Journal of
Blood Disorders & Transfusion

Hanna, J Blood Disorders Transf 2014, 6:1
DOI: 10.4172/2155-9864.1000250

Thesis Open Access

Expression of CD_{95} in Acute Lymphocytic Leukemia (ALL) in Egyptian Children before and after Treatment

Jackleen Raafat Awadallah Hanna*

Assistant Researcher, Medical Biochemistry Department, Medical Division, National Research Center, Egypt

Abstract

Acute lymphocytic leukemia (ALL) is a malignant disorder common among children. Apoptosis is a morphological process that leads to controlled cellular self-destruction and defective apoptotic pathways are greatly involved in tumor formation, progression, and metastasis. Apoptosis is the primary mechanism through which most chemotherapeutic agents induce tumor cell death. The purpose of this study was to monitor the expression of pro- and anti-apoptotic proteins CD_{95} and Bcl-2 as well as copper and zinc levels in the peripheral blood of children with acute lymphocytic leukemia prior to, and six months after starting chemotherapy. The study was performed on twenty five children attending the outpatient clinic of the National Cancer Institute – Cairo University. Ten normal children were included in the study and considered as control group. Results showed that Total Leukocyte Count (TLC) and bone marrow blast count were significantly higher in ALL children than controls while after treatment TLC was normalized whereas bone marrow blast count was significantly decreased, hemoglobin and platelet count were significantly decreased when compared to controls while significantly increased after treatment. CD_{95}% was significantly decreased before treatment relative to controls while it was significantly increased after treatment whereas Bcl-2 concentration showed significant increase before treatment compared to controls while it was significantly decreased after treatment. Serum Cu level showed significant increase in ALL cases at presentation compared to controls while it was not significantly changed after treatment. Serum Zn level was significantly decreased before treatment compared to controls while it was normalized after treatment. Cu/Zn was significantly higher in newly diagnosed ALL children than controls while it showed significant decrease after treatment. Negative significant correlation was found between CD_{95}% and Bcl-2 on one hand and between serum Cu and Zn levels on the other hand. It could be concluded that CD_{95}% and Bcl-2 might be useful diagnostic markers not only in the diagnosis but also in the follow up of ALL cases.

Keywords: Acute lymphocytic leukemia; Apoptosis; CD_{95}; Bcl-2; Copper; Zinc; Chemotherapy

List of abbreviations

ALL: Acute Lymphocytic Leukemia ; AML: Acute Myeloid Leukemia; MLL: Mixed Lineage Leukemia; TLC: Total Leukocyte Count; CD: Cluster of Differentiation; Bcl-2: B Cell Leukemia Lymphoma Gene; Bax: Bcl-2 Associated X Protein; Fas: Fatal Associated; TNF: Tumor Necrosis Factor; TRAIL: Tumor Necrosis Factor Related Apoptosis Inducing Ligand; DD: Death Domain; DED: Death Effector Domain; DISC: Death Inducing Signaling Complex; Apaf-1: Apoptotic Peptidase Activating Factor -1; Smac: Second Mitochondria Derived Activator Of Caspase; IAP: Inhibitor of Apoptosis Proteins; Bak: Bcl-2 Homologous Antagonist Killer; Bik: Bcl-2 Interacting Killer; Bcl-Xl: B Cell Lymphoma Extra Large

Introduction and Aim of the Work

Acute Lymphocytic Leukemia (ALL) is the malignant disorder resulting from the clonal proliferation of lymphoid precursors with arrested maturation and it is the most common type of leukemia found in children, that's why it is commonly called childhood leukemia [1]. Advances in treatment and prognosis of childhood leukemia are considered as a remarkable success of modern medicine, once considered a universally fatal disease with cure rates ranging from 75% to 85% by Faye and Chordas.

The immune system continually surveyed the body for the presence of abnormal cells which were destroyed when recognized. The immune response to a tumor was an early event leading to the destruction of the majority of tumor before it become clinically apparent, it plays an important role in delaying the growth or causing the regression of established tumors. The fact that tumors occur more frequently in the childhood period and in old age when the immune system functions less effectively [2]. Apoptosis is a programmed and controlled mode of cell death. Abnormalities in control of programmed cell death (apoptosis) play a critical role in tumourigenesis. Inhibitors of programmed cell death aberrantly prolong cell viability so contributing to occurrence and growth of tumors [3].

One of the markers of apoptosis is CD_{95} (cell surface molecule expressed on various cell types in the immune system and are designated by the cluster of differentiation or CD nomenclature) which is a surface glycoprotein receptor also called (APO -1/ Fas) expressed by a substantial minority of resting T & B cells and about 5% of resting Natural Killer cells (NK). The interaction of this receptor with its natural ligand CD_{95}L or agonistic antibodies induces the formation of a Death Inducing Signaling Complex (DISC) activating caspases leading to cleavage of cellular substrates [4]. The oncogene Bcl-2 is also involved in the regulation of cell death. Overexpression of Bcl-2 results in an inhibition of programmed cell death of hematopoietic cells [5]. B-cell precursor ALL cells overexpress Bcl-2, resulting in prolonged survival of leukemic cells [6].

***Corresponding author:** Jackleen Raafat Awadallah Hanna, Assistant Researcher, Medical Biochemistry Department, Medical Division, National Research Center, Egypt, Tel: 002-01223492341; E-mail: dr_jackleen@hotmail.com

Received January 05, 2015; **Accepted** January 08, 2015 **Published** January 12, 2015

Citation: Hanna JRA (2015) Expression of CD_{95} in Acute Lymphocytic Leukemia (ALL) in Egyptian Children before and after Treatment. J Blood Disorders Transf 6: 250. doi:10.4172/2155-9864.1000250

Blood zinc and copper concentrations as well as copper/zinc ratio may be useful parameters to demonstrate whether the nutritional status of children affects the disease progression or if the disease itself has an effect on nutritional status of patients [7].

Aim of the work

The aim of the present study is to identify the changes occurring in newly diagnosed ALL children regarding the expression of CD_{95} receptor and Bcl-2 level in blood as markers of apoptosis in addition to serum zinc and copper levels prior to and during the maintenance phase of the specified treatment protocol.

Review of Literature

Acute lymphocytic leukemia

Acute leukemia: Acute leukemia is a marrow-based neoplasm composed predominantly of minimally or partially differentiated haematopoietic/ lymphoid precursors. It is broadly classified into myeloid and lymphoid cell types, and subdivided using a combination of biologic, genetic, and pathogenetic features. Optimal diagnosis and classification require a variety of sophisticated immunophenotyping, cytogenetic and molecular biologic techniques [8] Clinical features are not definitive for differentiation of ALL and AML. The age profiles of acute leukemia differ, but overlap completely. ALL is predominant in childhood and AML in adults [9].

Acute lymphocytic leukemia: Acute Lymphocytic Leukemia (ALL) is the most common childhood cancer [10]. ALL results from the clonal proliferation and accumulation of progenitors that exhibit cell markers associated with the earliest stage of lymphoid maturation, this leukemic clone may exhibit features of either B-cell or T-cell commitment [11].

Epidemiology

The incidence of ALL is higher in boys than in girls, except during infancy, when there is a slight female predominance. Age-specific incidence patterns are characterized by a peak between the ages of 2 and 5 years, followed by falling rates during later childhood, adolescence and young adulthood [12].

Etiology

The causes of the vast majority of cases remain to be clarified, but likely involve interaction between the host inherited susceptibility, environmental and genetic factors [13].

(1) Enviromental and occupational factors: Low dose ionizing radiation [14], chronic benzene exposure [15], therapy related leukemia e.g. alkylating agents and topoisomerase II inhibitors [16] and smoking [17].

(2) Predisposing genetic diseases and genetic polymorphisms: A minority (5%) of cases of acute leukemia are associated with inherited predisposing genetic syndromes often involving genes whose encoded protein affect genomic stability and DNA repair [18].

Genetic syndromes have been related to leukemia such as:

Down's syndrome (trisomy 21) results in 10 to 18 fold elevated risk for leukemia [12], ataxia telangiectasia [19], bloom's syndrome [20], congenital X-linked agammaglobulinemia, immunoglobulin A deficiency and common variable immunodeficiency are also at increased risk [12].

Genetic polymorphisms of carcinogen-detoxifying enzymes have been variously associated with the development of leukemia. For example, deficiency of glutathione S-transferases, enzymes that detoxify electrophilic metabolites by catalyzing their conjugation to glutathione, is associated with infant leukemias without Mixed Lineage Leukemia gene (MLL) rearrangement and with ALL in black children. Polymorphisms of another enzyme, reduced nicotinamide adenine dinucleotide phosphate:quinine oxidoreductase, which converts benzoquinones to less toxic hydroxyl metabolites, have been associated with the development of infant and childhood ALL [12].

(3) Prenatal origin of some leukemias: The retrospective identification of leukemia-specific fusion genes (e.g. Mixed Lineage Leukemia fusion gene MLL/AF4) in the neonatal blood spots and the development of concordant leukemia in identical twins indicate that some leukemias have a prenatal origin. ALL with the t (4;11) (MLL/AF4) has a high concordance rate in identical twins (nearly 100%) and a very short latency period (a few weeks to a few months), suggesting that this fusion alone is either leukemogenic or requires only a small number of cooperative mutations to cause leukemia [12].

Clinical features

Symptoms may be insidious and slowly progressive over weeks to months, or they may be acute and explosive. In general, more the indolent onset, the better outcome. Symptoms and signs result from either bone marrow failure or the involvement of extra-medullary sites by leukemia. Easy fatigue, lethargy, fever, bone and joint pain are the most common presenting complaints. Bone pain results from bone erosion or leukemic involvement of the periosteum. Central nervous system (CNS) involvement by leukemia occurs in approximately 2% of patients at diagnosis and may be manifested by headache and vomiting or cranial nerve palsy. Cerebrospinal fluid (CSF) involvement is usually asymptomatic [21].

Physical examination often reveals pallor, petechiae, ecchymoses in the skin or mucous membranes and bone tenderness. Liver, spleen and lymph nodes are the most common sites of extramedullary involvement and are enlarged in more than half the patients. Testicular involvement occurs in only 2% of patients, mostly infants or adolescents with T-cell ALL [12].

Classification

(1) French-American-British (FAB) classification of ALL:

According to FAB classification; three subtypes of ALL are distinguished on basis of cell size, nuclear chromatin and shape, number and prominence of nucleoli, and relative amount and appearance of the cytoplasm [22]. FAB subtypes of ALL are summarized in Table 1.

(2) World Health Organization (WHO) proposed classification of ALL:

There was a consensus that FAB terms (L_1, L_2 and L_3) are no longer relevant, because L_1 and L_2 morphology do not predict immunophenotype, genetic abnormalities or clinical behaviour. The proposed WHO classification is based on the incorporation of data from morphology, immunophenotying, molecular and cytogenetic studies in the initial evaluation of leukemias [23]. The classification of ALL proposed by WHO is shown in Table 2.

Diagnosis

The diagnosis of acute leukemia usually follows the presentation

Cytology	L_1	L_2	L_3
Cell size	**Small**	Large	Large
Nuclear chromatin	Fine or clumped	Fine	Fine
Nuclear shape	Regular, may have cleft or indentation	Irregular, may have cleft or indentation	**Regular, oval to round**
Nucleoli	**Indistinct or not visible**	**One or more per cell; large, prominent**	One or more per cell; large, prominent
Amount of cytoplasm	**Scanty**	**Moderately abundant**	Moderately abundant
Basophilia of cytoplasm	Slight	Slight	**Prominent**
Cytoplasmic vacuolation	Variable	Variable	**Prominent**

Table 1: FAB classification of ALL The most helpful features for differentiating subtypes is in bold type.

1) Acute lymphoblastic leukemia/lymphoma
Synonyms: Former FAB L_1/L_2
(a) Precursor B-cell acute lymphoblastic leukemia (cytogenetic subgroups)
• t(12;21)(p13;q22) *TEL/AML1*
• t(9;22)(q34;q11) *BCR/ABL*
• t(V;11)(V;q23) *V/MLL*
• t(1;19)(q23;p13) *E2A/PBX1*
• Hypodiploid
• Hyperdiploid
(b) Precursor T-cell acute lymphoblastic leukemia
2) Burkitt cell leukemia/lymphoma
Synonyms: Former FAB L_3

V: variables **[23].**

BCR: Breakpoint cluster gene ABL: Abelson gene

AML 1: Acute myeloid leukemia gene 1

MLL: Mixed lineage leukemia gene

Table 2: WHO proposal for reclassification of ALL

of a patient with clinical features suggestive of the disease and relevant laboratory evaluation should be performed [24].

Blood count and blood smear: Laboratory evaluation reveals decreased hemoglobin concentration and haematocrit, reflecting massive replacement of the marrow by lymphoblasts. Red cell morphology is mildly abnormal, with exaggerated variation in cell size. Nucleated red cells or stippled erythrocytes may be present. Thrombocytopenia is present at the time of diagnosis. The mechanism of thrombocytopenia is a combination of inadequate production and decreased survival of platelets [25].

A White Blood Cell (WBC) count in excess of 50 x 10^3/mm^3 is frequently associated with prominent lymphadenopathy, hepatosplenomegaly, and the T-cell immunophenotype. Unlike the situation in AML, leucocytosis in ALL is rarely complicated by intracerebral haemorrhages or pulmonary insufficiency [26].

(2) Bone marrow examination: Bone marrow aspiration is the standard method of establishing the diagnosis and provides cells for immunophenotypic and cytogenetic analysis. The diagnosis of ALL is based on the demonstration of lymphoblasts in the bone marrow. By convention, the minimum number of bone marrow lymphoblasts required for diagnosis is set at 20% [21].

The most important morphologic characteristic in identifying lymphoblasts is their nuclear chromatin pattern; the chromatin in lymphoblasts is more clumped and irregularly distributed than in myeloblasts. The presence of nucleoli varies and cytoplasm is scant without granules [27].

(3) Cytochemical characterization: Special stains were more important in the identification of ALL before the widespread use of immunophenotyping, which has now become the primary diagnostic tool. Cytochemical stains on bone marrow smears are helpful in distinguishing ALL from AML and in subclassifying ALL [28]. Table 3 shows the cytochemistry of ALL.

(4) Immunophenotyping: The immunophenotye of leukemic cells reflects the cell lineage and differentiation stage of the transformed clone [29]. Immunophenotyping of blasts is used to distinguish between ALL and AML and in the subclassification of ALL [16]. The immunophenotypic classification of ALL, as demonstrated in Table 4, is based primarily on the surface and cytoplamsic marking characteristics of leukemic blasts and recognizes two lineages of lymphoblasts (T and B), each of which can be sub-classified into several maturational stages [21,30].

(5) Cytogenetic and molecular classification: Probably genetic alteration is present in every ALL case. Proto-oncogene activation, a new fusion gene creation or a deletion of a tumour suppressor gene are the mechanisms most often implicated. Clonal chromosomal abnormalities can be identified in 80-90% of childhood ALL. Recently, molecular genetics and cytogenetic findings have been able to classify 75-80% of ALL.

Karyotypically, ALL cases can be classified according to chromosome number (hyperploidy and hypoploidy), specific rearrangement and structural chromosomal abnormalities [12].

(6) Chemistry: A large leukemic cell burden having a high rate of cell turnover may produce several metabolic disturbances; chief among these is elevation of the serum uric acid level. Acute renal failure resulting from urate nephropathy may be a presenting feature, even in the absence of a large leukemic cell burden. Increased cell destruction also is responsible for hyperphosphatemia and hypocalcemia. Serum levels of lactate dehydrogenase (LDH) are increased because of an increased turnover of leukemic cells [31].

Prognosis

The prognostic factors of ALL are emphasized in Table 5. The ability to identify biologic and clinical features that influence prognosis has led to the use of different treatment regimens for different risk groups, which in turn has resulted in both improved overall outcomes and decreased toxicities for selected subsets of patients. Currently utilized schemes for classification of risk rely primarily on clinical, immunophenotypic, cytogenetic and response features [21].

Treatment strategy

The identification of reliable prognostic factors in ALL and the recognition of ALL as a heterogenous disease have led to the use of risk-directed therapy. Remission induction followed by intensification (consolidation) therapy to eliminate residual leukemia, eradication of CNS leukemia and continuation treatment to ensure maintainance of remission [12].

(1) Remission induction therapy: The goal of remission induction therapy is to induce a complete remission by eradicating more than 99% of leukemic burden, and by restoring normal haematopoiesis. Induction therapy typically includes a glucocorticoid (prednisone,

Reactions	Primary Normal Cells Manifesting Reaction	Major Diagnostic Utility
Myeloperoxidase (MPO)	Neutrophil series+, eosinophils+, monocytes+/-	L_1, L_2, L_3 -
Sudan Black B (SBB)	Neutrophil series+, monocytes+/-	L_1, L_2, L_3 -
Non Specific Esterase (α-naphthyl acetate or α-naphthyl butyrate)	Monocytes + (inhibited by sodium fluoride)	L_1, L_2 (focal)+/- L_3-
Periodic Acid Schiff (PAS)	Neutrophil series, monocytes and lymphocytes +	L_1, L_2 (70-75%) + (coarse granules or clumps in cytoplasm) L_3-
Acid Phosphatase (AP)	Neutrophil series, monocytes, lymphocytes, megakaryocytes and platelets +	L_1, L_2 (T cell)+ (strong focal paranuclear) L_3-

Table 3: Cytochemistry for ALL [28].

Type of reagent	Marker	B-Lineage				T-Lineage	
		Pro-B	Early-Pre-B CALLA	Pre-B	B-ALL	Pre-T	T-ALL
Against	HLA-DR	+	+	+	+	+/-	-
Precursor	TdT (n)	+	+	-	-	+	-
Cells	CD34	+	+	-	-	-	-
	CD19(m)	+	+	+	+	-	-
	CD22(c)	+	+	+	+	-	-
Against	CD10(m)	-	+	+	-/+	+/-	-
B-cell	CD20	-	-	+	+	-	-
Antigens	CD79a(c)	+	+	+	+	-	-
	clg (c)	-	-	+	+	-	-
	slg(m)	-	-	-	+	-	-
	CD7 (m)	-	-	-	-	+	+
Against	CD3(c)	-	-	-	-	+	+
T-cell	CD5(m)	-	-	-	-	+/-	+
Antigens	CD2	-	-	-	-	-	+

HLA-DR: Human leukocyte antigen class II

TdT: Terminal deoxynucleotide transferase

clg: cytoplasmic immunoglobulin

slg: surface immunoglobulin

CD: cluster of differentiation

Table 4: Immunological classification of ALL [30].

Determinants	Favourable	Unfavourable
White blood cell count	<10 x 10^9/L	>20 x 10^9/L
Age	3-7years	<1, > 10 years
Gender	Female	Male
Ethnicity	White	Black
Node, liver, spleen enlargement	Absent	Massive
Testicular enlargement	Absent	Present
CNS leukemia	Absent	Present
FAB morphologic features	L_1	L_2
Ploidy	Hyperploidy	Hypoploidy
Cytogenetic markers	Trisomies 4, 10, and/or 17	t(9;22)
	t(12;21)	t(4;11)
Time to remission	<14 days	>28 days
Minimal residual disease	< 10^{-4}	>10^{-3}

Table 5: Prognostic factors of ALL [21]

prednisolone or dexamethasone), vincristine and at least a third agent (asparaginase, anthracycline, or both) [21].

(2) Intensification (consolidation) therapy: Once normal heamatopoiesis is restored, patients can tolerate intensification (consolidation) therapy. There is no consensus on the best regimens and their duration. Intensification therapy is basically a repetition of the induction therapy at 3 months after remission induction. Effective components of intensification treatment include the intensive use of asparaginase and high-dose methotrexate [12].

(3) CNS-directed therapy: Treatment for subclinical or overt CNS leukemia is an integral part of successful therapy for ALL. Patients with one or more of the following features are at increased risk of CNS relapse and require more intensive CNS-directed therapy: large leukaemic cell burden, T-cell ALL, high-risk genetic abnormality, and the presence of leukemic cells in cerebrospinal fluid. Dexamethasone and intrathecal therapy were shown to improve CNS control. Whether

triple (methotrexate, hydrocortisone and cytarabine) intrathecal therapy is more efficacious than intrathecal methotrexate alone remains to be determined [32].

(4) Continuation treatment: The standard backbone of continuation treatment is the combination of methotrexate and mercaptopurine. The addition of intermittent pulses of vincristine and a glucocorticoid to the continuation treatment regimens improves results [12].

(5) Supportive care: At diagnosis, all febrile (with fever) patients should be given as empiric broad spectrum antibiotics until an infection can be excluded. Most patients require packed red blood cell transfusions. Patients are transfused with platelets to keep count close to 100 x 10^9/L before diagnostic lumbar puncture to reduce the risk of traumatic tap. All blood products should be irradiated in patients who are receiving chemotherapy to prevent graft-versus-host disease. Careful attention of fluid and electrolyte balance is essential, both at diagnosis and for 48-72 hours after the start of chemotherapy. All patients require intravenous hydration. Specific measures to treat or prevent hyperuricaemia include allopurinol [12].

(6) Allogeneic haematopoietic stem-cell transplantation: Because improvements in transplantation and chemotherapy are occurring in parallel, the indications for transplantation in newly diagnosed and relapsed ALL patients should be re-evaluated periodically. At present, Philadelphia-positive ALL [t(9;22)(q34;q11)], induction failure and early marrow relapse are clear indications for transplantation. Transplantation has not been shown to improve outcome in other types of very high-risk ALL whose outcome may be improved by intensive therapy containing high-dose cytarabine [33].

Apoptosis (Programmed cell death)

The term apoptosis had been coined in order to describe the morphological processes leading to controlled cellular self-destruction [34].

Apoptosis is of Greek origin, having the meaning "falling off or dropping off", in analogy to leaves falling off trees or petals dropping off flowers. This analogy emphasizes that the death of living matter is an integral and necessary part of the life cycle of organisms. The apoptotic mode of cell death is an active and defined process which plays an important role in the development of multicellular organisms and in the regulation and maintenance of the cell populations in tissues upon physiological and pathological conditions [35].

Apoptotic cells can be recognized by morphological changes: the cell shrinks, shows deformation and loses contact to its neighboring cells. Its chromatin condenses and marginates at the nuclear membrane, the plasma membrane is blebbing or budding, and finally the cell is fragmented into compact membrane-enclosed structures, called 'apoptotic bodies' which contain cytosol, the condensed chromatin and organelles (Figure 1). The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response [36].

Apoptosis is in contrast to the necrotic mode of cell death in which case the cells suffer a major insult, resulting in a loss of membrane integrity, swelling and disrupture of the cells. During necrosis, the cellular contents are released uncontrolled into the cell's environment which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue [35].

A breakthrough in apoptosis research comes from the genetic study of genes that controls apoptotic cell death in the nematode Caenorhabditis elegans (C.elegans) (Figure 2). In the worm three genes CED-9 (C.elegans death-9), CED-3 and CED-4 are directly involved in controlling the execution of apoptosis during development [37]. CED-3 is a caspase that is a cysteine protease that cleaves certain proteins after specific aspartic acid residues [38];

It exists as a zymogen, which is activated through self-cleavage [39]. CED-4 binds to CED-3 and promotes CED-3 activation, whereas CED-9 binds to CED-4 and prevents it from activating CED-3 [40]. Normally, CED-9 is complexed with CED-4 and CED-3, keeping CED-3 inactive. Apoptosis stimuli cause CED-9 dissociation, allowing CED-3 activation and thereby committing the cell to die by apoptosis [41]. Mammalian caspases are similar to CED-3 [39]. Apaf-1 (apoptotic protease activating factor) was the only mammalian CED-4 homolog known so far [42]. The products of the mammalian Bcl-2 gene family are related to CED-9 but include two subgroups of proteins that either inhibit or promote apoptosis [43,44].

Molecularly, apoptosis is a complex series of events with multiple positive and negative feedback loops and integration into other critical intracellular pathways including cell cycle progression and phospho-signaling pathways. Classically, apoptosis culminates in the activation of caspases, cysteine proteases that cleave critical intracellular proteins and thereby induce the final stages of cell death [45].

Activation of the apoptotic pathway: Apoptosis can be triggered by various stimuli from outside or inside the cell, e.g. by ligation of cell surface receptors, by DNA damage as a cause of defects in DNA repair mechanisms, treatment with cytotoxic drugs or irradiation, by a lack of survival signals, contradictory cell cycle signaling or by developmental death signals. Death signals of such diverse origin nevertheless appear to eventually activate common cell death machinery leading to the characteristic features of apoptotic cell death [46].

(1) Role of Caspases in apoptosis: The caspases, cysteine proteases homologous to C. elegans CED-3, are of central importance in the apoptotic signaling network which is activated in most cases of apoptotic cell death [47]. Seven different caspases have been identified in Drosophila, and 14 different members of the caspase family have been described in mammals, with caspase-11 and caspase–12 only identified in the mouse [48,49].

In the cell, caspases are synthesized as inactive zymogens, the so called procaspases, which at their N-terminus carry a prodomain followed by a large and a small subunit which sometimes are separated

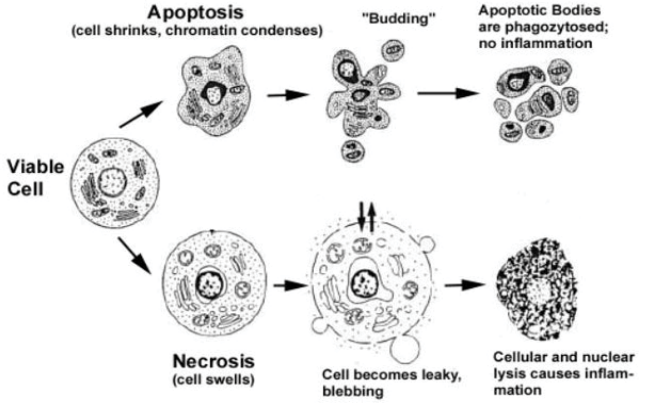


Figure 1: Hallmarks of the apoptotic and necrotic cell death process [38].

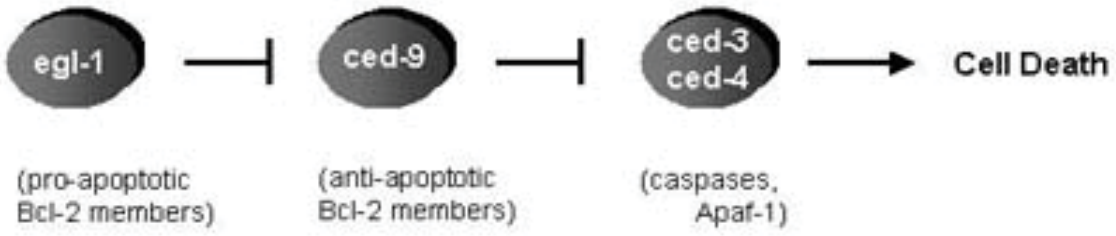


Figure 2: Role of C. elegans as a model system containing basic components of the cell death machinery [44].

by a linker peptide. Upon maturation, the procaspases are proteolytically processed between the large and small subunit, resulting in a small and a large subunit. The prodomain is also frequently but not necessarily removed during the activation process. A heterotetramer consisting of each two small and two large subunits then forms an active caspase. The proapoptotic caspases can be divided into the group of initiator caspases including procaspases-2, -8, -9 and –10, and into the group of executioner caspases including procaspases-3, -6, and –7. Whereas the executioner caspases possess only short prodomains, the initiator caspases possess long prodomains, containing Death Effector Domains (DED) in the case of procaspases -8 and -10 or caspase recruitment domains (CARD) as in the case of procaspase-2 and procaspase-9. Via their prodomains, the initiator caspases are recruited to and activated at death inducing signaling complexes either in response to the ligation of cell surface death receptors (extrinsic apoptosis pathways) or in response to signals originating from inside the cell (intrinsic apoptosis pathways) [50]. Multiple pathways lead to caspase activation and several of these pathways are already being targeted for therapeutic intervention. One of these pathways is the death-receptor-mediated or extrinsic pathway and the mitochondrial or intrinsic pathway illustrated in Figure 3 [45].

(2) Extrinsic apoptosis pathway: The extrinsic pathway is activated when ligands such as FAS (fatal associated) and TRAIL (Tumor Necrosis Factor (TNF) - related apoptosis-inducing ligand) bind to death receptors on the cell surface [45]. These receptors contain a cytosolic domain called Death Domain (DD) which recruits adaptor proteins to the receptor complex after binding to their ligands [51]. The recruited adaptor proteins have a death domain end and a Death Effector Domain (DED). Once bound to the receptor, the DED binds to upstream (initiator) caspase -8 and -10, which then become activated. Procaspase-8 is recruited by its DEDs to the Death Inducing Signaling Complex (DISC), a membrane receptor complex formed following to the ligation of a member of the Tumor Necrosis Factor Receptor (TNFR) family (Figure 4) [45]. When bound to the DISC, several procaspase-8 molecules are in close proximity to each other and therefore are assumed to activate each other by auto-proteolysis [48]. Once activated, caspase -8 is released into the cytoplasm where it cleaves and activates downstream (effector) caspases, such as caspase-3 leading to cell death and apoptosis [52].

(3) Intrinsic apoptosis pathway: The mitochondrial or intrinsic pathway of caspase activation is initiated by damage to the mitochondria that results in the release of a number of proteins into the cytoplasm including cytochrome c (Figures 3 and 5). When released into the cytoplasm, cytochrome c complexes with apoptotic protease activating factor -1 (Apaf-1) and this complex recruits upstream caspases, such as caspase - 9. Upon recruitment, caspase-9 dimerizes, activates and in turn cleaves and activates downstream effector caspase -3 causing cell death and apoptosis [53]. With cytochrome c, second mitochondria-derived activator of caspase (Smac) is also released from the mitochondria and induces apoptosis by binding to the inhibitor of apoptosis proteins (IAP) , which are family of proteins normally inactivate a number of caspases , including caspase -3 [54].

Initiator caspases are responsible for the first proteolytic events e.g. cleavage of cytoskeletal and related proteins including actin and fodrin (membrane-associated cytoskeletal proteins). These early apoptotic events are thought to be responsible for the characteristic blebbing of the cell surface. Cleavage of translocase (flippase) and/or activation of scramblase (floppase) lead to a subsequent flip of phosphatidylserine from the inner to the outer leaflet of the plasma membrane. Externalization of phosphatidylserine is used by cells as a signal for the attraction of macrophages [55].

Once the initiator caspases have been activated, they can proteolytically activate the effector procaspases-3, -6, and -7 which subsequently cleave a specific set of protein substrates, including procaspases themselves, resulting in the mediation and amplification of the death signal and eventually in the execution of cell death with all the morphological and biochemical features usually observed [56,57].

Apoptosis and cancer

Malfunction of the death machinery results from the mutation of genes that code for factors directly or indirectly involved in the initiation, mediation, or execution of apoptosis, and several mutations in apoptosis genes have been identified as a causing or contributing factor in human diseases [58].

Regulation of normal cell growth and turnover is balanced between cell proliferation, cell differentiation and apoptosis, a disruption of this balance is thought to be an important event leading to carcinogenesis [59].

Regulation of apoptosis: The apoptotic process can be set in motion by diverse stimuli such as genotoxic damage (e.g. chemotherapy, radiation) or deprivation of cytokines (e.g. erythropoietin). DNA single or double strand breaks activates a cascade beginning with the DNA-binding transcription factor p53 whose targets induce either growth arrest or entry of the cell into the apoptotic pathway [60].

One of the effector molecules in apoptosis is Fas antigen. Cross linking of Fas by its ligand (Fas L) or agonistic anti- Fas antibodies induces apoptosis of cells expressing Fas on the membrane by triggering cascade of caspases [59]. Bcl-2 emerged as the first example of an intracellular apoptosis-suppressor and the first identified proto-oncogene which contributed to neoplasia through effects on cell life span regulation rather than cell division [61]. p53 tumour suppressor gene plays a critical role in regulation of cell proliferation, mainly through induction of growth arrest or apoptosis. The p53 gene maintains the integrity of the genome, responding to damaged DNA by overexpression and induction of G1 arrest, before initiation of DNA repair. Alternatively, if DNA is not repaired, p53 may induce

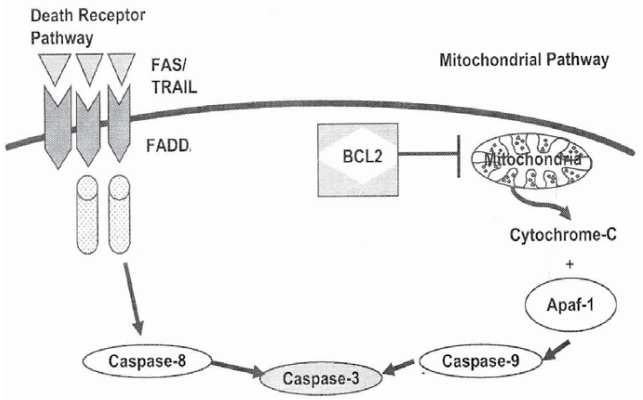


Figure 3: Intrinsic pathway.

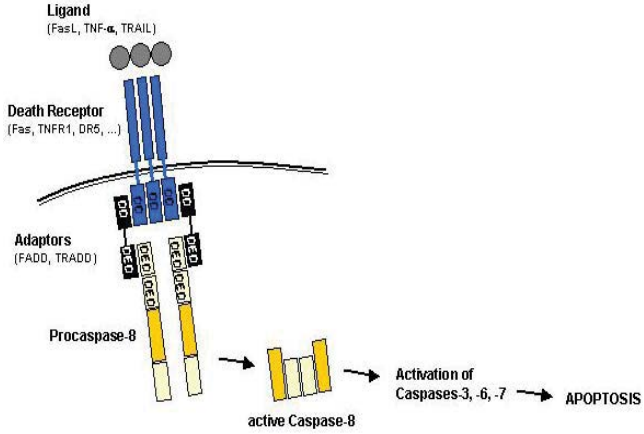


Figure 4: Receptor mediated caspase activation at the death inducing signaling complex (DISC) [52].

apoptosis [62]. p53 also induces the expression of several pro-apoptotic molecules such as Fas (Apo-l/CD_{95}) receptor, as well as the Bax protein .So p53 either induces growth arrest or apoptosis [63].

Inhibitor of Apoptosis Proteins (IAP) are a family of specific proteins known for their ability to inhibit apoptosis induced through mitochondria- dependent and independent pathways by preventing activation of pro-caspase - 9 and inhibition of caspase - 3 and caspase - 7 [64]. One of the members of this family is the survivin gene, survivin suppresses apoptosis induced by Fas, Bax, caspases and anticancer drugs [65]. survivin blocks apoptosis by inhibiting members of caspase cysteine protease family such as caspase-9, caspase-3 and caspase-7 [66]. Overexpression of survivin has been documented in leukemias [67].

(1) CD_{95} receptor/ligand system

The CD_{95}, a 48 kDa type1-transmembrane receptor, is a member of the tumor necrosis factor/nerve surface molecules, which includes various molecules involved in immune regulation, such as TNF receptor I and II, CD_{27}, CD_{30} and CD_{40} [68-71] CD_{95} also called Fas or Apo-1 [71]. Some researchers demonstrated that Fas was expressed on majority of human leukemia cells, although the intensity of expression was variable [72].

Physiological apoptosis can be induced by a multitude of stimulants, including withdrawal of growth factors, hormones, and activation of death receptors such as CD_{95} [69,70]. Defects in Fas-Fas ligand pathway (usually Fas but occasionally FasL germline mutations) cause autoimmune lymphoproliferative syndrome, a rare childhood disorder associated with B cell lymphomas, classic Hodgkin's lymphoma and nodular lymphocyte predominant Hodgkin's lymphoma [71], polymorphisms in Fas or FasL associated with increased risk of autoimmune hepatitis [73]. Increased risk of type II diabetes [74].

The CD_{95} receptor/ ligand (CD_{95}/ CD_{95}L) system has been described as a key signal pathway involved in regulation of apoptosis in several cell types [75]. The Fas receptor contains a conserved cytosolic domain known as death domain (DD) that is responsible for recruiting adaptor proteins such as FADD (Fas Associated Protein and Death Domain) to the receptor complex after binding of the ligand. The FADD protein binds certain caspases prodomains such as caspase-8 and -10 [76]. Processing of caspase-8 releases the activated protease into the cytosol, where it can cleave and activate other downstream procaspases [77].

(2) B cell leukemia lymphoma gene (Bcl-2)

Bcl-2 is an oncogenic protein that inhibits apoptosis [78]. Bcl-2 is encoded by a 230 kb gene that results in a 26 kDa protein [79]. Bcl-2 oncoprotein family of cytoplasm proteins have been identified in mammalian cells, viruses and other organisms. Most are integral membrane proteins that associate with organelles throughout the cytoplasm, including the endoplasmic reticulum, outer nuclear envelop, and inner plasma membrane, as well as with mitochondria. All Bcl-2 family members share conserved amino acid sequences [80].

Members of the Bcl-2 family

Bcl-2 family of proteins falls into two classes with opposite biologic effects (Figure 6). Some of these proteins actively induce or promote apoptosis such as Bcl-2 associated x protein (Bax), Bcl-2 homologous antagonist killer (Bak), Bcl-2 interacting killer (Bik), whereas others inhibit apoptosis such as Bcl-2 and B cell lymphoma extra large (Bcl-xL) [81,82].

Function

Bcl-2 has been identified as an apoptotic proto-oncogene while studying chromosomal translocations in human follicular lymphomas [83]. The Bcl-2 gene was found at the breakpoints of t(14;18) in low grade B-cell lymphomas, these lymphomas represent examples of human malignancies in which the neoplastic cell expansion can be attributed primarily to failed programmed cell death rather than rapid cell division [84]. The Bcl-2 gene is moved from its normal chromosomal location at 18q21 into juxtaposition with powerful enhancer elements in the immunoglobulin heavy-chain (IgH) locus at 14q32, with the result being deregulation of the translocated Bcl-2 gene and overproduction of Bcl-2 mRNAs and their encoded proteins [85].

Bcl-2 supresses apoptosis in two ways; direct action on mitochondria to prevent increased permeability and by effects mediated by interactions with other proteins. The release of cytochrome c from mitochondria triggers caspase activation by binding to Apaf-1 [86]. The Apaf-1 protein normally resides in an inactive conformation in the cytosol, but on binding cytochrome c, an ATP oligomerization domain within this protein mediates Apaf-1 aggregation [87]. The oligomerizated complex then binds pro-caspase - 9, and facilitates trans-processing of caspase - 9 zymogen via induced proximity mechanism [88]. It has been shown that anti-apoptotic Bcl-2 proteins block the release of cytochrome c from the mitochondria, while the pro-apoptotic Bcl-2 proteins (especially bax) lead to the release of cytochrome c and subsequent Apaf-1 activtion. The pro-apoptotic Bcl-2 proteins are forming pores in the outer mitochondrial membrane

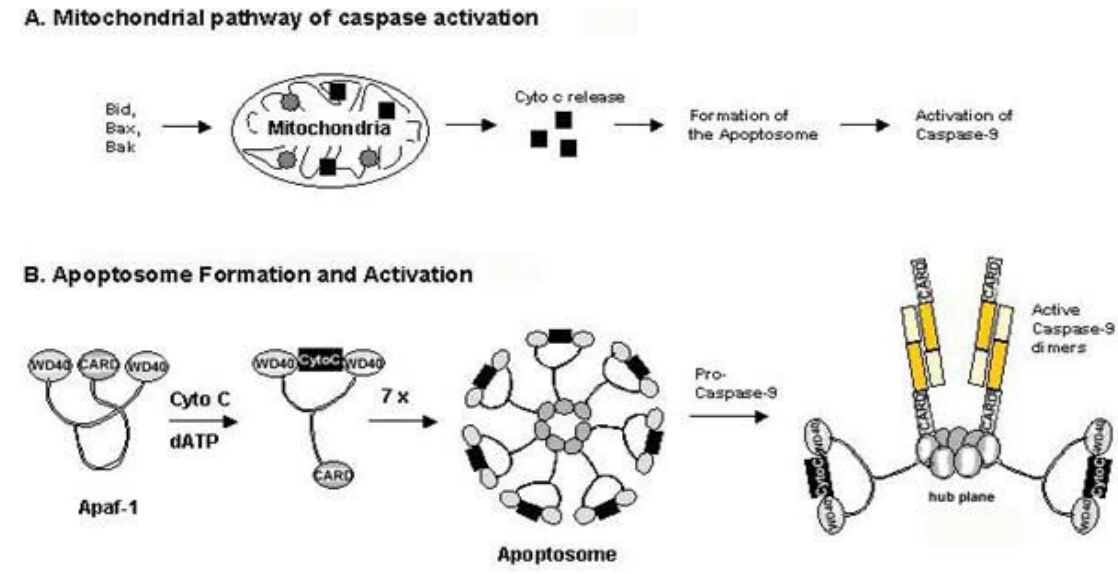


Figure 5: Mitochondria-mediated caspase activation at the apoptosome [57].

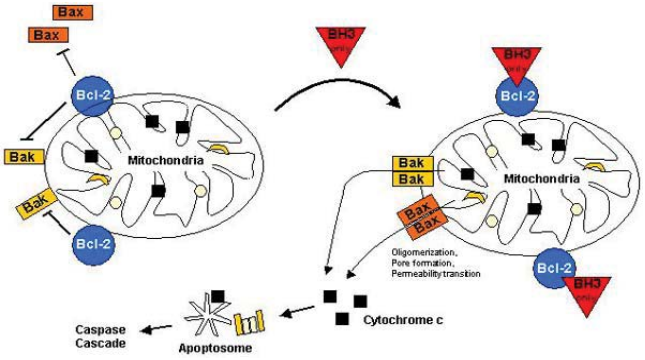


Figure 6: Regulation of apoptosis by the Bcl-2 family [82].

releasing cytochrome c, since they have a transmembrane domain that anchor them in the outer mitochondrial membrane. The anti-apoptotic Bcl-2 proteins prevent the release of cytochrome c from the mitochondria by forming heterodimers with the pro-apoptotic family members preventing them from forming pores [89]. Indeed, it is thought that mitochondrial permeability is determined by the ratio of pro-apoptotic and anti-apoptotic members of the Bcl-2 family in the membrane [78]. Bcl-2 heterodimerizes with Bax to prevent apoptosis, while Bax homodimers accelerate cell death and counteract the survival function of Bcl-2/Bax heterodimers, relative amount are more important than individual protein level to regulate apoptosis and susceptibility to a given apoptotic stimulus [89].

Minerals

Malnutrition is one of the major problems in children with cancer. The trace elements, vitamins and some fatty acids and amino acids are essential nutrients in that they cannot be synthesized or they cannot be produced in an adequate amount under normal conditions so they must be derived from the diet [90]. Severe weight loss and abnormally low concentrations of certain plasma proteins such as albumin have been recognized for a long time in patients with cancer, including children [91]. These changes might result from inadequate energy and protein intakes, as side effects associated with chemotherapy and/or the disease itself [92].

Zinc (Zn): Zinc is an essential mineral that is found in almost every cell. It stimulates the activity of approximately 100 enzymes catalyzing many reactions in body [93]; also it supports a healthy immune system [94,95].

1) Distribution

There are 2–4 grams of zinc distributed throughout the human body. Most zinc is in the brain, muscle, bones, kidney, and liver, with the highest concentrations in the prostate and parts of the eye [96]. Plasma zinc has a rapid turnover rate and it represents only about 0.l% of total body zinc content [97]. Zinc is widely distributed in a variety of foods particularly protein foods, such as milk, meat, fish, eggs, nuts, whole grains and legumes [98].

2) Functions

Zinc is found in nearly 100 specific enzymes , serves as structural ions in transcription factors and is stored and transferred in metallothioneins. It is typically the second most abundant transition metal in organisms after iron and it is the only metal which appears in all enzyme classes [99]. In proteins, Zn ions are often coordinated to the amino acid side chains of aspartic acid, glutamic acid, cysteine and histidine.

Zinc serves a purely structural role in zinc fingers, twists and clusters. Zinc fingers form parts of some transcription factors, which are proteins that recognize DNA base sequences during the replication and transcription of DNA. Each of the nine or ten Zn2+ ions in a zinc finger helps maintain the finger's structure by coordinately binding to four amino acids in the transcription factor. The transcription factor wraps around the DNA helix and uses its fingers to accurately bind to the DNA sequence [100]. Zinc stabilizes the molecular structure of cellular components and membranes and in this way contributes to the maintenance of cell and organ integrity. Zinc plays a central role in the immune system, affecting a number of aspects of cellular and humoral immunity [101].

Mechanisms underlying the relationship between zinc and immunity are still open to speculation. Zinc is necessary for the activity of some immunity mediators. This has been clearly shown for thymulin, a nonapeptidic hormone secreted by thymic epithelial cells, and requiring the presence of zinc for its biological activity. This peptide promotes T lymphocyte maturation, cytotoxicity and IL-2 production. Zinc is bound to thymulin in a 1:1 stoichiometry via the side chains of asparagine and the hydroxyl groups of the 2 serines. Thymulin activity, in vitro and in vivo, in both animals and humans, is dependent on plasma zinc concentrations such that marginal changes in zinc intake or availability affect thymulin activity [102]. Thymulin is detectable in the serum of zinc-deficient patients, but is not active. The binding of zinc to the peptide results in a conformational change that produces the active form of thymulin. The use of thymulin as an indicator of zinc deficiency has been suggested and the assay of serum thymulin activity with or without zinc addition in vitro may be used as a sensitive criterion for diagnosing mild zinc deficiency in humans. Zinc could also be critical for some cytokine activity. For instance, it has been demonstrated that the production or the biological activity of IL-1, IL-2, IL-3, IL-4, IL-6, IFNα and TNFα was affected by zinc deficiency [103].

3) Zinc deficiency

Zinc deficiency is usually due to insufficient dietary intake, but can be associated with malabsorption, chronic liver disease, chronic renal disease, sickle cell disease, diabetes, malignancy, and other chronic illnesses [104]. Symptoms of mild zinc deficiency are diverse. Clinical outcomes include depressed growth, diarrhea, impotence and delayed sexual maturation, alopecia, eye and skin lesions, impaired appetite, altered cognition, impaired host defense properties, defects in carbohydrate utilization, and reproductive teratogenesis. Mild zinc deficiency depresses immunity [97,105].

4) Supplementation

Nearly two billion people in the developing world are deficient in zinc. In children it causes an increase in infection and diarrhea, contributing to the death of about 800,000 children worldwide per year [103]. The World Health Organization advocates zinc supplementation for severe malnutrition and diarrhea. Zinc supplements help prevent disease and reduce mortality, especially among children with low birth weight or stunted growth [104]. However, zinc supplements should not be administered alone, since many in the developing world have several deficiencies, and zinc interacts with other micronutrients [105].

Copper (Cu)

1) Distribution

Copper is essential in all plants and animals. The human body normally contains copper at a level of about 1.4 to 2.1 mg for each kg of body weight.Copper is distributed widely in the body and occurs in liver, muscle and bone. Copper is transported in the bloodstream on a plasma protein called ceruloplasmin. When copper is first absorbed in the gut it is transported to the liver bound to albumin. Copper metabolism and excretion is controlled by the delivery of copper to the liver by ceruloplasmin, where it is excreted in bile. The copper content of foods is variable and depends on the copper content of the soil and on the copper loss or contamination throughout processing. Some types of hepatic cirrhosis cause hypocupremia because of the inability of the damaged liver to synthesize ceruloplasmin [106-108]. Serum or plasma levels of copper vary with age and are higher in adult women (80 to 155) than in men (70 to 140) μg/dL [109]

2) Functions

Divalent copper forms complexes with proteins, many of which are enzymes, a group of these enzymes constitute copper metalloenzymes with oxidase activity. In biological systems, copper has the ability to induce the synthesis of Metallothionein (MT) and is intermediate between cadmium and zinc in this activity. Approximately 50% of dietary copper is absorbed and about 1.5 to 3.0 mg/day of dietary copper has been determined to be safe and adequate [109].

3) Copper deficiency

Copper deficiency can occur through copper-deficient parenteral nutrition or as a result of gastric bypass surgery. Zinc competes with copper for absorption by the gut. Chronic excessive zinc consumption can cause copper deficiency (and is hence used to treat excessive copper levels as in Wilson's disease).

Because of its role in facilitating iron uptake, copper deficiency can often produce anemia-like symptoms. In humans chronic copper depletion leads to abnormalities in metabolism of fats, high triglycerides, non-alcoholic steatohepatitis (NASH), fatty liver disease and poor melanin and dopamine synthesis causing depression and sunburn. Food rich in copper should be eaten away from any milk or egg proteins as they block absorption, copper deficiency results in hypercholesterolemia and decreased antioxidant protection [109].

However, Wilson disease is a disease characterized by the excess hepatic deposition of copper and it produces oxidative stress and increases the risk of liver cancer [110]. Also elevation of serum copper was seen in cancer of the stomach, liver, biliary tract, lung and in leukemia [111].

Copper/zinc ratio

It is believed that zinc and copper compete for absorption in the digestive tract so that a diet that is excessive in one of these minerals may result in a deficiency in the other. It was found that the copper to zinc ratio was significantly higher in patients with lymphoma or acute and chronic leukemias compared to control subjects. A person at increased risk of one of these cancers should check blood levels of copper and zinc to rule out abnormalities and make adjustments accordingly. Since zinc and copper are antagonistic, and zinc deficiency is relatively common, supplemental zinc is often used to improve this ratio. Zinc helps block the absorption of copper and acts to remove accumulated copper from the body as well as prevent its accumulation [112,113].

Flowcytometric analysis

Flow cytometry is the most practiced member of a family of technologies known variously as automated, analytical and quantitative cytology. As the term implies, flow cytometry (FCM) is the measurement (-metry) of cellular (cyto-) properties (physical or chemical) as they are moving in a fluid stream (flow) through a stationary set of detectors [114].

The use of flow cytometry in the clinical laboratory has grown substantially in the past decade. This is attributable in part to the development of smaller, userfriendly instruments and a continuous increase in the number of clinical applications. Flow cytometry measures multiple characteristics of individual particles flowing in single file in a stream of fluid. Light scattering at different angles can distinguish differences in size and internal complexity, whereas light emitted from fluorescently labeled antibodies can identify a wide array of cell surface and cytoplasmic antigens. This approach makes flow cytometry a powerful tool for detailed analysis of complex populations

in a short period of time [115]. It is a method to quantify the expression and activities of a variety of proteins and enzymes for diagnostic and therapeutic purposes [116].

General principles: Flowcytometry measures optical and fluorescence characteristics of the single cells (or any other particle, including nuclei, microorganisms, chromosome preparations, and latex beads). Physical properties, such as size (represented by forward angle light scatter) and internal complexity (represented by right-angle scatter) can resolve certain cell populations. Fluorescent dyes may bind or intercalate with different cellular components such as DNA or RNA. Additionally, antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside cells. When labeled cells are passed by a light source, the fluorescent molecules are excited to a higher energy state. Upon returning to their resting states, the fluorochromes emit light energy at higher wavelengths. The use of multiple fluorochromes, each with similar excitation wavelengths and different emission wavelengths (or "colors"), allows several cell properties to be measured simultaneously. Commonly used dyes include propidium iodide, phycoerythrin, and fluorescein. Tandem dyes with internal fluorescence resonance energy transfer can create even longer wavelengths and more colors [117-119].

Inside a flow cytometer, cells in suspension are drawn into a stream created by a surrounding sheath of isotonic fluid that creates laminar flow, allowing the cells to pass individually through an interrogation point. At the interrogation point, a beam of monochromatic light, usually from a laser, intersects the cells. Emitted light is given off in all directions and is collected via optics that direct the light to a series of filters and mirrors that isolate particular wavelength bands. The light signals are detected by photomultiplier tubes and digitized for computer analysis (Figure 7). The resulting information usually is displayed in histogram or two dimensional dot-plot formats [120].

When a cell passes through a laser beam, it scatters light. That light is measured on a detector and the resulting signal can provide information about the cells. Forward angle Scatter (FS) is a measure of cell size. Side scatter (90_ scat) is a measure of cellular components or granularity. In the dotplot of forward-versus-side scatter, human white blood cells can be differentiated. Figure 8 shows the separation of lymphocytes, monocytes, and granulocytes.

Clinical application of flowcytometry

Diagnosis of hematological malignancies

The identification and quantitation of cellular antigens with fluorochrome labeled monoclonal antibodies (immunophenotyping) is one of the most important applications of the flow cytometer. Immunophenotypic analysis is critical to the initial diagnosis and classification of the acute leukemias, chronic lymphoproliferative diseases, and malignant lymphomas since treatment strategy often depends upon antigenic parameters [121,122]. In addition, immunophenotypic analysis provides prognostic information not available by other techniques, provides a sensitive means to monitor the progress of patients after chemotherapy or bone marrow transplantation, and often permits the detection of minimal residual disease. Flow cytometric analysis of apoptosis, multidrug resistance, leukemia-specific chimeric proteins, cytokine receptors and other parameters may provide additional diagnostic or prognostic information in the near future [123].

Subjects and Methods

Subject groups

Group (1):-

Ten normal children of matched age and sex with the patient group were taken as control group.

Group (2):-

Twenty five pediatric patients newly diagnosed of acute lymphocytic leukemia attending the outpatient clinic of the pediatric oncology unit - National Cancer Institute – Cairo University, were enrolled in the study.

Group (3):-

Twenty patients of group 2 (5 patients were missed) received treatment according to the treatment protocol of the National Cancer Institute – Cairo University (Total XV) and served as Group 3.

Age Range: 3-18 years **Sex:** 18 M and 7 F

Informed medical consent was obtained from the parents after full explanation of the aim and procedures of the study.

Diagnostic criteria

Diagnosis was carried on according to the WHO classification of tumor regarding the B and T-cell Neoplasms. The cytogenetic profiles, genotypes and immunophenotype of the malignant cell have had considerable impact on prognostic stratification with recognition of low and high risk groups. These stratifications have resulted in more specific therapeutic regimens with higher remission rates for unfavorable prognostic groups [124].

All patients were subjected to the diagnostic work up which included history, clinical and radiological (chest X-ray) examination, laboratory evaluation including complete blood picture, blood chemistry, bone marrow aspirate examination, CSF examination, determination of leukemic surface markers by immunophenotyping using flowcytometry and chromosomal pattern detection (Karyotyping) [125]

Common features of ALL include:

-Patients presented with persistent fever and pallor, anemia, thrombocytopenia, blasts in the peripheral blood and bone marrow.

-Bone marrow aspirate showed negative sudan black stain which confirm ALL diagnosis whereas positive sudan black stain indicates AML (acute myelogenous leukemia).

Chemicals:

Monoclonal antibodies for CD_{95} and IgG were purchased from Dako Group (Glostrup, Denmark).

ELISA kit for human Bcl-2 was supplied from DRG International, Inc., (East Mountainside,NJ).

Experimental design

Freshly taken blood samples were used for determination of CD_{95}, as a marker of apoptosis, using Moflow Flowcytometer and for determination of Bcl-2 using ELISA technique.

Serum was separated from another blood sample and used for determination of zinc and copper levels using Atomic Absorption Spectrophotometer.

Before treatment and during the maintenance phase (after six months from the beginning of the treatment) routine laboratory

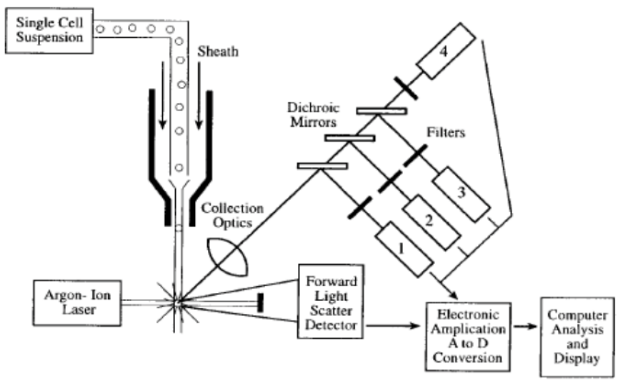


Figure 7: Schematic diagram of a flow cytometer.
(A single cell suspension is hydrodynamically focused with sheath fluid to intersect an argon-ion laser. Signals are collected by a forward angle light scatter detector, a sidescatter detector (1), and multiple fluorescence emission detectors (2–4). The signals are amplified and converted to digital form for analysis and display on a computer screen [115].)

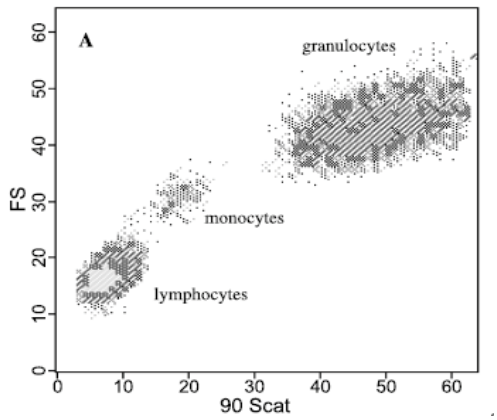


Figure 8: Dot plot of human white blood cells [121].

investigation results were obtained from the patients medical sheets including total leukocytic count, platelet count , hemoglobin concentration and bone marrow blast count. Regarding the normal group CBC and hemoglobin concentration only were performed.

Procedure for assessment of blood parameters

One ml of blood was delivered into a vacutainer EDTA tube for determination of total leukocyte count (TLC) and hemoglobin concentration using automated cell counter (Sysmex-k 1000 Japan).

Blood films were assessed and stained by Leishman's stain for morphological examination of total leukocyte count, blast cells and platelets [126].

Treatment protocol used for children with acute lymphocytic leukemia (Pediatric National Cancer Institute Cairo University) Total XV

Patients received the standard pediatric ALL chemotherapy protocol applied at the NCI, Cairo University [127]. The protocol is composed of three phases:

The first induction of remission phase (6-7 weeks) is composed of the administration of:-

1) Vincristine (VCR) 1.5 mg/m^2 I.V 4 doses (max 2 mg) in day 1, 8, 15, 22.

2) Doxorubicin (DOX) IV, 25 mg/m^2 given on days 1, 8.

3) Prednisone 40 mg/m^2 PO (t.i.d) started on day 1 to day 28.

4) L-asparaginase 10,000 I.M IU/m^2 alternating days for 9 doses.

5) 6-Mercaptopurine (6-MP) 60 mg/m^2 daily PO for 2 weeks day 22- 35.

6) Ara-C 75 mg/m^2 I.V. in days 23-26 and 30-33.

7) Cyclophosphamide (Cyclo) 1000 mg/m^2 over 15 – 45 min day 22.

8)Trimethoprime + Sulphamethoxazole 5-10 mg/kg/day or 150 mg/m^2 /day in divided doses every 12 hours three days per week starting from day 15.

9)Triple intrathecal (Ith): **Methotrexate** 8-12 mg/m^2, **Hydrocortisone** 16-24 mg/m^2 , **Ara-C** 24-36 mg/m^2 (dose adjusted according to age) in day 1, 15 and some cases may need additional triple intrathecal in days 8, 22 according to results of CSF examination (blasts in CSF).

N.B CSF examination done in day 1, 8, 15, 22.

Bone marrow examination for re-evaluation was done on day 43 to determine remission status. Patients who achieved complete remission were promoted to:-

The second phase of therapy (consolidation) (8 weeks) and were offered high dose **Methotrexate (HD-MTX)** IV 5 g/m^2 for high/ standard risk patients and 2.5 for low risk patients over 24 hours infusion at day 1, 15, 29, 43.

6-MP 50 mg/m^2 daily for 8 weeks.

Triple intrathecal with CSF in days 1, 15, 29, 43.

Ca leucovorin 15 mg/m^2 for high risk and 10 mg/m^2 for low risk patients is given as antidote for methotrexate toxicity.

The third continuation (maintenance) phase is based on using different drug combinations given on weekly bases for a total of 120 weeks.

Dexamethasone (DEX): 12 mg/m^2 (t.i.d) for 5 days given in week 1, 4, 7, 9, 14, 17, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68, 72, 76,80, 84, 88, 92, 96, 100.

Vincristine (VCR): 2 mg/m^2 I.V on day 1 given in weeks 1, 4, 7, 8, 9, 11,14, 17, 18, 19, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68,72, 72, 76, 80, 84, 88, 92, 96, 100.

Doxorubicin (DOX): 30 mg/m^2 I.V on day 1 given in weeks 1, 4, 7, 8, 11, 14.

6-Mercaptopurine (6-MP): 50 mg/m^2 PO daily till week 20 then 75 mg/m^2 from week 21 till end of treatment.

L-Asparaginase (ASP): 25,000 IU/m^2 I.M on day 1 till week 19.

Triple intrathecal (Ith): in weeks **7,** 12, 17, 24, 28, 32, 36, 40, 44, 48.

Methotrexate (MTX): 40 mg/m^2 I.V or I.M on day 1 in weeks 21, 22, 25, 26, 29, 30, 33, 34, 37, 38, 41, 42, 45, 46, 49, 50, 53, 54,57, 58, 61, 62, 65, 66, 69, 70, 71, 73, 74, 75, 77, 78, 79,81, 82, 83, 85, 86, 87, 89, 90, 91, 93, 94, 95, 97, 98, 99,101 then weekly till the end of treatment.

Cyclophosphamide (Cyclo): 300 mg/m^2 IV or I.M on day 1 given every 4th week starting from week 23 till week 67.

Ara-C: 300 mg/m^2 IV on day 1 given every 4th week starting from week 19 till week 67.

First reinduction done in weeks 7, 8, 9.

Second reinduction done in weeks 17, 18, 19.

N.B

Reinduction consist of Dex 8 mg/m^2 daily (t.i.d) PO day 1-8 and day 15-21, VCR 1.5 mg/m^2/week – max 2 mg day 1, 8, 15, high dose Ara-C 2 g/m^2 IV days 15, 16.

Triple intrathecal consists of Methotrexate 8-12 mg/m^2, Hydrocortisone 16-24 mg/m^2 , Ara-C 24-36 mg/m^2 (dose adjusted according to age).

Bone marrow aspirate examination was done in day 1, 15, 22, 43 in induction phase and in week 7, 48, 120, 146 in continuation phase.

Methods

Determination of CD_{95}% using Flowcytometer:-

Principle:-

Flowcytometry is a method of measuring cell characteristics as they flow through special detectors while being illuminated by an intense laser beam. Cells to be analyzed by this system have to be prepared into single cell suspension; hence they might be forced to flow within a fluid sheath while they are interrogated by the laser beam. Every cell entering the beam will scatter light in all directions. The light scattered in the forward direction will be proportional to the size of the cell whereas the light scattered at 90 degree angle will provide information of its intracytoplasmic granularity [128].

Expression of cell surface antigens on human lymphocytes can be analyzed by flowcytometry according to the following methodology:-

Direct immunofluorescence against T subsets using flow cytometry and conjugated monoclonal antibodies that are labeled with Fluorescin Isothiocyanate (FITC).

Detection of cell expression of CD_{95} receptor molecules was done using flowcytometer Moflow Dako cytomation. Flow cytometry analysis operation started by Laser alignment using fluorochrome labeled microspheres (Immuno-check Beads, Coulter Co. Hialeah FL. USA). Then single colour immunoflourscence protocol was defined. Sample acquisition was done through running 10.000 events, then a gate was set to include the population of interest, [129]. Data analysis was done to determine percentage positivity for the antibody [130].

Reagents:

1- Lysing solution:

2.25 g NH_4Cl, 0.25 g $KHCO_3$ and 0.00925 g EDTA-disodium salt were dissolved in 200 ml distilled water and used for lysing RBCs in the specimen.

2- Phosphate buffered saline (PBS):

1.81 g Na_2HPO_4 , 0.51 g NaH_2PO_4 and 7.41 g NaCl were dissolved in one liter distilled water, pH = 7.4.

3- Monoclonal antibody for IgG1 (Anti IgG1) conjugate FITC:

It is an antibody of the same isotype of the test antibody (CD_{95}), to determine whether fluorescence that is observed is due to non-specific binding of the fluorescent antibody.

4- Monoclonal antibody for CD_{95} (Anti CD_{95}) conjugate FITC.

5- Standardized vacutainer EDTA tubes.

Procedure:

Fresh 2.5 ml whole blood was taken in a vacutainer EDTA tube.

Ten µl monoclonal antibody (Anti IgG) conjugate FITC was added to 100 µl of whole blood and served as a positive control which was used with each batch of the sample, test tube number (1).

Ten µl monoclonal antibody (Anti CD_{95}) conjugate FITC was added to 100 µl of whole blood, test tube number (2).

Test tubes were incubated for 30 min at 4°C in dark.

4 ml lysing solution were added to both test tubes and left for 7 min at 4°C in dark.

Both test tubes were centrifuged at 1800 rpm. for 10 min.

The supernatant was decanted and the resulting pellets was washed three times using PBS.

Each pellet was suspended in 200 µl PBS and subjected to flowcytometric analysis.

Quantitative determination of Bcl-2 protein in cell lysate using ELISA:-

Bcl-2 assay was performed according to the method of [131].

Principle

Mouse monoclonal antibody to human Bcl-2 immobilized on a microtiter plate to bind the Bcl-2 in the standards or samples. After 1 hour incubation, the excess sample or standard is washed out and a biotinylated monoclonal antibody to Bcl-2 is added. This antibody binds to the Bcl-2 captured on the plate. The excess antibody is washed out after 1 hour incubation and streptavidin conjugated to Horseradish peroxidase is added, which binds to the biotinylated monoclonal Bcl-2 antibody. Excess conjugate is washed out and substrate is added. The enzyme reaction is stopped after 30 minutes and the color generated is read at 450 nm. Quantitation is achieved by the construction of a standard curve using known concentrations of Bcl-2.

Material

Human Bcl-2 microtiter plate (96 wells)

Wells coated with mouse monoclonal antibody specific for human Bcl-2 protein.

Bcl-2 EIA antibody

A solution of biotinylated monoclonal antibody to Bcl-2

Assay buffer

Tris buffered saline containing detergents.

Bcl-2 EIA conjugate

A solution of streptavidin conjugated to Horseradish peroxidase.

Wash buffer concentrate

Tris buffered saline containing detergents.

Human Bcl-2 standard

Two vials each contain 1,200 pg of lyophilized recombinant human Bcl-2.

TMB substrate

A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide.

Stop solution : 1N HCl.

Cell lysis buffer 1 mM EDTA, 6 M Urea, 0.5% Triton X-100, 0.005% Tween 20 in phosphate buffered saline.

Plate sealers: used to cover the plate during incubation.

Reagent preparation

Wash buffer

The wash buffer was prepared by diluting 50 mL of the supplied concentrate with 950 mL of deionized water.

Assay buffer plus protease inhibitors

Protease Inhibitor Cocktail (PIC) provided from Sigma (Sigma-Aldrich®) was added to the buffer (0.5 mL/mL) immediately prior to use. This modified Assay Buffer was used for standard reconstitution and all sample and standard dilutions to ensure optimal integrity of human Bcl-2.

N.B: PIC is a mixture of protease inhibitors with broad specificity for the inhibition of serine, cysteine, aspartic acid proteases and aminopeptidases.

Human Bcl-2 standards

The lyophilized human Bcl-2 Standard is allowed to warm to room temperature. 1 mL of Assay Buffer plus Inhibitors was added to the lyophilized Bcl-2 vial and vortexed. Vortexed again prior to use. The vial was labeled standard #1. Six 12 x 75 mm polypropylene tubes are labeled #2 through #7.

500 µL of Assay Buffer plus Inhibitors was pipetted into tube #2 through #7.

500 µL of reconstituted standard #1 was added to tube #2 and vortexed.

500 µL of tube #2 was added to tube #3 and vortexed thoroughly. This was continued for tubes #4 through #7.

The concentrations of human total Bcl-2 in standard vial#1 and tubes #2 through #7 will be 1,200, 600, 300, 150, 75, 37.5 and 18.8 pg/mL, respectively. Assay buffer plus inhibitors was used as standard 0 pg/mL.

Cell lysis buffer Sigma Protease Inhibitor Cocktail (PIC) (0.5 mL/mL) was added to the buffer prior to use.

Sample preparation

Blood samples were collected on vacutainer EDTA tubes, centrifuged at 1,500 rpm for 5 minutes and the supernatant was discarded, the cell pellets were re-suspended and washed with PBS, cells were pelleted at 1,500 rpm for 5 minutes and the supernatant was discarded, the cell pellets were re-suspended with modified cell lysis buffer, vortexed and were placed on ice for 5 minutes.

The lysates were vortexed and centrifuged at 16,000 rpm for 15 minutes and the supernatants separated, divided into several aliquots and stored frozen at -70°C for further analysis.

Assay procedure

100 µL of Assay Buffer plus protease inhibitors were pipetted into the blank and S0 (0) pg/mL standard wells.

100 µL of standards and samples were pipetted into the appropriate wells.

The plate was tapped gently, sealed and incubated at room temperature on a plate shaker (Varishaker- British) for 1 hour at ~500 rpm.

The contents of the wells were discarded and washed by adding 400 µL of wash buffer to each well. Washing was repeated 3 more times for a total of 4 washes. After the final wash, the plate was firmly tapped on a lint free paper towel to remove any remaining wash buffer.

100 µL of the biotinylated monoclonal Bcl-2 antibody was pipetted into each well, except the blank.

The plate was sealed and incubated at room temperature on a plate shaker for 1 hour at ~500 rpm.

The contents of the wells were discarded and washed by adding 400 µL of wash buffer to each well. Washing was repeated 3 more times for a total of 4 washes. After the final wash, the wells were firmly tapped on a lint free paper towel to remove any remaining wash buffer.

100 µL of the conjugate was added to each well, except the Blank.

The plate was sealed and incubated at room temperature on a plate shaker for 30 minutes at ~500 rpm.

The contents of the wells were discarded and washed by adding 400 µL of wash solution to each well. Washing was repeated 3 more times for a total of 4 washes. After the final wash, the wells were firmly tapped on a lint free paper towel to remove any remaining wash buffer.

100 µL of Substrate Solution was pipetted into each well.

The wells were incubated for 30 minutes at room temperature on a plate shaker at ~500 rpm.

100 µL Stop Solution was pipetted to each well.

The absorbance was read against blank using microplate reader (MRX- British) at 450 nm with correction at 590 nm (reference wavelength).

Calculation of results

Linear graph paper was used to plot the optical density versus Bcl-2 concentration in each standard, the concentration of Bcl-2 in the samples was determined from the standard curve (Figure 9).

Determination of zinc and copper by Atomic Absorption Spectrophotometry:-

The determination of zinc and copper by atomic absorption spectrophotometry is done according to the method of [132] and as they are so similar they are estimated together in the same dilution, but at different wavelengths. The absorption for zinc and copper are markedly free from interference effects.

The assessment was done using Perken-Elmer 2380 Atomic absorption spectrophotometer.

Procedure:-

1- Preparation of stock standard solutions

Zinc stock standard solution (200 µg of Zn^{2+} /ml):-

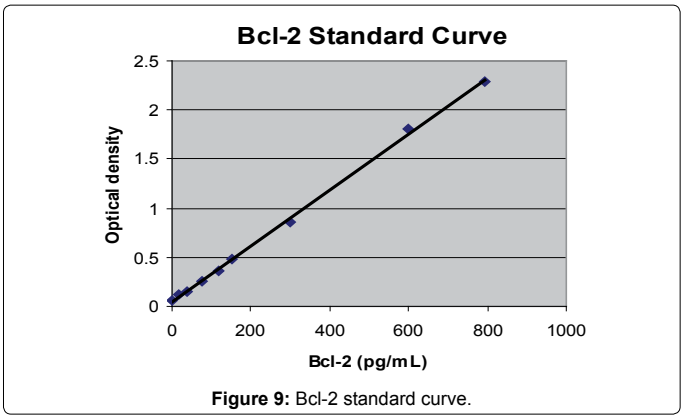


Figure 9: Bcl-2 standard curve.

0.176 gm of $ZnSO_4.7H_2O$ was dissolved in deionized water then 2 drops of conc. H_2SO_4 were added and diluted to 200 ml H_2O.

Copper stock standard solution (1000 µg of Cu^{2+} /ml):-

0.3928 gm of $CuSO_4.5H_2O$ was dissolved in deionized water and diluted to 1000 ml. This solution contains 1000 µg of Cu/ml. 10 ml of stock copper were diluted to 100 ml. This solution contains 100 µg of Cu/ml.

2- Preparation of working standard solutions

Zinc working standard solution:-

It was prepared by taking 0.5, 1.0, 1.5 and 2.0 ml of stock zinc standard solution and diluting each to 200 ml with deionized water to provide working standard solutions of 50, 100, 150 and 200 µg Zinc / 100ml, respectively.

Copper working standard solution:-

It was prepared by adding 1, 2, 3 and 4 ml of diluted copper stock solutions and diluting each to 100 ml to provide working standard solutions of 100, 200, 300 and 400 µg of copper / 100 ml, respectively.

Recommended instrument settings

Spectrophotometer was adjusted to wavelength 324 nm for Cu and 213 nm for Zn, the slit width was kept at 0.5 nm for both Cu and Zn, the current lamp was adjusted at 5 mA for Cu and 10 mA for Zn while the fuel system was composed of air-acetylene with air pressure adjusted to 20 lb/in^2 and acetylene flow to 5 lb/in^2.

Technique

One ml of serum samples was mixed with 4 ml of deionized water.

Working standard solutions of zinc and copper were prepared as previously described.

The Perken-Elmer 2380 Atomic absorption spectrophotometer was operated according to the standard instrument conditions for zinc and copper.

The blank and the different working standards for zinc and copper then the unknown samples were aspirated.

Serum zinc and copper concentrations were calculated from the corresponding calibration curve (Figure 10).

The statistical analysis

Statistical analysis using SPSS (Statistical package for social science) version 12, software package for data analysis [133] was done.

The quantitative data were presented in the form of mean, and Standard Error (SE) and the following tests were used:-

1. Test of significance:

One way ANOVA was used to compare between the means followed by Tukey's test. P value < 0.05 is considered to be significant.

2. Correlation coefficient:

Pearson correlation coefficient was done between each two variables to study the relation between them.

Results

Blood parameters and bone marrow blast count in all studied groups.

As shown in Table 6 and Figure 11a-11d), blood parameters and bone marrow blast count for children with Acute Lymphocytic Leukemia (ALL) at presentation showed a significant elevation in TLC and bone marrow blast count while hemoglobin and platelet count were significantly decreased when compared to controls.

Administration of the treatment protocol resulted in normalization in TLC whereas bone marrow blast count has been significantly reduced; a significant elevation of hemoglobin and platelet count has been demonstrated when compared to values before treatment.

Data shown in Table 7, Figure 12a and 12b) revealed that in newly diagnosed ALL children, CD_{95}% was significantly decreased and Bcl-2 concentration was significantly increased when compared to controls.

After receiving treatment CD_{95}% showed significant elevation while Bcl-2 concentration showed significant decrease on comparing to values before treatment.

Serum copper and zinc levels and Cu/Zn ratio in all studied groups

Table 8 and Figure 13a-13c) show that in newly diagnosed ALL children serum Cu level and Cu/Zn ratio showed significant elevation and serum Zn level showed significant decrease when compared to controls.

After administration of the treatment protocol, serum Zn level was normalized and Cu/Zn ratio was significantly decreased while serum Cu level showed no significant change when compared to values before treatment.

Correlation between different parameters in ALL children after treatment

By performing Pearson Correlation between parameters in newly diagnosed ALL children, negative significant correlations was found between CD95 and Bcl-2 and between serum Cu and Zn levels.

Also, it was found that CD95 is negatively correlated with TLC and bone marrow blast count while positively correlated with hemoglobin and platelet count, but Bcl-2 was oppositely correlated to the same parameters by being positively correlated with TLC and bone marrow blast count and negatively correlated with hemoglobin and platelet count. (Table 9, Figures 14 and 15).

TLC is negatively correlated with hemoglobin and platelet count while positively correlated with bone marrow blast count.

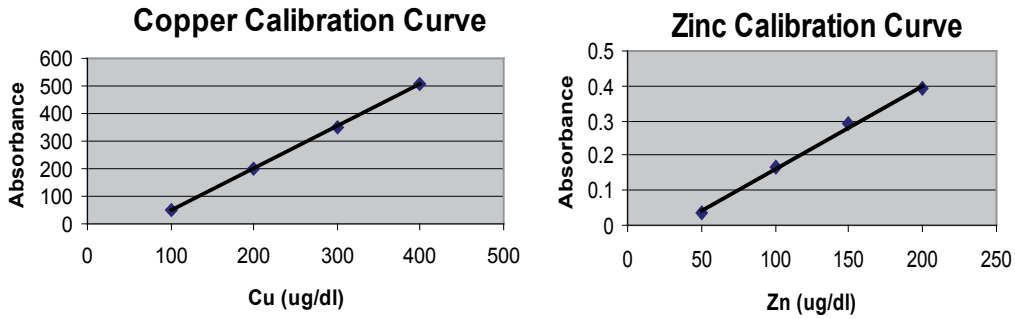


Figure 10: Cu and Zn calibration curves.

parameters / Groups	Blood Picture			Bone marrow Blasts%
	TLC x 10^3 (mm^3)	Hemoglobin (gm%)	Platelets x 10^3 (mm^3)	
Group1 (Control)	6.1 ± 0.52	11.9 ± 0.37	252.7 ± 17.13	0
Group 2 (ALL before treatment)	212.16 ± 5.8 [a]	6.412 ± 0.25 [a]	43.4 ± 4.5 [a]	89.16 ± 1.22 [a]
Group 3 (ALL after treatment)	9.88 ± 1.26 [b]	8.56 ± 0.51 [a,b]	113.3 ± 10.09 [a,b]	76.42 ± 2.85 [a,b]

Table 6: Blood parameters and bone marrow blast count in all studied groups (Values are means ± S.E.M. Group 1 (n)=10, Group 2 (n)=25, Group 3 (n)=20, [a] $P < 0.05$ compared to control group and [b] $P < 0.05$ compared to ALL cases before treatment.)

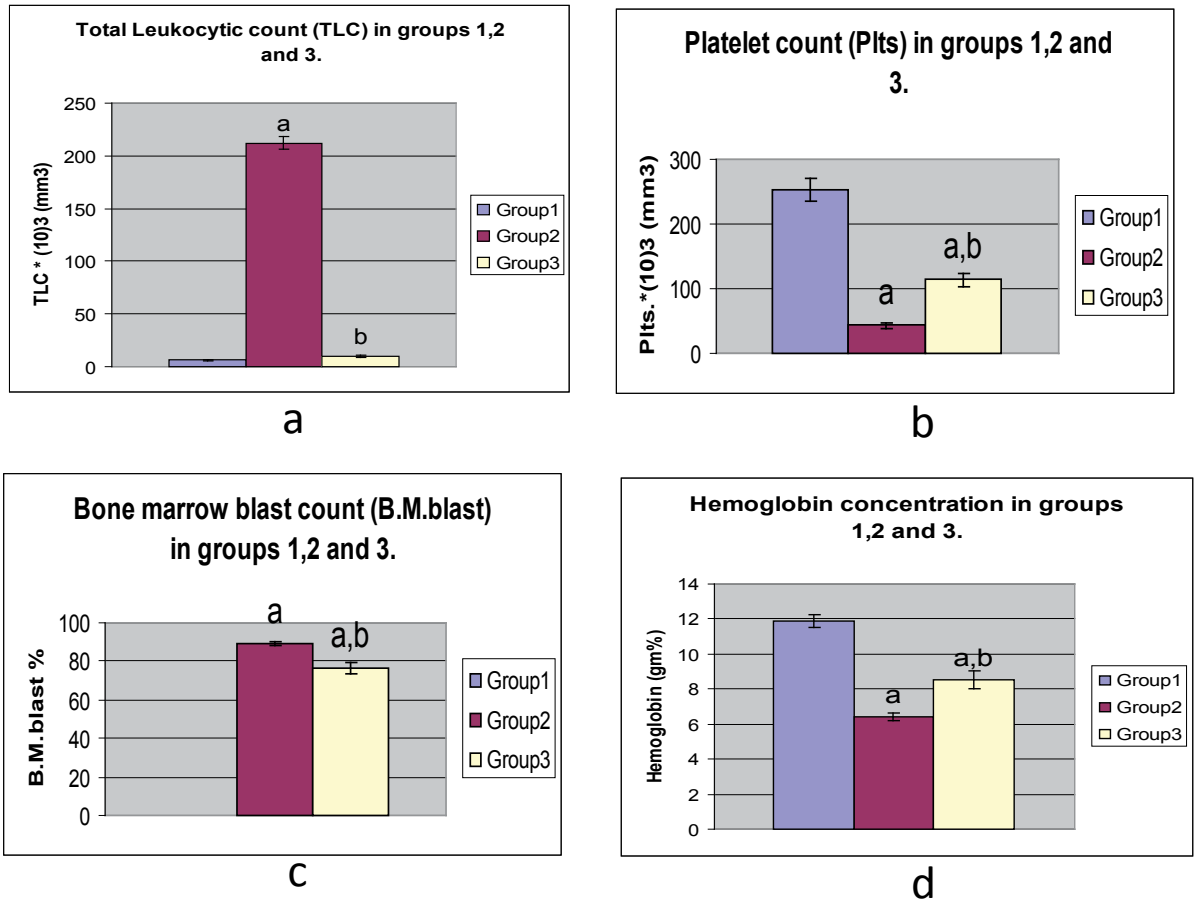


Figure 11(a-d): Total Leukocytic Count (TLC) (a), platelet count (b), bone marrow blast count (c) and hemoglobin concentration (d) in control (Group 1), ALL children before treatment (Group 2) and ALL children after treatment(Group 3).

a: significantly different compared to control group, b: significantly different compared to ALL cases before treatment.

Parameters / Groups	CD_{95}%	Bcl-2 pg/ml
Group1 (Control)	0.55 ± 0.08	67.2 ± 8.93
Group 2 (ALL before treatment)	0.06 ± 0.005 [a]	707.95 ± 29.6 [a]
Group 3 (ALL after treatment)	0.27 ± 0.05 [a,b]	272.2 ± 22 [a,b]

Table 7: CD_{95} % and Bcl-2 concentration in all studied groups (Values are means ± S.E.M. Group 1 (n)=10, Group 2 (n)=25, Group 3 (n)=20, [a] $P < 0.05$ compared to control group and [b] $P < 0.05$ compared to ALL cases before treatment.)

Correlation between different parameters in ALL children after treatment

By performing Pearson Correlation between parameters in ALL children receiving the treatment protocol, negative significant correlation was found between CD95 and Bcl-2.

Also, it was found that CD95 is negatively correlated with bone marrow blast count while positively correlated with platelet count, on the other hand Bcl-2 is positively correlated with bone marrow blast count and negatively correlated with platelet count (Table 10, Figures 16 and 17).

Discussion

Acute lymphocytic leukemia (ALL), also called acute lymphoblastic leukemia is a quickly progressing disease in which too many immature white blood cells (lymphoblasts) are found in the blood and bone marrow [134]. Leukemia is the most common pediatric cancer and is the most common cause of disease-related death in childhood. Acute lymphoblastic leukemia accounts for 80% of pediatric leukemias [135]. Childhood cancer incidence is around 120-150/million/year in subjects with age range (0-14) years old and the number of new cases/year worldwide is approximately 250,000. About 3,000 children in the United States and 5,000 children in Europe are diagnosed with ALL each year. The peak incidence of ALL occurs between age 2 and 5 years. The incidence of ALL is higher among boys than girls, and this difference is greatest among pubertal children, in the United States, ALL is more common among white children than black children, and this is related probably to geographical variation in biology or different environmental exposure [136].

The present study is concerned with certain markers of acute lymphocytic leukemia in children that are useful in diagnosis and follow up of the disease. The studied markers include CD_{95}% and Bcl-2 concentration as markers of apoptosis as well as serum copper and zinc levels and consequently Cu/Zn ratio to demonstrate whether the nutritional status of children affect the disease progression or if the disease itself has an effect on nutritional status of patients.

The current results showed that Total Leukocytic Count (TLC) and bone marrow blast count were significantly higher while hemoglobin concentration and platelet count were significantly lower in newly diagnosed cases when compared to controls. Similar results are reported by [137] who indicated that in newly diagnosed patients with ALL, bone marrow morphology always shows replacement by blasts (immature blood cells) with decreased or absence of normal hematopoietic elements, a finding that could explain the decreased hemoglobin concentration in newly diagnosed patients in our study.

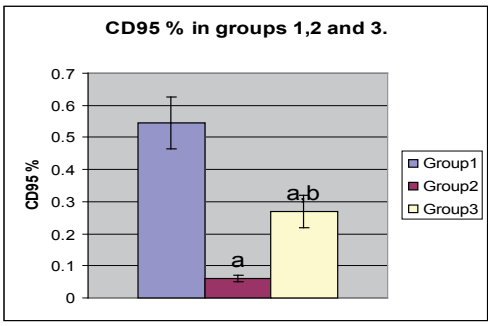
A study done by Kantarjian and Faderl [138] showed that leukemia cells crowd out the normal blood-making cells in the bone marrow which results in shortage of normal blood cells and this is responsible for most signs and symptoms of ALL. Moreover, Conter et al. [136] found that the degree of leukocyte elevation at diagnosis is a very strong predictor of prognosis in ALL, also decreased platelet count in usually present at diagnosis. They also demonstrated that more than 75% of patients presents with anemia.

After receiving treatment, TLC was normalized whereas bone marrow blast count was significantly decreased. Regarding hemoglobin concentration and platelet count, they showed significant rise reflecting a good response for the treatment protocol. Same finding was reported by Schwartz et al. [139] who demonstrated that higher dexamethasone doses improve the initial response to therapy and with increasing dexamethasone dose, the percent reduction in marrow blasts increased. Moreover, it has been reported that L-asparaginase has an inhibitory effect on lymphocyte blastogenesis [140]. The folate antagonist, methotrexate (MTX), is one of the most active antileukemic drugs accounting in part for the success that has been achieved in our study. Intracellularly, methotrexate is retained in the form of polyglutamates. Methotrexate polyglutamates (MTXPGs) in nonleukemic cells contain predominantly 3 glutamyl residues ($MTXGlu_3$). In contrast, lymphoblasts from children accumulate predominantly MTXPGs with 5 glutamyl residues ($MTXGlu_5$). MTXPGs, particularly those with more than 3 glutamyl residues (long chain MTXPGs), are retained in cells for long intervals, inhibiting DNA synthesis. MTXPGs are equipotent with MTX as inhibitors of Dihydrofolate Reductase (DHFR). Indeed their formation in cells appears critical to MTX cytotoxicity [141].

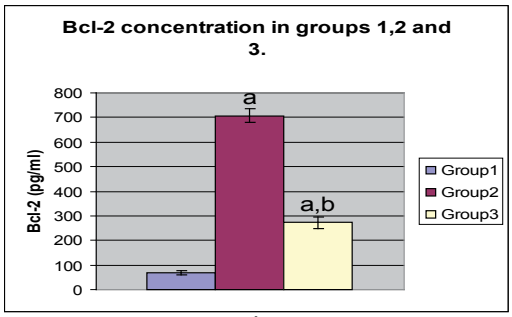
In the current study, we investigated the role of apoptosis and activation of apoptosis signaling molecules in the treatment of ALL. Apoptosis is a physiological mechanism that eliminates excessive, damaged or unwanted cells; it is a highly regulated pathway important for maintaining homeostasis in multicellular organisms. It can be initiated through various signals via the extrinsic pathway which involves death receptors, or via the intrinsic pathway which is initiated by intracellular damage and involves the mitochondria and release of cytochrome c to further activate caspases [142]. It has been shown that deregulation of apoptotic cell death can disrupt the delicate balance between cell proliferation and cell death and contributes to the pathogenesis of a number of human diseases including cancer, viral infections, autoimmune diseases, neurodegenerative disorders, and AIDS (acquired immunodeficiency syndrome) [143]. One of the effector molecules in apoptosis is Fas antigen. Crosslinking of Fas by its ligand (Fas L) or agonistic anti Fas antibodies induces apoptosis of cells expressing Fas on the membrane by triggering cascade of caspases, Fas (APO-1/CD_{95}) is a 45-kDa membrane protein which regulates apoptosis in many lymphoid cell types [144]. Ligation of the CD_{95} death receptor in the plasma membrane leads to a direct caspase 8-dependent activation of downstream effector caspases, leading to apoptotic cell death [145].

In the present study, results revealed that CD_{95}% in blood of newly diagnosed ALL cases is significantly low when compared to controls while after treatment it showed significant increase. Consistent with our results, Tamiya et al. [146] found defects in expression of Fas antigen on leukemia cells due to mutations in Fas genes of these cells. Moreover, Muschen et al. [147] revealed that CD_{95}/Fas (receptor-ligand system), is mutated and downregulated in lymphoid and solid tumors and loss of its function is associated with resistance to drug induced cell death. This finding was supported by Inaba et al. [148].

Molecular analysis of Fas transcripts in leukemic cells from infants with ALL revealed no detectable expression of full-length Fas mRNA after a single round of reverse transcription and polymerase chain



a



b

a: significantly different compared to control group,

b: significantly different compared to ALL cases before treatment.

Figure 12: (a,b): CD95 % (a) and Bcl-2 concentration (b) in control (Group 1), ALL children before treatment (Group 2) and ALL children after treatment (Group 3).

Parameters / Groups	Cu µg/dl	Zn µg/dl	Cu/Zn
Group 1 (Control)	134.3 ± 5.52	99.43 ± 4.41	1.39 ± 0.12
Group 2 (ALL before treatment)	296.4 ± 6.05 [a]	67.4 ± 3.5 [a]	4.7 ± 0.3 [a]
	292.5±15.99 [a]		2.87 ± 0.23 [a,b]

Table 8: Serum copper and zinc levels and Cu/Zn ratio in all studied groups (Values are means ± S.E.M. Group 1 (n)=10, Group 2 (n)=25, Group 3 (n)=20, [a] P < 0.05 compared to control group and [b] P < 0.05 compared to ALL cases before treatment.)

reaction amplification (RT-PCR). However, a more sensitive nested RT-PCR analysis revealed alternatively spliced Fas transcripts in three of five infants (60%) with the remaining two infants showing no detectable Fas mRNA expression. The primary sequence variation of Fas mRNA seen in the samples was a previously described variant lacking exon 6 encoding soluble Fas. However, several novel alternatively spliced Fas transcripts in the ALL cells have been detected. These variants lack intact transmembrane domains and thus are predicted to encode soluble Fas variants [149].

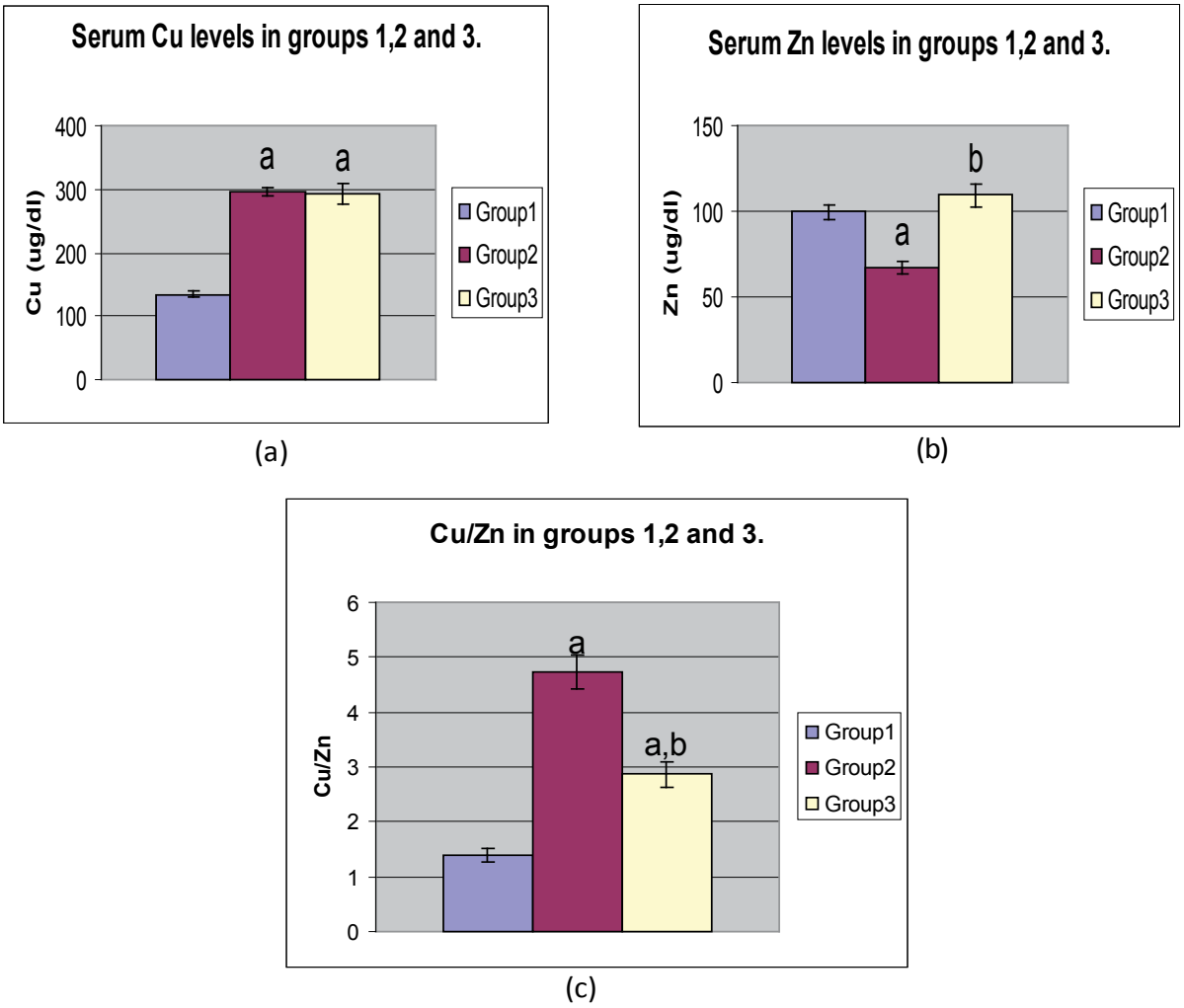
Chemotherapeutic agents exert their action by multiple mechanisms, including intercalation into DNA, inhibition of DNA replication, cell membrane damage, or free radical generation [150,151]. Although the primary intracellular targets of drug action are rather distinct, it has become evident that drug-induced cytotoxicity ultimately converges on a common pathway, causing apoptosis. Cells exposed to anticancer drugs display apoptotic alterations, such as cell shrinkage, chromatin condensation, and internucleosomal DNA fragmentation [152]. Recently, De Vries and Froukje [153] reported that several anti-cancer drugs induce upregulation of Fas receptor (FasR) and Fas ligand (FasL), followed by subsequent induction of Fas-mediated apoptosis.

Zandieh et al. [59] supported the present results by stating that the expression of Fas antigen in most leukemic cells was low and increased above 20% after treatment. The study recommended the evaluation of this antigen before, during and after treatment as it may help to predict the susceptibility to trigger apoptosis by cytotoxic chemotherapy. In addition, Hannun [151] found that treatment of leukemia with doxorubicin results in upregulation of the ligand to the Fas receptor mediating the ability of the drug to kill these cells, this mechanism accounts for the sensitivity of leukemia cells to chemotherapy. Moreover, Kalivendi et al. [154] demonstrated that reactive oxygen species generated from doxorubicin metabolism in mitochondria result in increased cytosolic calcium levels by stimulating the calcium-release channels. Elevated levels of cytosolic calcium activate the calcium-dependent phosphatase, calcineurin, which dephosphorylates NFAT (nuclear factor of activated T-lymphocytes), calcium/calcineurin-dependent transcription factor. Dephosphorylated NFAT rapidly translocates to the nucleus and activates target genes, including Fas L. NFAT has been implicated as an important transactivator of the Fas L promoter, thus initiating Fas/FasL mediated pathway of apoptosis [155].

The observed increase in CD_{95}%, in the current study, might be referred to the inclusion of corticosteroids in the treatment protocol where Uckan et al. [156] demonstrated that the administration of high-dose methylprednisolone to ALL children was associated with a rise in Fas and FasL expression, suggesting their contributory role in the induction of apoptosis. Wesselborg et al. [4] showed that different antineoplastic drugs, such as daunorubicin, doxorubicin, etoposide, and mitomycin C induce caspase-dependent apoptosis to a similar extent in both CD_{95}-sensitive and resistant leukemic T cells where caspase-8 was activated in both cell types by these drugs such finding was recently supported by the study of Stankovic and Marston [157]. Moreover, Jeanine et al. [158] found that cells resistant to CD_{95} induced apoptosis were cross-resistant to apoptosis induction by etoposide and radiation and suggested that apoptosis signaling induced by these three stimuli (CD_{95}, etoposide and gamma radiation) has a common aspect. Since CD_{95} signaling involves activation of caspase-8, anticancer drugs would be able to induce synthesis of CD_{95} ligand and its receptor.

On the contrary, Mc Gahon et al. [159] demonstrated that flowcytometric analysis of doxorubicin treated leukemic cells failed to show significant increase in Fas or Fas ligand expression despite the induction of significant levels of apoptosis in these cells. The authors demonstrated that low-dose anti-Fas IgM treatment in combination with doxorubicin, methotrexate, camptothecin and etoposide produced an augmented cytoxicity in leukemic cells, concluding that although recruitment of the Fas/APO-1/ CD_{95} receptor/ligand system is not a necessary requirement for chemotherapeutic drug-induced apoptosis, combination of anti-Fas IgM and drug treatment produces a synergistic cytotoxic effect which may be useful in the treatment of human leukemias and this supports the finding of Wieder et al. who showed that in vitro apoptosis of pre-B blasts in response to epirubicin was CD_{95}/Fas independent even though caspase-8 was activated.

However, Lam et al. [160] found that Fas levels in ALL cells were



a: significantly different compared to control group,

b: significantly different compared to ALL cases before treatment.

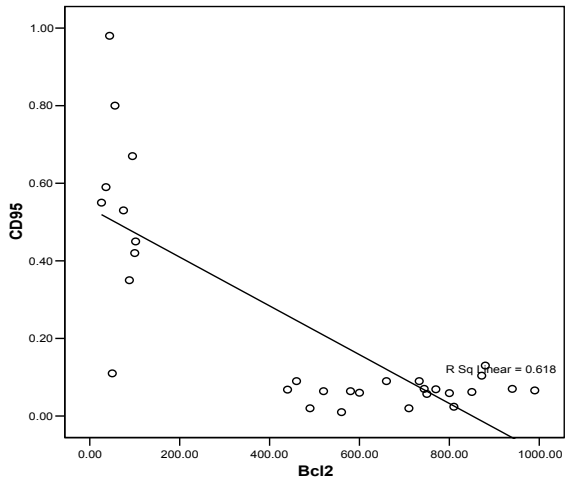
Figure 13(a-c): Serum levels of Cu (a) , Zn (b) and copper-zinc ratio (Cu/Zn) (c) in control (Group 1), ALL children before treatment (Group 2) and ALL children after treatment (Group 3).

Parameter	TLC	Hemoglobin	Platelets count	Bone marrow blast count	CD_{95}	Bcl-2	Cu	Zn	Cu/Zn
TLC	1	(-) 0.870**	(-) 0.902**	0.943**	(-) 0.828**	0.967**	0.906**	(-) 0.669**	0.752**
Hemoglobin	……..	1	0.862**	(-) 0.901**	0.831**	(-) 0.813**	(-) 0.911**	0.659**	(-) 0.763**
Platelets count	……..	……..	1	(-) 0.942**	0.925**	(-) 0.862**	(-) 0.905**	0.654**	(-) 759**
Bone marrow blast count	……..	……..	……..	1	(-) 0.856**	0.894**	0.936**	(-) 0.680**	0.778**
CD_{95}	……..	……..	……..	……..	1	(-) 0.786**	(-) 0.820**	0.560**	(-) 0.675**
Bcl-2	……..	……..	……..	……..	……..	1	0.865**	(-) 0.596**	0.688**
Cu	……..	……..	……..	……..	……..	……..	1	(-) 0.717**	0.869**
Zn	……..	……..	……..	……..	……..	……..	……..	1	(-) 0.914**
Cu/Zn	……..	……..	……..	……..	……..	……..	……..	……..	1

** Correlation is significant at the 0.01 level (2-tailed).

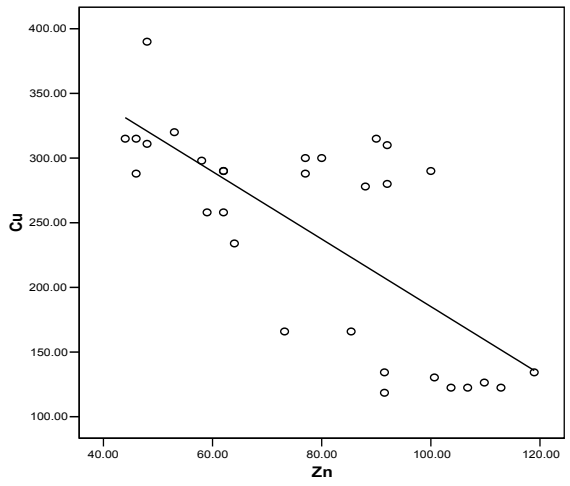
Table 9: Correlation between different parameters in ALL children before treatment

not significantly affected by treatment with vincristine, methotrexate, or dexamethasone. However, following treatment with ADR (Adriamycin®, Doxorubicin) and VP-16 (VePesid®, Etoposide), Fas was found to be induced and the increase was observed predominately in ADR- and VP-16-sensitive cell lines, the authors concluded that expression of Fas was increased after treatment with ADR and VP-16 which are two



Bcl-2 Concentration (pg/ml)

Figure 14: Correlation between CD_{95} % and Bcl-2 concentration in ALL children before treatment.



Bcl-2 Concentration (pg/ml)

Figure 15: Correlation between serum Cu and Zn levels in ALL children before treatment.

topoisomerase II-interactive antineoplastic agents known to cause DNA damage (Corbett and Osheroff,), [161] but not after treatment with vincristine, methotrexate, or dexamethasone suggests that induction of Fas in ALL cells is triggered by DNA damage.

Bcl-2 being another marker of leukemia is very important for evaluating the effect of chemotherapy, it's an oncogenic protein that inhibits apoptosis. It is located within the mitochondrial membrane, endoplasmic reticulum and nuclear envelope [162,163]. Bcl-2 is appreciated to belong to a family of related and interacting molecules such as Bax, Bcl-x, Bad, Bag, Bak, and Bik, some of which are antiapoptotic, whereas other members of the family, such as Bax, display pro-apoptotic function [164,165]. Interestingly, Bcl-2 and related antiapoptotic proteins seems to dimerize with the proapoptotic molecule, Bax, inhibiting its function [166].

Studies on the Bcl-2 oncogene led to the identification of an important antiapoptotic function for this protein that therefore gave survival advantage to lymphomas that overexpressed the protein product [167,168]. Unlike other oncogenes, the Bcl-2 gene is unique in that the principal mechanism by which it contributes to neoplastic cell expansion is by prolonging cell survival rather than by accelerating the rate of cell proliferation [5]. Studies in transgenic mice that contain Bcl-2/Ig fusion genes indicate that this survival advantage conferred by Bcl-2 allows cells to accumulate due to a lack of normal turnover, and can set the stage for the acquisition of additional genetic lesions that can act in concert with Bcl-2 and thus lead to more aggressive disease [168,169].

Expression levels of proteins involved in the induction or inhibition of apoptosis have been described to contribute to the response against a variety of drugs used for the treatment of leukemia [170]. It was found that the anti-apoptotic protein Bcl-2 is overexpressed in many cancers [171] and one of the mechanisms of Bcl-2 to inhibit apoptosis is prevention of cytochrome c release from the mitochondria thereby blocking caspase activation and apoptosis [160,172].

Modzelelwska et al.) and Srinivas et al. [173,174] support the findings that overexpression of Bcl-2 inhibits apoptosis and is responsible for resistance to chemotherapy. Moreover, it has been found that high levels of expression of Bcl-2 have been shown to correlate with poor treatment outcome in some hematological malignancies [175,176].

The current study revealed that blood Bcl-2 concentration was significantly higher in newly diagnosed ALL cases compared to controls whereas it exhibited a significant decrease after receiving treatment. In harmony with the present results, Volm et al. [176] and Maksoud et al. [177,178] demonstrated that there was significant increase of blood levels of Bcl-2 among leukemic patient compared to the controls. Hanada et al. [179] suggested that the increase in Bcl-2 concentration in newly diagnosed cases of leukemia was due to DNA hypomethylation in the 5' end of the Bcl-2 gene. The hypomethylation is associated with transcriptional upregulation of gene expression. Activation of this proto-oncogene with consequent upregulation of gene expression leads to inhibition of apoptosis.

Regarding the effect of treatment, Fadeel et al. [180] found that chemotherapy induce caspase dependent cleavage of endogenous Bcl-2 and this caspase mediated cleavage following chemotherapy is responsible for the attenuation of Bcl-2 function leading to apoptosis induction. Moreover, Haldar et al. [181] found that drugs such as taxol, vinblastine and vincristine induce apoptosis by phosphorylation of Bcl-2 at serine residues leading to loss of its antiapoptotic function as well as decreased binding of Bcl-2 to the proapoptotic bax protein therefore increased free bax and consequently apoptosis induction. Schimmer et al. [45] showed that Bcl-2 is an attractive therapeutic target and small molecules that target receptor-mediated pathway induce caspase-3 activation and overcome the blockage of the mitochondrial-mediated pathway caused by over expression of Bcl-2 and this temporary inhibition of Bcl-2 may not be toxic to normal cells. The authors also suggested that creating Bcl-2 inhibitors has focused on developing small molecules that mimic the action of the endogenous Bcl-2-binding death agonist.

Hafez et al. [182] demonstrated that $CD_{95}L$ was significantly lower in cases with no remission after treatment than those who achieved remission, also the anti-apoptotic factor Bcl-2 was found to be higher in cases than controls and in cases with no remission than those who achieved remission thus the authors suggested that, inhibitors for the activity of Bcl-2 as well as stimulators to CD_{95} could have a potential therapeutic benefit.

Parameter	TLC	Hemoglobin	Platelets count	Bone marrow blast count	CD_{95}	Bcl-2	Cu	Zn	Cu/Zn
TLC	1	(-) 0.327	(-) 0.229	0.416	(-) 0.218	0.246	0.313	0.049	0.295
Hemoglobin	.....	1	0.579**	(-) 0.650**	0.167	(-) 0.544**	(-) 0.439*	(-)0.029	(-) 0.389*
Platelets count	.....	.....	1	(-) 0.793**	0.453*	(-) 0.647**	(-) 0.696**	(-) 0.039	(-) 0.627**
Bone marrow blast count	.....	.....	.....	1	(-) 0.428*	0.845**	0.907**	0.116	0.806**
CD_{95}	.....	.....	.....	.....	1	(-) 0.380*	(-) 0.395*	(-) 0.056	(-) 0.359
Bcl-2	.....	.....	.....	.....	.....	1	0.831**	0.076	0.719**
Cu	.....	.....	.....	.....	.....	.....	1	0.109	0.817**
Zn	.....	.....	.....	.....	.....	.....	.....	1	(-) 0.459*
Cu/Zn	.....	.....	.....	.....	.....	.....	.....	.....	1

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Table 10: Correlation between different parameters in ALL children after treatment.

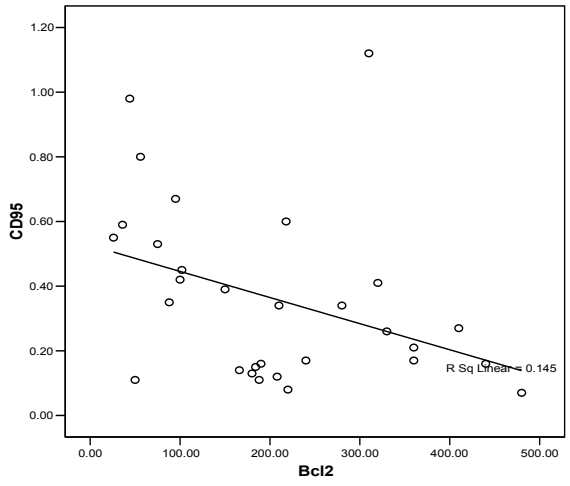


Figure 16: Correlation between CD95% and Bcl-2 concentration in ALL children after treatment.

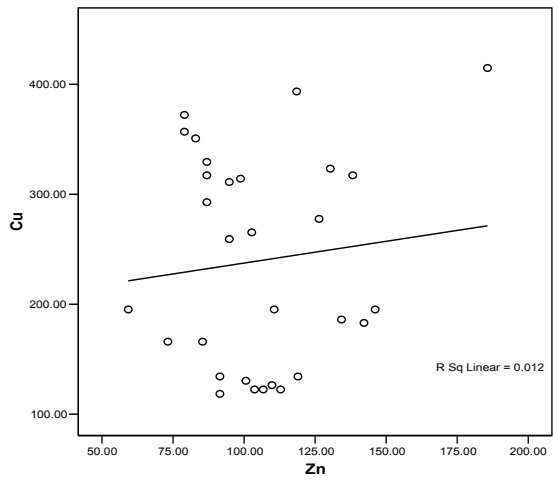
Lam et al. [160] found that, in eleven ALL cell lines following ADR treatment, a decrease in the level of both Bax and Bcl-2 protein was observed at drug concentrations of 5–10 μM. The study demonstrated that, in addition to the parental forms of Bax and Bcl-2, a second lower molecular weight form of both Bax and Bcl-2 termed Bax(s) and Bcl-2(s), respectively were also detected following ADR treatment and the levels of these shorter forms, were increased with increasing concentrations of ADR. Such result could be explained on the basis that Bax and Bcl-2 are cleaved during drug-induced apoptosis by caspases, yielding species similar in size to Bax(s) and Bcl-2(s) and this cleavage constitutes part of a positive feedback loop which amplifies apoptotic signaling and accelerates apoptosis [183,184].

On correlating CD_{95}% and Bcl-2 concentration to each other, CD_{95}% and Bcl-2 concentration exhibited a negative significant correlation. In accordance with our findings, Wuchter et al. [185] observed a negative correlation between Bcl-2 and CD_{95} expression and that complete remission was associated with higher CD_{95} expression levels compared to non-responders while high Bcl-2 expression levels is correlated with poor response to chemotherapy. The observed negative correlation between CD_{95}% and Bcl-2 could be referred to the fact that Bcl-2 molecule has been shown to inhibit FasL transcription in activated T lymphocytes, to downregulate Glucocorticoid (GC)-induced apoptosis, and to affect chemotherapy sensitivity [186-189].

The present study showed that CD_{95} is negatively correlated with TLC and bone marrow blast count while positively correlated with hemoglobin and platelet count. In line with the current results, Ramalingam et al. [190] found that sFas antigen levels were negatively correlated with total white blood cell count. However, Bcl-2 was found to be positively correlated with TLC and bone marrow blast count and negatively correlated with hemoglobin and platelet count. Supporting the findings of the current study, Maksoud et al. [178] demonstrated that patient's blood Bcl-2 was positively correlated with TLC and percent of blast cells in bone marrow but inversely correlated with hemoglobin concentration and platelets in children with ALL at diagnosis and in relapse.

Another aim of the current study was to investigate whether the disease or the chemotherapeutic treatment has an adverse effect on ALL cases regarding their serum mineral levels such as Cu and Zn concentrations. The current study revealed that in newly diagnosed ALL children, serum copper levels were significantly elevated while serum zinc levels were significantly lowered when compared to controls, as a result Cu/Zn ratio was significantly elevated. After administration of the treatment protocol serum Zn levels were normalized and Cu/Zn ratio was significantly decreased while serum Cu level showed no significant change when compared to values before treatment. Among abnormalities of mineral metabolism in ALL children at diagnosis, elevation of circulating copper, reduction in circulating zinc and consequently an alteration in copper : zinc ratio have been reported [191]. In harmony with the current study finding, Galan et al. [192] demonstrated that zinc levels in serum and/or inside white blood cells were often lower in patients with head and neck cancer or childhood leukemia. Moreover, **Eby** [193] showed that low blood levels of zinc are often noted in ALL cases and reported that zinc treatment is vital for rapid and permanent recovery as leukemic cells contain much less zinc than normal lymphocytes suggesting an error in zinc metabolism which appears correctable with zinc treatment.

Prasad [194] demonstrated that zinc deficiency causes impairment of T-cell functions and that impaired cellular immune functions played a key role in the occurrence of malignant processes. Moreover, Sahin et al. [195] reported that zinc deficiency seems to be associated with the development of ALL in children and suggested that chronic zinc deficiency might have played a role in the development of an altered lymphoid system associated with a malignant disorder by causing some degree of immune dysfunction and increasing the susceptibility to oncogenic viral infection leading to uncontrolled lymphoproliferation. They concluded that avoiding chronic zinc deficiency should be taken into consideration in the prevention of childhood leukemia



Bcl-2 Concentration (pg/ml)

Figure 17: Correlation between serum Cu and Zn level in ALL children after treatment.

in developing countries. Cvijanovich et al. [196] demonstrated that plasma zinc concentrations, among critically ill children, correlated inversely with measures of inflammation namely, C-reactive protein and interleukin-6 (IL-6) which were significantly elevated at initial diagnosis in acute leukemic children as demonstrated by Minamishima et al. [197].

Also, Akkus et al. [198] demonstrated that serum copper levels were found to be higher in ALL cases than those of controls and that was attributed to high percentage of blood cells in bone marrow or in peripheral circulation. In addition, Sgarbieri et al. [199] revealed that chemotherapy given to children has an important effect on their linear growth rate and nutritional status as well as their serum copper levels. The authors also showed that mean serum copper levels at diagnosis were significantly higher than in healthy children, and that there is a slightly decreased zinc concentration.

Gokhale et al. [199] indicated that the mean serum zinc levels in the post therapy profile of the patients were comparable to controls and majority of the patients had acceptable levels of zinc. The authors suggested that blood components which were administered as supportive care to the patients during therapy may have contributed to maintaining serum zinc levels in optimal or in higher range, even after completion of therapy. In contrast to our results, Malvy et al. [200] reported that chemotherapy induced a significant reduction in serum zinc level which may be attributed to low dietary intake.

The present study also showed negative significant correlation between serum copper and zinc levels. Supporting the current findings, [201-205] showed that copper concentration in serum was higher and zinc concentration was lower in leukemic patients than in healthy controls and also showed that there is a negative significant correlation between copper and zinc. They suggested that there is a relationship between changes in copper and zinc concentrations and modifications of the immune response associated with haematologic cancers.

Although based on a small number of patient and control samples, it can be concluded that the assessment of the apoptotic markers might be useful in monitoring the response to treatment protocol. However, long-term studies with large number of cases are still needed.

Summary and Conclusion

Acute Lymphocytic Leukemia (ALL) is a malignant disorder common among children. Apoptosis or programmed cell death is greatly involved in the mechanism of cancer development as well as drug resistance to cancer and it is the primary mechanism through which most chemotherapeutic agents induce tumor cell death.

In the present work, the aim was to study some markers of apoptosis which can help in diagnosing as well as following up of children with ALL after receiving treatment. Serum zinc and copper levels were also studied to give an indication about the effect of chemotherapy as well as the nutritional status of the children.

The study was carried out on children attending the outpatient clinic of the National Cancer Institute - Cairo University. Blood samples were collected from twenty five children with ALL prior to, and six months after starting chemotherapy according to the treatment protocol used in the National Cancer Institute – Cairo University (Total XV). Ten normal children were studied as control group. An informed consent was obtained from parents.

The present study included three groups:

Group 1:- Ten normal children considered as the control group.

Group 2:- Twenty five newly diagnosed children with ALL before receiving treatment.

Group 3:- Twenty children with ALL after receiving treatment where five cases were missed.

Routine laboratory tests were done for all the studied groups including Blood picture (Total Leukocytic Count (TLC), hemoglobin and platelet count) and Bone marrow blast count. Freshly taken blood samples were used in determination of CD_{95}% (using flowcytometer) and Bcl-2 concentration (using ELISA). Serum was separated and used in determination of serum zinc and copper levels (using atomic absorption spectrophotometer).

Results of the Current Study

TLC and bone marrow blast count were significantly higher in ALL cases at presentation than the control group while after treatment TLC was normalized whereas bone marrow blast count was significantly decreased and this may be attributed to the inhibitory effect of L-asparginase on lymphocyte blastogenesis. However, hemoglobin and platelet count before treatment were significantly decreased when compared to controls due to absence of normal hematopoietic elements while significantly increased after treatment reflecting a good response to the treatment protocol.

CD_{95}% was significantly decreased in newly diagnosed ALL children relative to controls because of the mutation and downregulation of the CD_{95}/Fas receptor- ligand system while it showed significant elevation after treatment where chemotherapy causes upregulation of the Fas receptor and Fas ligand followed by subsequent induction of Fas-mediated apoptosis. As for Bcl-2 it showed significant increase before treatment compared to controls which may be due to DNA hypomethylation in the 5' end of the Bcl-2 gene leading to transcriptional upregulation of gene expression while it was significantly decreased after treatment due to chemotherapeutic caspase dependent cleavage of endogenous Bcl-2 as well as phosphorylation of Bcl-2 at serine residues leading to loss of its antiapoptotic function. Negative significant correlation was found between CD_{95}% and Bcl-2 concentration which may be referred to the fact that Bcl-2 molecule inhibits FasL transcription in activated T lymphocytes.

Serum Cu level showed significant increase in ALL cases before treatment compared to controls which may be attributed to the high percentage of blood cells in bone marrow or in peripheral circulation while it showed no significant change after treatment. Serum Zn level was significantly decreased before treatment compared to controls due to error in zinc metabolism and was normalized after treatment. Blood components which were administered as supportive care to the patients during therapy may have contributed to maintaining serum zinc levels in optimal or in higher range, even after completion of therapy. Consequently, Cu/Zn ratio was significantly higher in newly diagnosed ALL children than controls while it showed significant decrease after treatment. Only at presentation, a negative significant correlation was found between serum Cu and Zn levels.

Recommendations

In conclusion, the current study demonstrated that both CD_{95}% and Bcl-2 concentration are useful diagnostic markers that may help not only in diagnosis but also in follow up of ALL cases. However, further studies are still needed involving larger number of patients and comparing values at diagnosis with values after completing the whole treatment course.

It's also recommended to give a Zn supplement to children with ALL in order to achieve rapid and permanent recovery.

Acknowledgment

First and foremost thanks to God

I would like to express my great appreciation and infinite gratitude to Prof. Dr. Maged Barakat Professor of Biochemistry, Faculty of Pharmacy, Cairo University, for his supervision, scientific guidance, and continuous help throughout the whole work.

Special gratitude and sincere thanks to Prof. Dr. Zakaria El-Khayat Professor of Biochemistry and Head of the Medical Biochemistry Department - National Research Center, for his great assistance, supervision, valuable suggestions and for his kind help throughout this work and during the preparation of the manuscript.

I would like to express my deepest gratitude and sincere appreciation to Dr.Manal Fouad Ismail Assistant Professor of Biochemistry, Faculty of Pharmacy - Cairo University, for helping me in every step of the work and for her valuable advice and keen supervision during writing the manuscript.

My deepest thanks and appreciation goes to Prof. Dr. Naglaa kholosy Professor of Clinical Pathology and Head of the Human Genetics Department - National Research Center for her supervision and great help during performing the study.

My special thanks to Prof Dr. Raafat Awadallah Hanna Professor of Medical Biochemistry - National Research Center (my dear father), for helping and supporting me in every step in the work and for encouraging me to go through the difficulties faced during my work.

I would like to express my deepest gratitude to Prof. Dr. Hala Gouda Professor of Child Health - National Research Center, for her help in the field of pediatrics and also my sincere thanks to Prof. Dr. Ashraf Zaghlol Professor of surgery - National Cancer Institute, for his great assistance in sample collection.

My thanks and gratitude to Dr. Mehrivan Mostafa Teacher of Medical Biochemistry - National Research Center, for helping and advising me throughout the study.

References

1. Ryschich E, Cebotari O, Fabian OV, Autschbach F, Kleeff J, et al. (2004) Loss of heterozygosity in the HLA class I region in human pancreatic cancer. Tissue Antigens 64: 696-702.
2. Boon T, Cerottini JC, Van den Eynde B, van der Bruggen P, Van Pel A (1994) Tumor antigens recognized by T lymphocytes. Annu Rev Immunol 12: 337-365.
3. Paydas S, Tanriverdi K, Yavuz S, Disel U, Sahin B, et al. (2003) Survivin and aven: two distinct antiapoptotic signals in acute leukemias. Ann Oncol 14: 1045-1050.
4. Wesselborg S, Engels IH, Rossmann E, Los M, Schulze-Osthoff K (1999) Anticancer drugs induce caspase-8/FLICE activation and apoptosis in the absence of CD95 receptor/ligand interaction. Blood 93: 3053-3063.
5. Korsmeyer SJ (1992) Bcl-2 initiates a new category of oncogenes: regulators of cell death. Blood 80: 879-886.
6. Campana D, Coustan-Smith E, Manabe A, Buschle M, Raimondi SC, et al. (1993) Prolonged survival of B-lineage acute lymphoblastic leukemia cells is accompanied by overexpression of bcl-2 protein. Blood 81: 1025-1031.
7. Sgarbieri UR, Fisberg M, Tone LG (1999) Nutritional assessment and serum zinc and copper concentration in leukemic children. Sao Paulo Med J 117: 13-18.
8. Gilliland DG (2002) Molecular genetics of human leukemias: new insights into therapy. Semin Hematol 39: 6-11.
9. Head DR (2004) Classification and differentiation of the acute leukemias. In: Greer JP, Rodgers GM, Foerster J, Paraskevas F, Lkens JN, et al. Wintrobe's clinical haematology (11thedn.) Lippincott Williams and Wilkins 2053-2076.
10. Bhatia S, Sather HN, Heerema NA, Trigg ME, Gaynon PS, et al. (2002) Racial and ethnic differences in survival of children with acute lymphoblastic leukemia. Blood 100: 1957-1964.
11. Pui CH, Evans WE (1998) Acute lymphoblastic leukemia. N Engl J Med 339: 605-615.
12. Liang DC, Pui CH (2005) Childhood acute lymphoblastic leukemia. In: Hoffbrand AV, Catovsky D and Tuddenham EC. Postgraduate Haematology 5th ed. by BlackWell Publishing Ltd: 542-560.
13. Pui CH, Campana D, Evans WE (2001) Childhood acute lymphoblastic leukemia – current status and future prospectives. Lancet Oncol 19: 697-704.
14. Schubauer-Berigan MK, Wenzl TB (2001) Leukemia mortality among radiation-exposed workers. Occup Med 16: 271-287.
15. Smith MA, Ries LA, Gurney JG (1999) Cancer incidence and survival among children and adolescents: United States SEER program 1975-1995. Bethesda, Md: National Cancer Institute. SEER Program.
16. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller Hermelink HK, et al. (2000) The World Health Organization classification of hematological malignancies. Report of the clinical advisory committee meeting. Mod Pathol 13: 193-207.
17. Shu XO, Ross JA, Pendergrass TW, Reaman GH, Lampkin B, et al. (1996) Parental alcohol consumption, cigarette smoking, and risk of infant leukemia: a Childrens Cancer Group study. J Natl Cancer Inst 88: 24-31.
18. Horwitz M (2001) Epidemiology and genetics of acute and chronic leukemia. In: American Cancer society. Atlas of Clinical Oncology Adult Leukemias 63-87.
19. Peris-Bonet R, Bad AF, Melchol I (1996) Childhood cancer incidence registration in the province of Valencia. Spain. J Epidemiol Biostar 1: 107-113.
20. Vanasse GJ, Concannon P, Willerford DM (1999) Regulated genomic instability and neoplasia in the lymphoid lineage. Blood 94: 3997-4010.
21. Whitlock JA, Gaynon PS (2004) Acute lymphoblastic leukemia in children. In: Greer JP, Rodgers GM, Foerster J, Paraskevas F, Lukens JN, et al. Windrobe's Clinical Haematology (11thedn.) by Lippincott and Wilkins: 2143-2167.
22. Behm FG (1990) Morphologic and cytochemical characteristics of childhood lymphoblastic leukemia. Hematol Oncol Clin North Am 4: 715-741.
23. Jaffe ES, Harris NL, Stein H, Vardiman JW (2001) World Health Organization classification of tumors, pathology and genetics: tumors of haematopoietic and lymphoid tissues. Lyon IARC Press.
24. Bain BJ (2001) Leukemia diagnosis (3rdedn.) BlackWell Publishing. Oxford 97-101.
25. Hoelzer S, Gokbuget N (2005) Adult acute lymphoblastic leukemia. In: Hoffbrand AV, Catovsky D, Tuddenham EC. Postgraduate haematology 5th ed. by BlackWell publishing Ltd. 525-560.
26. Bunin NJ, Pui CH (1985) Differing complications of hyperleukocytosis in children with acute lymphoblastic or acute nonlymphoblastic leukemia. J Clin Oncol 3: 1590-1595.
27. Kinney MC, Lukens JN (1999) Acute leukemia. In: Lee GR, Foerster J, Lukens JN, Paraskevas F, Greer JB, Rodgers GM. Wintrobe's Clinical Haematology 10th ed. Lipincott Williams and Wilkins: 2209-2230.

28. Rosenthal NS, Farhi DC (2000) Special stains in the diagnosis of acute leukemia. IN: Crisan d. Clinics in laboratory medicine. Philadelphoa. Pennyselvania. WB Saunders 29-39.

29. Lichtman MA, Beutler E, Kpps T, Williams JW (2003) Williams Manual of Haematology. McGraw-Hill: 303-315.

30. Catovsky D, Hoffbrand AV (1999) Acute leukemia. Postgraduate haematology.5th edition by Black Well Publishing Ltd. ch.18:p.372, 509-518.

31. Jones DP, Stapleton FB, Kalwinsky D, McKay CP, Kellie SJ, et al. (1990) Renal dysfunction and hyperuricemia at presentation and relapse of acute lymphoblastic leukemia. Med Pediatr Oncol 18: 283-286.

32. Clarke M, Gaynon P, Hann I, Harrison G, Masera G, et al. (2003) CNS-directed therapy for childhood acute lymphoblastic leukemia: Childhood ALL Collaborative Group overview of 43 randomized trials. J Clin Oncol 21: 1798-1809.

33. Wheeler KA, Richards SM, Baily CC, et al. (2000) Bone marrow transplantation versus chemotherapy in the treatment of very high risk childhood acute lymphoblastic leukemia in first remission: results from medical research council. Blood 96: 2412-2418.

34. Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26: 239-257.

35. Leist M, Jäättelä M (2001) Four deaths and a funeral: from caspases to alternative mechanisms. Nat Rev Mol Cell Biol 2: 589-598.

36. Saraste A, Pulkki K (2000) Morphologic and biochemical hallmarks of apoptosis. Cardiovasc Res 45: 528-537.

37. Hengartner MO (1996) Programmed cell death in invertebrates. Curr Opin Genet Dev 6: 34-38.

38. Van Cruchten S, Van Den Broeck W (2002) Morphological and biochemical aspects of apoptosis, oncosis and necrosis. Anat Histol Embryol 31: 214-223.

39. Salvesen GS, Dixit VM (1997) Caspases: intracellular signaling by proteolysis. Cell 91: 443-446.

40. Chinnaiyan AM, O'Rourke K, Lane BR, Dixit VM (1997) Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. Science 275: 1122-1126.

41. Ashkenazi A, Dixit VM (1998) Death receptors: signaling and modulation. Science 281: 1305-1308.

42. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X (1997) Apaf-, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90: 405-413.

43. Yang E, Korsmeyer SJ (1996) Molecular thanatopsis: a discourse on the BCL2 family and cell death. Blood 88: 386-401.

44. Gewies A (2003) Introduction to Apoptosis. Apo Review 1-26.

45. Schimmer AD, Hedley DW, Penn LZ, Minden MD (2001) Receptor- and mitochondrial-mediated apoptosis in acute leukemia: a translational view. Blood 98: 3541-3553.

46. Hengartner MO (1999) Programmed cell death in the nematode C. elegans. Recent Prog Horm Res 54: 213-222.

47. Bratton SB, MacFarlane M, Cain K, Cohen GM (2000) Protein complexes activate distinct caspase cascades in death receptor and stress-induced apoptosis. Exp Cell Res 256: 27-33.

48. Denault JB, Salvesen GS (2002) Caspases: keys in the ignition of cell death. Chem Rev 102: 4489-4500.

49. Richardson H, Kumar S (2002) Death to flies: Drosophila as a model system to study programmed cell death. J Immunol Methods 265: 21-38.

50. Sartorius U, Schmitz I, Krammer PH (2001) Molecular mechanisms of death-receptor-mediated apoptosis. Chembiochem 2: 20-29.

51. Harada H (1999) Regulation of apoptosis by BH3 domain only proteins. Cell Cycle. 17: 1603-1606.

52. Jacobs-Helber SM, Roh KH, Bailey D, Dessypris EN, Ryan JJ, et al. (2003) Tumor necrosis factor-alpha expressed constitutively in erythroid cells or induced by erythropoietin has negative and stimulatory roles in normal erythropoiesis and erythroleukemia. Blood 101: 524-531.

53. Schimmer AD (2008) Apoptosis in leukemia: from molecular pathways to targeted therapies. Best Pract Res Clin Haematol 21: 5-11.

54. James B (2004) CLL. In: Greer JP, Rodgers GM, Foerster J, Paraskevas F, Lukens JN, et al. Windrobe's Clinical Haematology 11th ed. by Lipincott Williams and Wilkins: 2145.

55. Sorrentino BP (2002) Gene therapy to protect haematopoietic cells from cytotoxic cancer drugs. Nat Rev Cancer 2: 431-441.

56. Earnshaw WC, Martins LM, Kaufmann SH (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 68: 383-424.

57. Acehan D, Jiang X, Morgan DG, Heuser JE, Wang X, et al. (2002) Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. Mol Cell 9: 423-432.

58. Müllauer L, Gruber P, Sebinger D, Buch J, Wohlfart S, et al. (2001) Mutations in apoptosis genes: a pathogenetic factor for human disease. Mutat Res 488: 211-231.

59. Zandieh T, Safari Fard A, Aghaipur M (2003) Expression of Fas Antigen (CD95) on Human Leukemic Cells and Assessment of Apoptosis on Fas+ and Fas- Samples by Flowcytometry Method. Iran J Allergy Asthma Immunol 2: 198-207.

60. Lyonel GI, Esther DI (1999) Apoptosis. Stem cells 17: 307-313.

61. BrajuskoviÄ G, VukosaviÄ S, DimitrijeviÄ‡ J, CeroviÄ‡ S, Usaj SK, et al. (2004) [Expression of the Bcl-2 family of proteins in peripheral blood B-lymphocytes in patients with chronic lymphocytic leukemia]. Vojnosanit Pregl 61: 41-46.

62. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, et al. (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397: 441-446.

63. Prives C (1998) Signaling to p53: breaking the MDM2-p53 circuit. Cell 95: 5-8.

64. Deveraux QL, Takahashi R, Salvesen GS, Reed JC (1997) X-linked IAP is a direct inhibitor of cell-death proteases. Nature 388: 300-304.

65. Tamm I, Wang Y, Sausville E, Scudiero DA, Vigna N, et al. (1998) IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res 58: 5315-5320.

66. Chandele A, Prasad V, Jagtap JC, Shukla R, Shastry PR (2004) Upregulation of survivin in G2/M cells and inhibition of caspase 9 activity enhances resistance in staurosporine-induced apoptosis. Neoplasia 6: 29-40.

67. Kamihira S, Yamada Y, Hirakata Y, Tomonaga M, Sugahara K, et al. (2001) Aberrant expression of caspase cascade regulatory genes in adult T-cell leukaemia: survivin is an important determinant for prognosis. Br J Haematol 114: 63-69.

68. Klas C, Debatin KM, Jonker RR, Krammer PH (1993) Activation interferes with the APO-1 pathway in mature human T cells. Int Immunol 5: 625-630

69. Friesen C, Fulda S, Debatin KM (1999) Cytotoxic drugs and the CD95 pathway. Leukemia 13: 1854-1858.

70. Belaud-Rotureau MA, Durrieu F, Labroille G, Lacombe F, Fitoussi O, et al. (2000) Study of apoptosis-related responses of leukemic blast cells to in vitro anthracycline treatment. Leukemia 14: 1266-1275.

71. Poppema S, Maggio E, van den Berg A (2004) Development of lymphoma in Autoimmune Lymphoproliferative Syndrome (ALPS) and its relationship to Fas gene mutations. Leuk Lymphoma 45: 423-431.

72. Takahashi T, Tanaka M, Inazawa J, Abe T, Suda T, et al. (1994) Human Fas ligand: gene structure, chromosomal location and species specificity. Int Immunol 6: 1567-1574.

73. Hiraide A, Imazeki F, Yokosuka O, Kanda T, Kojima H, et al. (2005) Fas polymorphisms influence susceptibility to autoimmune hepatitis. Am J Gastroenterol 100: 1322-1329.

74. Nolsøe RL, Hamid YH, Pociot F, Paulsen S, Andersen KM, et al. (2006) Association of a microsatellite in FASL to type II diabetes and of the FAS-670G>A genotype to insulin resistance. Genes Immun 7: 316-321.

75. Krammer PH, Dhein J, Walczak H, Behrmann I, Mariani S, et al. (1994) The role of APO-1-mediated apoptosis in the immune system. Immunol Rev 142: 175-191.

76. Han Z, Pantazis P, Wyche Jh, Kouttab VJ, Hendrickson EA (2001) A Fas-associated death domain protein- dependent mechanism mediates the apoptotic action of non-steroidal anti-inflammatory drugs in the human leukemic Jurkat cell line. J. Biol. Chem 276: 38748-38754.

77. Maher S, Toomey D, Condron C, Bouchier-Hayes D (2002) Activation-induced cell death: the controversial role of Fas and Fas ligand in immune privilege and tumour counterattack. Immunol Cell Biol 80: 131-137.

78. Reed JC (1997) Double identity for proteins of the Bcl-2 family. Nature 387: 773-776.

79. Hockenery DM (1995) bcl-2, a novel regulator of cell death. Bioessays 17: 631-638.

80. Borner C (2003) The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions. Mol Immunol 39: 615-647.

81. Gélinas C, White E (2005) BH3-only proteins in control: specificity regulates MCL-1 and BAK-mediated apoptosis. Genes Dev 19: 1263-1268.

82. Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, et al. (2002) Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell 2: 183-192.

83. Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, et al. (1985) Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell 41: 899-906.

84. Nelson DA, Tan TT, Rabson AB, Anderson D, Degenhardt K, et al. (2004) Hypoxia and defective apoptosis drive genomic instability and tumorigenesis. Genes Dev 18: 2095-2107.

85. Nowell PC, Croce CM (1987) Cytogenetics of neoplasia. In: Greene MI, Hamaoka T, (eds.) Development and Recognition of the Transformed Cell. Plenum Publishing Corporation. New York.

86. Reed JC (1997) Cytochrome c: can't live with it--can't live without it. Cell 91: 559-562.

87. Zou H, Li Y, Liu X, Wang X (1999) An APAF-1. cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J Biol Chem 274: 11549-11556.

88. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, et al. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91: 479-489.

89. Reed JC (1998) Bcl-2 family proteins. Oncogene 17: 3225-3236.

90. Linder MC (1991) Nutritional Biochemistry and Metabolism with Clinical Applications. Second edition. Elsevier, New York.

91. Fleta Zaragozano J, Moreno Aznar L, Bueno Lozano M (1989) [Nutrition and cancer in pediatrics. A complex interaction]. An Esp Pediatr 30: 333-339.

92. Mauer AM, Burgess JB, Donaldson SS, Rickard KA, Stallings VA, et al. (1990) Special nutritional needs of children with malignancies: a review. JPEN J Parenter Enteral Nutr 14: 315-324.

93. Sandstead HH (1994) Understanding zinc: recent observations and interpretations. J Lab Clin Med 124: 322-327.

94. Prasad AS (1995) Zinc: an overview. Nutrition 11: 93-99.

95. Solomons NW (1998) Mild human zinc deficiency produces an imbalance between cell-mediated and humoral immunity. Nutr Rev 56: 27-28.

96. Rink L, Gabriel P (2000) Zinc and the immune system. Proc Nutr Soc 59: 541-552.

97. Hambridge KM, Casey CE, Krebs NF (1987) Zinc. In: Mertz W (ed.) Trace elements in human and animal nutrition, (5thedn). Vol. 2. Orlando, FL, Academic Press 1-137.

98. Prasad AS (1966) Zinc metabolism. CC Thomas, Springfield, IL. (QP535. Z6 P7).

99. Prasad AS (2008) Zinc in human health: effect of zinc on immune cells. Mol Med 14: 353-357.

100. Greenwood NN, Earnshaw A (1997) Chemistry of the Elements (2ndedn). Oxford: Butterworth-Heinemann.

101. Shankar AH, Prasad AS (1998) Zinc and immune function: the biological basis of altered resistance to infection. Am J Clin Nutr 68: 447S-463S.

102. Dardenne M, Pléau JM, Nabarra B, Lefrancier P, Derrien M, et al. (1982) Contribution of zinc another metals to the biological activity of the serum thymic factor. Proc Natl Acad Sci U S A 79: 5370-5373.

103. Prasad AS (2003) Zinc deficiency. BMJ 326: 409-410.

104. Prasad AS (2000) Effects of zinc deficiency on immune functions. J Trace Elements Exp Med 13: 1-20.

105. Ibs KH, Rink L (2003) Zinc-altered immune function. J Nutr 133: 1452S-6S.

106. WHO contributors (2007). The impact of zinc supplementation on childhood mortality and severe morbidity (PDF). World Health Organization. http://www.who.int/child_adolescent_health/documents/zinc_mortality/en/index.html. Retrieved 2009-03-01.

107. Shrimpton R, Gross R, Darnton-Hill I, Young M (2005) Zinc deficiency: what are the most appropriate interventions? BMJ 330: 347-349.

108. Burtis CA, Ashwood ER (1999) Trace element. In: "Tietz textbook of clinical chemistry". (3rdedn), Chp. 30.

109. Kaplan LA, Pesce AJ, Kazmierczak SC (2003) Trace elements. In: Kaplan LA, Pesce AJ, Kazmierczak SC (eds.) "Clinical chemistry", (4thedn), Chp.38.

110. Hussain SP, Raja K, Amstad PA, Sawyer M, Trudel LJ, et al. (2000) Increased p53 mutation load in nontumorous human liver of Wilson disease and hemochromatosis: oxyradical overload diseases. Proc Natl Acad Sci 97: 12770.

111. Imamura M (1963) Shirutsu Sapporo Byorn Ishi, 23(1-2), 6-27. From chem. Abst. 61: 13694.

112. Cohen Y, Epelbaum R, Haim N, McShan D, Zinder O (1984) The value of serum copper levels in non-Hodgkin's lymphoma. Cancer 53: 296-300.

113. Gupta SK, Shukla VK, Gupta V, Gupta S (1994) Serum trace elements and Cu/Zn ratio in malignant lymphomas in children. J Trop Pediatr 40: 185-187.

114. Mc Coy JP (1994) Basic principles in clinical flowcytometry In: Keren DF, Hanson CA, Hurtbise PE (eds.) Flow cytometry and clinical diagnosis. ASCP press. Chicago P. 26.

115. Brown M, Wittwer C (2000) Flow cytometry: principles and clinical applications in hematology. Clin Chem 46: 1221-1229.

116. Kevin JM, Fletcher MA (2005) Quantitative flowcytometry in clinical lab. , Clin. Appl Immunol Rev 5: 353-372.

117. Orfao A, Ruiz-Arguelles A, Lacombe F, Ault K, Basso G, et al. (1995) Flow cytometry: its applications in hematology. Haematologica 80: 69-81.

118. Shapiro H (1995) Practical flow cytometry, 3rd ed. New York: Wiley-Liss, 542 pp.

119. Watson JV (1999) The early fluidic and optical physics of cytometry. Cytometry 38: 2-14.

120. Karen DF, Hanson CA, Hurtubise PE (1994) Flow cytometry and clinical diagnosis. Chicago: ASCP Press, 664.

121. Robinson JP (2004) Flowcytometry. Encyclopedia of Biomaterials and Biomedical Engineering 630-640.

122. Comazzi S, Gelain ME, Riondato F, Paltrinieri S (2006) Flow cytometric expression of common antigens CD18/CD45 in blood from dogs with lymphoid malignancies: a semi-quantitative study. Vet Immunol Immunopathol 112: 243-252.

123. Bleesing JJ, Fleisher TA (2001) Immunophenotyping. Semin Hematol 38: 100-110.

124. Brunning R, Flandrin G, Borowitz M, Swerdlow S, Matutes E, Bennett j and Head D (2001) Precursor B lymphoblastic leukemia/lymphoblastic lymphoma. In World Health Organization classification of Tumors. Published by IARC press lyon Washington. Pp 109 – 117.

125. Khairy A, El-Sissy A (2003) Karyotype in Acute Lymphoblastic. Leukemia: Impact on Clinical Presentation and Duration of First remission. Journal of Egyptian Nat. Cancer Inst 15: 121-128.

126. Lee GR (1999) Wintrobe's Hematology, Lippincott.23-25.

127. Pui CH, Relling MV, Sandlund JT, Downing JR, Campana D, et al. (2004) Rationale and design of Total Therapy Study XV for newly diagnosed childhood acute lymphoblastic leukemia. Ann Hematol 83 Suppl 1: S124-126.

128. Ruiz-Arguelles A (1992) Flow cytometry in the clinical laboratory. Principles, applications and problems. Clin Chim Acta 211: S13-27.

129. Tran A, Yang G, Doglio A, Ticchioni M, Laffont C, et al. (1997) Phenotyping of intrahepatic and peripheral blood lymphocytes in patients with chronic hepatitis C. Dig Dis Sci 42: 2495-2500.

130. Jaroszesk MJ (1998) Flow cytometry Protocols, Humana Press.

131. Chard T (1990) An Introduction to Radioimmunoassay & Related Techniques 4th Ed. Amsterdam: Elsevier.

132. Sinha SN, Gabrieli ER (1970) Serum copper and zinc levels in various pathologic conditions. Am J Clin Pathol 54: 570-577.

133. Saeys Y, Inza I, Larrañaga P (2007) A review of feature selection techniques in bioinformatics. Bioinformatics 23: 2507-2517.

134. Lanzkowisky P (2000) Incidence and epidemiology of acute leukemias, manual of pediatric hematology and oncology. chapter 14: 359.

135. Pui CH, Relling MV, Downing JR (2004) Acute lymphoblastic leukemia. N Engl J Med 350: 1535-1548.

136. Conter V, Rizzari C, Sala A, Chiesa R, Citterio M, et al. (2004) Acute Lymphoblastic Leukemia. Orphanet Encyclopedia 1-13

137. Estrov Z, Freedman MH (1991) Acute lymphoblastic leukemia blast cells do not inhibit bone marrow hematopoietic progenitor colony formation. Exp Hematol 19: 221-225.

138. Kantarjian HM, Faderl S (2004) Acute lymphoid leukemia in adults. In: Abeloff MD, Armitage JO, Lichter AS, Niederhuber JE, Kastan MB, McKenna WG, eds. Clinical Oncology. 3rd ed. Philadelphia, Pa: Elsevier 2793-2824.

139. Schwartz CL, Thompson EB, Gelber RD, Young ML, Chilton D, Cohen HJ and Sallan SE (2001) Improved Response With Higher Corticosteroid Dose in Children With Acute Lymphoblastic Leukemia. J Clin Oncol. Vol 19, No 4 , pp 1040-1046.

140. Fidler IJ, Montgomery PC, Ferguson E (1971) L-asparaginase and lymphocyte blastogenesis. Lancet 2: 1091-1092.

141. Whitehead VM, Rosenblatt DS, Vuchich MJ, Beaulieu D (1987) Methotrexate polyglutamate synthesis in lymphoblasts from children with acute lymphoblastic leukemia. Dev Pharmacol Ther 10: 443-448.

142. Singh N (2007) Apoptosis in health and disease and modulation of apoptosis for therapy: An overview. Indian J Clin Biochem 22: 6-16.

143. Danial NN, Korsmeyer SJ (2004) Cell death: critical control points. Cell 116: 205-219.

144. Tahereh Z, Asghar SF, Mahnaz A (2003) Expression of Fas Antigen (CD95) on Human Leukemic Cells and assessment of Apoptosis on Fas+ and Fas- Samples by Flowcytometry Method, Department of Research Center of Iranian Blood Transfusion Organisation Tehran 2: 4.

145. Nagata S, Golstein P (1995) The Fas death factor. Science 267: 1449-1456.

146. Tamiya S, Etoh K, Suzushima H, Takatsuki K, Matsuoka M (1998) Mutation of CD95 (Fas/Apo-1) gene in adult T-cell leukemia cells. Blood 91: 3935-3942.

147. Müschen M, Warskulat U, Beckmann MW (2000) Defining CD95 as a tumor suppressor gene. J Mol Med (Berl) 78: 312-325.

148. Inaba H, Shimada K, Zhou YW, Ido M, Buck S, et al. (2005) Acquisition of Fas resistance by Fas receptor mutation in a childhood B-precursor acute lymphoblastic leukemia cell line, MML-1. Int J Oncol 27: 573-579.

149. Wood CM, Goodman PA, Vassilev AO, Uckun FM (2003) CD95 (APO-1/FAS) deficiency in infant acute lymphoblastic leukemia: detection of novel soluble Fas splice variants. Eur J Haematol 70: 156-171.

150. Barry MA, Behnke CA, Eastman A (1990) Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. Biochem Pharmacol 40: 2353-2362.

151. Hannun YA (1997) Apoptosis and the dilemma of cancer chemotherapy. Blood 89: 1845-1853.

152. Fisher DE (1994) Apoptosis in cancer therapy: crossing the threshold. Cell 78: 539-542.

153. De Vries, Froukje J (2008) Complexity of apoptotic pathways in leukemia treated with chemotherapy or cellular immunotherapy, Doctoral thesis, Leiden University.

154. Kalivendi SV, Konorev EA, Cunningham S, Vanamala SK, Kaji EH, et al. (2005) Doxorubicin activates nuclear factor of activated T-lymphocytes and Fas ligand transcription: role of mitochondrial reactive oxygen species and calcium. Biochem J 389: 527-539.

155. Schulz RA, Yutzey KE (2004) Calcineurin signaling and NFAT activation in cardiovascular and skeletal muscle development. Dev Biol 266: 1-16.

156. Uçkan D, Yetgin S, Cetin M, Ozyürek E, Okur H, et al. (2003) The effects of high dose methylprednisolone on apoptosis in children with acute lymphoblastic leukemia. Clin Lab Haematol 25: 35-40.

157. Stankovic T, Marston E (2008) Molecular mechanisms involved in chemoresistance in paediatric acute lymphoblastic leukaemia. Srp Arh Celok Lek 136: 187-192.

158. Jeanine GR, Boesen-de Cock, Annemiek D, Evert de Vries, Wim J, et al. (1999) Common Regulation of Apoptosis Signaling Induced by CD95 and the DNA-damaging Stimuli Etoposide and g-Radiation Downstream from Caspase-8 Activation; J Biol Chem 274: 14255–14261.

159. McGahon AJ, Costa Pereira AP, Daly L, Cotter TG (1998) Chemotherapeutic drug-induced apoptosis in human leukaemic cells is independent of the Fas (APO-1/CD95) receptor/ligand system. Br J Haematol 101: 539-547.

160. Lam V, Findley HW, Reed JC, Freedman MH, Goldenberg GJ (2002) Comparison of DR5 and Fas expression levels relative to the chemosensitivity of acute lymphoblastic leukemia cell lines. Leuk Res 26: 503-513.

161. Corbett AH, Osheroff N (1993) When good enzymes go bad: conversion of topoisomerase II to a cellular toxin by antineoplastic drugs. Chem Res Toxicol 6: 585-597.

162. Kroemer G (1997) The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat Med 3: 614-620.

163. Reed JC (1995) Bcl-2: prevention of apoptosis as a mechanism of drug resistance. Hematol Oncol Clin North Am 9: 451-473.

164. Yang E, Zha J, Jockel J, Boise LH, Thompson CB, et al. (1995) Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell 80: 285-291.

165. Wang HG, Takayama S, Rapp UR, Reed JC (1996) Bcl-2 interacting protein, BAG-, binds to and activates the kinase Raf-1. Proc Natl Acad Sci U S A 93: 7063-7068.

166. Sato T, Hanada M, Bodrug S, Irie S, Iwama N, et al. (1994) Interactions among members of the Bcl-2 protein family analyzed with a yeast two-hybrid system. Proc Natl Acad Sci U S A 91: 9238-9242.

167. Korsmeyer SJ, Yin XM, Oltvai ZN, Veis-Novack DJ, Linette GP (1995) Reactive oxygen species and the regulation of cell death by the Bcl-2 gene family. Biochim Biophys Acta 1271: 63-66.

168. McDonnell TJ, Korsmeyer SJ (1991) Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18). Nature 349: 254-256.

169. Katsumata M, Siegel RM, Louie DC, Miyashita T, Tsujimoto Y, et al. (1992) Differential effects of Bcl-2 on T and B cells in transgenic mice. Proc Natl Acad Sci U S A 89: 11376-11380.

170. Solary E, Droin N, Bettaieb A, Corcos L, Dimanche-Boitrel MT, et al. (2000) Positive and negative regulation of apoptotic pathways by cytotoxic agents in hematological malignancies. Leukemia 14: 1833-1849.

171. Schena M, Larsson LG, Gottardi D, Gaidano G, Carlsson M, et al. (1992) Growth- and differentiation-associated expression of bcl-2 in B-chronic lymphocytic leukemia cells. Blood 79: 2981-2989.

172. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, et al. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275: 1129-1132.

173. Modzelewska M, Sikorska-Fic B, Kowalska H, Rokicka-Milewska R, Wasik M (1998) [BCL-2 antigen expression in blood cells in children suffering from leukemia]. Wiad Lek 51 Suppl 4: 119-123.

174. Srinivas G, Kusumakumary P, Nair MK, Panicker KR, Pillai MR (1998) Bcl-2 protein and apoptosis in pediatric acute lymphoblastic leukemia. Int J Mol Med 1: 755-759.

175. Matsushima H, Kitamura T, Goto T, Hosaka Y, Homma Y, et al. (1997) Combined analysis with Bcl-2 and P53 immunostaining predicts poorer prognosis in prostatic carcinoma. J Urol 158: 2278-2283.

176. Campos L, Sabido O, Sebban C, Charrin C, Bertheas MF, et al. (1996) Expression of BCL-2 proto-oncogene in adult acute lymphoblastic leukemia. Leukemia 10: 434-438.

177. Volm M, Zintl F, Sauerbrey A, Koomägi R (1999) Proliferation and apoptosis in newly diagnosed and relapsed childhood acute lymphoblastic leukemia. Anticancer Res 19: 4327-4331.

178. Maksoud NA, Abdellatif MMS, Saad IM (2005) Assesment of Bcl-2 and Interferon Gamma expression in children with acute lymphoblastic leukemia at different stages of the disease. Kasr El Aini Medical Journal 11: 307-317.

179. Hanada M, Delia D, Aiello A, Stadtmauer E, Reed JC (1993) bcl-2 gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia. Blood 82: 1820-1828.

180. Fadeel B, Hassan Z, Hellström-Lindberg E, Henter JI, Orrenius S, et al. (1999) Cleavage of Bcl-2 is an early event in chemotherapy-induced apoptosis of human myeloid leukemia cells. Leukemia 13: 719-728.

181. Haldar S, Basu A, Croce CM (1997) Bcl2 is the guardian of microtubule integrity. Cancer Res 57: 229-233.

182. Hafez M, Al-Tonbary Y, El-Bayoumi MA, Hatem N, Hawas S, et al. (2007) Markers of apoptosis and proliferation related gene products as predictors of treatment outcome in childhood acute lymphoblastic leukemia. Hematology 12: 209-218.

183. Fujita N, Tsuruo T (1998) Involvement of Bcl-2 cleavage in the acceleration of VP-16-induced U937 cell apoptosis. Biochem Biophys Res Commun 246: 484-488.

184. Kirsch DG, Doseff A, Chau BN, Lim DS, de Souza-Pinto NC, et al. (1999) Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome c. J Biol Chem 274: 21155-21161.

185. Wuchter C, Karawajew L, Ruppert V, Buchner T, Schoch C, Haferlach T, Ratei R, Dorken B and Ludwig W (1999) clinical significance of CD95, Bcl-2 and Bax expression and Cd95 function in adult de novo myeloid leukemia in contxt of p-glycoprotien function, maturation stage and cytogenetics. Leukemia 13: 1943-1953.

186. Coustan-Smith E, Kitanaka A, Pui CH, McNinch L, Evans WE, et al. (1996) Clinical relevance of BCL-2 overexpression in childhood acute lymphoblastic leukemia. Blood 87: 1140-1146.

187. DiGiuseppe JA, LeBeau P, Augenbraun J, Borowitz MJ (1996) Multiparameter flow-cytometric analysis of bcl-2 and Fas expression in normal and neoplastic hematopoiesis. Am J Clin Pathol 106: 345-351.

188. Smets LA, van den Berg JD (1996) Bcl-2 expression and glucocorticoid-induced apoptosis of leukemic and lymphoma cells. Leuk Lymphoma 20: 199-205.

189. Srivastava RK, Sasaki CY, Hardwick JM, Longo DL (1999) Bcl-2-mediated drug resistance: inhibition of apoptosis by blocking nuclear factor of activated T lymphocytes (NFAT)-induced Fas ligand transcription. J Exp Med 190: 253-265.

190. Ramalingam S, Kannangai R, Abraham OC, Subramanian S, Rupali P, et al. (2008) Investigation of apoptotic markers among human immunodeficiency virus (HIV-1) infected individuals. Indian J Med Res 128: 728-733.

191. Sgarbieri UR, Fisberg M, Tone LG, Latorre Mdo R (2006) Nutritional assessment and serum zinc and copper concentration among children with acute lymphocytic leukemia: a longitudinal study. Sao Paulo Med J 124: 316-320.

192. Galan P, Briançon S, Favier A, Bertrais S, Preziosi P, et al. (2005) Antioxidant status and risk of cancer in the SU.VI.MAX study: is the effect of supplementation dependent on baseline levels? Br J Nutr 94: 125-132.

193. Eby GA (2005) Treatment of acute lymphocytic leukemia using zinc adjuvant with chemotherapy and radiation--a case history and hypothesis. Med Hypotheses 64: 1124-1126.

194. Prasad AS (1997) Zinc and immunity. In trace elements in humans 1-11.

195. Sahin G, Ertem U, Duru F, Birgen D, Yüksek N (2000) High prevelance of chronic magnesium deficiency in T cell lymphoblastic leukemia and chronic zinc deficiency in children with acute lymphoblastic leukemia and malignant lymphoma. Leuk Lymphoma 39: 555-562.

196. Cvijanovich NZ, King JC, Flori HR, Gildengorin G, Wong HR (2009) Zinc homeostasis in pediatric critical illness. Pediatr Crit Care Med 10: 29-34.

197. Minamishima I, Ohga S, Ishii E, Matsuzaki A, Kai T, et al. (1993) Serum interleukin-6 and fever at diagnosis in children with acute leukemia. Am J Pediatr Hematol Oncol 15: 239-244.

198. Akkus I, Gürbilek M, Çaliskan U, Kaptanoglu B, Uner A (1998) Blood and fingernail copper and magnesium levels of children with acute lymphoblastic leukemia. Tr J of Medical Sciences 28: 155-156.

199. Gokhale CD, Udipi SA, Ambaye RY, Pai SK, Advani SH (2007) Post-therapy profile of serum total cholesterol, retinol and zinc in pediatric acute lymphoblastic leukemia and non-Hodgkin's lymphoma. J Am Coll Nutr 26: 49-56.

200. Malvy DJ, Arnaud J, Burtschy B, Sommelet D, Leverger G, et al. (1997) Antioxidant micronutrients and childhood malignancy during oncological treatment. Med Pediatr Oncol 29: 213-217.

201. Carpentieri U, Myers J, Thorpe L, Daeschner CW 3rd, Haggard ME (1986) Copper, zinc, and iron in normal and leukemic lymphocytes from children. Cancer Res 46: 981-984.

202. Cortes JE, Kantarjian HM (1995) Acute lymphoblastic leukemia. A comprehensive review with emphasis on biology and therapy. Cancer 76: 2393-2417.

203. Griffith DP, Liff DA, Ziegler TR, Esper GJ, Winton EF (2009) Acquired copper deficiency: a potentially serious and preventable complication following gastric bypass surgery. Obesity (Silver Spring) 17: 827-831.

204. Brandt EG, Hellgren M, Brinck T, Bergman T, Edholm O (2009) Molecular dynamics study of zinc binding to cysteines in a peptide mimic of the alcohol dehydrogenase structural zinc site. Phys Chem Chem Phys 11: 975-983.

205. Colby-Graham MF, Chordas C (2003) The childhood leukemias. J Pediatr Nurs 18: 87-95.

Citation: Hanna JRA (2015) Expression of CD_{95} in Acute Lymphocytic Leukemia (ALL) in Egyptian Children before and after Treatment. J Blood Disorders Transf 6: 250. doi:10.4172/2155-9864.1000250