondan geçmişe doğru sıralayın)

	Görevi	**Kurum**	**Süre (Yıl - Yıl)**
1.			-
2.			-
3.			-

Yabancı Dilleri	**Okuduğunu Anlama***	**Konuşma***	**Yazma***	**KPDS/ÜDS Puanı**	**(Diğer) Puanı**
English	iyi	iyi	iyi		IELTS 5.5
Turksh	orta	zayıf	zayıf		TOMER

*Çok iyi, iyi, orta, zayıf olarak değerlendirin

	Sayısal	**Eşit Ağırlık**	**Sözel**
LES Puanı			
(Diğer) Puanı			

Bilgisayar Bilgisi

Program	**Kullanma becerisi**

Yayınları/Tebligleri Sertifikaları/Ödülleri

Özel İlgi Alanları (Hobileri):

Kitap okumak, sinemaya gitmek.