# IN VITRO DRUG SENSITIVITY, APOPTOSIS AND PROGNOSIS IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

**Project Completion Report**
**(G.O (Ms) No. 103/99/STED dated 11/10/1999)**


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# PROJECT COMPLETION REPORT

## TITLE OF PROJECT: IN VITRO DRUG SENSITIVITY, APOPTOSIS AND PROGNOSIS IN PEDIATRIC ACUTE LYMPNOBLASTIC LEUKEMIA

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### *Summary of Work Done:*

This study looked for any associations between *in vitro* drug sensitivity and clinical outcome in pediatric acute lymphoblastic leukemia (ALL) with the standard drugs used for leukemia therapy. A total of seventy-two samples were analyzed. *In vitro* sensitivity to drugs was tested by a methyl-thiazol-tetrazolium (MTT) assay in six serial fold dilutions. Apoptosis was determined by TUNEL assay and apoptotic index was calculated for each sample. Patients sensitive to Prednisone, Asparginase, Vincristine and 6-Mercapto Purine (6-MP) had higher overall survival compared to patients whose tumor cells were resistant to these drugs ($P < 0.01$). For the other drugs tested, overall survival (OS) did not vary from that of the resistant patients. For Doxorubicin, Asparginase, Vincristine, Prednisone combination (DAVP) sensitivity, there was a significant worsening of prognosis from the extremely sensitive patients through an intermediate sensitive group to a most resistant group. The present study thus shows that *in vitro* drug sensitivity testing provides significant prognostic information in childhood ALL.

# CHAPTER 1

## *INTRODUCTION TO PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA*

Acute Lymphoblastic Leukemia (ALL) is a malignant disorder characterized by clonal proliferation and excessive accumulation of lymphoblasts and their progenitors with arrested maturation. This lymphoid malignancy represents a heterogeneous group of diseases that vary with respect to morphological, cytogenetic and immunologic features of transformed cells. The disease is characterized by uncontrolled growth of immature hemopoietic cells at the expense of normal marrow function. Leukemia cells rapidly accumulate in the bone marrow cavity, ultimately replacing most of the normal hematopoietic cells. These cells then circulate into blood and infiltrate into other tissues throughout the body, thus resulting in signs and symptoms of the disease.

ALL is the most common malignancy in children accounting for 25% of all childhood cancers and approximately 75% of all cases of childhood leukemias (Pui, 1995). The genesis of childhood leukemia remains a mystery. It is not known how a hemopoietic progenitor becomes leukemic, but damage to the genetic program of the cells is thought to accumulate as a result of multiple separate events. Some of these events may be stochastic (i.e., random or chance damage to cell's DNA, occurring during cell division). The interactions

between host, environment and random factors are difficult to determine, and the cause of leukemia in an individual usually remains obscure. However, association with epidemiologic factors including maternal and paternal exposure to radiation, history of maternal fetal loss or fertility problems, higher birth weight and use of exogenous growth hormone, race and children with higher socio-economic status have been considered (Ross et al., 1994; Swensen et al., 1997; Gomez et al., 1998). Recent reviews find no relationship between exposure to electro magnetic field radiation and incidence of ALL while involvement of any virus is also controversial (Ben David and Bernstein, 1991). A highly randomized study has suggested that ALL in children occurs in a seasonal manner suggesting a role for a still unidentified infectious agent (Badrinath et al., 1997). Certain chemicals such as benzene and its derivatives are capable of damaging bone marrow progenitors and may give rise to myelodysplasia or acute leukemia. The use of cytotoxic and immunosuppressive agents is associated with a small, but definite risk of leukemic transformation (Sandler and Ross, 1997). Acute leukemia may also arise de novo (primary leukemia) or evolve from a pre-existing clonal blood disorder, when it is known as secondary leukemia (Ross et al., 1994).

## INCIDENCE, PREVALENCE AND SURVIVAL

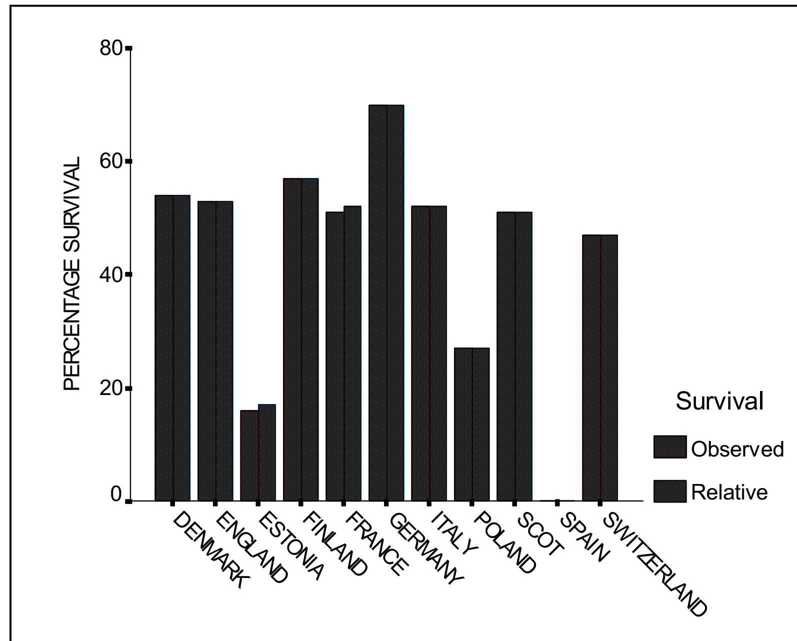
### *International patterns*

There are substantial geographic differences in the incidence of childhood leukemia, and these may help provide clues to etiology. In North Africa and the

Middle East, ALL is relatively rare, while in India and China ALL is somewhat common, but its incidence is less than the industrialized West. The rates range from 4.3, 4.5, 0.7 and 1.2 per 100,000 in US, Costa Rica, India and Kuwait respectively (Boring et al., 1994). In United States and other developed countries, there is a significant peak in childhood ALL incidences that occur between the ages of 2 and 5 (Gurney et al., 1995). This peak has been apparent since the 1930's, and it is absent in many developing countries (Ross et al., 1994). Higher socio economic status has been associated with an increased risk of ALL in a majority of studies. Based on these observations, it is possible that aspects associated with modernization may account for this age peak.

Survival of ALL patients in countries differs widely (Figure 1) (Berrino et al., 1995).

**FIGURE 1- OBSERVED vs. RELATIVE FIVE YEAR SURVIVAL OF ALL PATIENTS FROM EUROPE (ADAPTED FROM IARC PUBLICATION NO.132, LYON, 1995)**




*[The total number of patients included for survival analysis were not the same from different countries and may be a reason for the wide variation for the survival rates seen. However, the overall five-year survival among the European registries was 59%. The relative five year survival of childhood ALL from Spain have been recently obtained as 83% (Jaume et al., 2001)]*

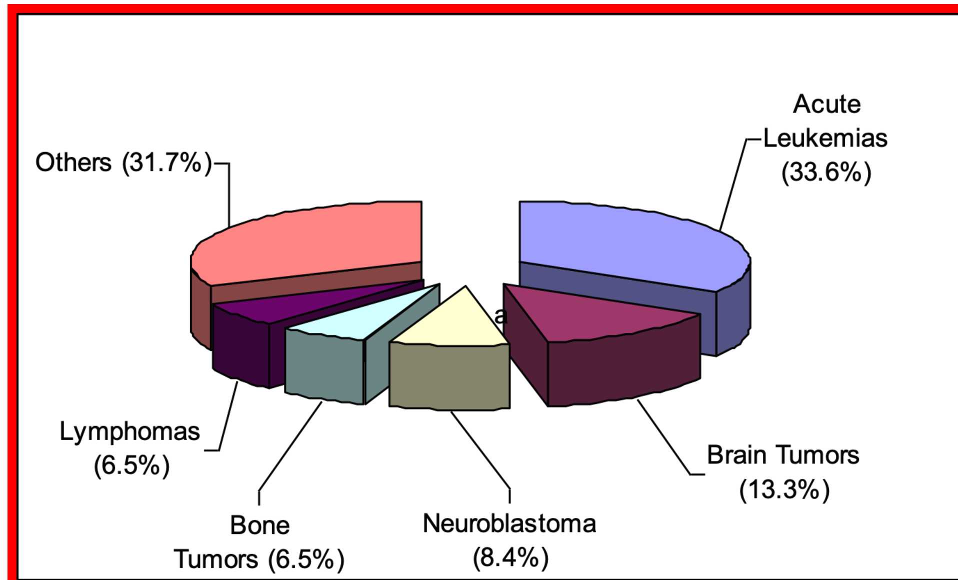
No data is available regarding mortality in India. However, the linear trend of risk of leukemia is 12.2 for males and 2.1 for females in India. *(Linear trend represents the percentage change in risk every five years over the entire data period).*

**Magnitude of the problem in India**

As per the report of National Cancer Registry Program (NCRP), ICMR, acute leukemia incidence in India varies among different registries. Childhood ALL has

a crude incidence affecting 2.13, 3.05 and 3.00 per 100000 children at Mumbai, Chennai and Thiruvananthapuram [Bombay Cancer Registry, NCRP (ICMR), 1994; Population based Cancer Registry, NCRP (ICMR), 1997, Cancer Institute (WIA); Hospital Tumor Registry, Regional Cancer Centre, 1997)]. At Regional Cancer Centre, Thiruvananthapuram, leukemia cases formed 3.9% (351/9002) of all cancers registered during the year 1998. Among this 126 (35.8%) fall in the pediatric age group. The leading sites in pediatric cases are Acute Leukemia (33.6%), Brain tumors (13.3%), Neuroblastomas (8.4%) and Lymphoma (6.5%). Acute leukemias form the most common malignancy reported and 87% of acute leukemias are acute lymphoblastic leukemias (Figure 2).

**FIGURE 2- DISTRIBUTION PATTERN OF PEDIATRIC PATIENTS (ADAPTED FROM THE REGIONAL CANCER CENTRE ANNUAL REPORT, 1998-99)**



## Epidemiology of childhood acute lymphoblastic leukemia

Although it is conceivable that etiologic mechanisms are similar for subsets of adult and childhood leukemia with similar morphologic and molecular characteristics, studies thus far have not had the ability to evaluate this hypothesis. Therefore, there have been many well-designed studies to unveil risk factors for childhood leukemia and several unifying hypothesis have been proposed (Sandler and Ross, 1997). In assessing leukemia risk factors, studies of childhood leukemia have a number of advantages. The interval between exposure to putative risk factors and leukemia onset may be shorter, recall of exposures is likely to be better, and intervening factors may be fewer.

## Etiology of childhood leukemia

### *Genetic syndromes*

Several genetic syndromes have been associated with an increased risk of childhood leukemia. Studies suggest a 10 to 20 fold increased risk of leukemia in children with Down's syndrome (Pui, 1995). Other genetic syndromes associated with childhood ALL and AML include Bloom's syndrome, Neurofibromatosis, Schwachmann syndrome, Ataxia Telangiectasia and Klinefelter's syndrome (Sandler and Ross, 1997). However, by and large, genetic syndromes account for a very small proportion of childhood leukemia.

### *Ionizing radiation*

In utero exposure to diagnostic x-rays is considered one of the few identified causes of childhood leukemia (Sorahan and Roberts, 1993). Recently, there has

been considerable interest concerning the role of maternal preconceptional radiation exposure and subsequent risk of leukemia in offspring (Roman et al., 1997).

***Nonionzing radiation***

Childhood exposure to low-level electromagnetic fields (EMF) has been investigated in several studies. The findings have been somewhat inconsistent and dependant on the particular measure of exposure used. Results of recent studies however do not show any association of increased leukemia with EMF exposure (Ross et al., 1994; Hardell et al., 1995).

***Chemical exposures and others***

Pesticide exposure has been associated with childhood leukemia in a number of studies. Paternal and maternal exposure to chemicals has also been associated with an increased risk of childhood leukemia (Leiss and Savitz, 1995).

## CLINICAL FEATURES OF PEDIATRIC ALL

The signs and symptoms of the child presenting with ALL reflect the degree of bone marrow infiltration with leukemic cells and the extent of extramedullary spread. The most common symptoms and clinical findings are usually manifestations of the underlying anemia, thrombocytopenia, and neutropenia, which reflect the failure of normal hematopoiesis. Pallor, fatigue, petechiae, purpura, bleeding and fever are most often present. Lymphadenopathy, hepatomegaly, and splenomegaly are manifestations of extramedullary leukemic spread. Hepatosplenomegaly occurs in two thirds of the patients and is usually

symptomatic. Lymphadenopathy usually is painless and may be localised or generalized.

The duration of symptoms in children presenting with ALL may vary from days to months. Anorexia, bone pain particularly affecting the long bones, are common and reflects leukemic involvement of the periosteum and bone. Signs and symptoms of central nervous system involvement are rarely observed at the time of initial diagnosis.

A number of presenting clinical features and laboratory findings have prognostic importance (Margolin and Poplack, 1997). Since the child with ALL typically may present with non-specific symptoms, ALL may mimic a variety of non-malignant conditions. Definitive diagnosis of ALL requires a bone marrow aspirate. Childhood ALL must be differentiated from other pediatric malignancies that may present with bone marrow involvement, including neuroblastoma, rhabdomyosarcoma, retinoblastoma, and non–Hodgkin's lymphoma. ALL and childhood non–Hodgkin's lymphoma are closely related disorders, and distinguishing between the two may often be difficult. Additional laboratory and clinical evaluation only can differentiate these two disorders.

## CHARACTERIZATION OF LEUKEMIA

### *Morphologic classification*

There have been several attempts to classify ALL cells morphologically using criteria such as cell size, nuclear cytoplasmic ratio, nuclear shape, number and

prominence of nucleolar nature and intensity of cytoplasmic staining, presence of granules and inclusions and changes in nuclear chromatin (Margolin and Poplack, 1997). One system, however, proposed by the French-American-British (FAB) Cooperative Working Group has been generally accepted (Bennet et al., 1976). This system defines three categories of lymphoblasts (Table 1).

**TABLE 1 - FAB CLASSIFICATION OF LYMPHOBLASTIC LEUKEMIA**

| Cytologic features | L1 | L2 | L3 |
|---|---|---|---|
| Cell size | Small cells predominate | Large, heterogeneous in size | Large homogenous |
| Nuclear chromatin | Homogenous in any one case | Variable, heterogeneous in any one case | Finely stippled and homogenous |
| Nuclear shape | Not visible or small and inconspicuous, more vesicular | Irregular clefting and indentation common | Regular-oval to round |
| Nucleoli | Regular, occasional clefting of indenting | One or more present, often large | Prominent, one or more |
| Amount of cytoplasm | Scanty | Variable, often moderately abundant | Moderately abundant |
| Basophila of cytoplasm | Slight or moderate, rarely intense | Variable, deep in some | Very deep |
| Cytoplasmic vacuolation | Variable | Variable | Often prominent |

L1 lymphoblasts are usually smaller with scanty cytoplasm and inconspicuous nucleoli. Cells of L2 variety are larger and they demonstrate considerable heterogeneity in size, prominent nucleoli and more abundant cytoplasm.

Lymphoblasts of the L3 type, notable for their deep cytoplasmic basophilia are large and frequently display prominent cytoplasmic vacuolation (Figure 3). Approximately 85% of children with ALL have predominant L1 morphology, 14% have L2 and almost 1% has L3 morphology. L1 morphology is associated with a higher remission induction rate and better event free survival compared to tumors with L2 morphology, which appear to convey poor prognosis. Patients with L3 morphology have the worst overall prognosis. Although the FAB classification system appears to have value as a prognostic indicator, no biologic basis for morphologic differences delineated by this system has been identified.

***Immunophenotypic classification***

This is a more clinically relevant classification of ALL and is based on the expression of certain antigens on the surface of leukemic cells. Normal lymphocytes express specific antigens in an orderly fashion through their different stages of differentiation. According to Greaves (Greaves, 1986), lymphoblasts represent an interruption at different steps of differentiation of normal lymphocytes. Therefore, expression of antigens on the cell surface indicates the specific step in differentiation where transformation occurred. Approximately 80% of the cases of ALL arise from B-cell lineage. Most of these cases express the common ALL antigen (CALLA), B-cell differentiation antigens and have undergone immunoglobulin gene rearrangement. Ten to twenty percent of ALL cases arise from the T-cell lineage. Such cases express CD2, CD3, CD5 and CD7 T cell antigens. CALLA is usually absent in cells of the T cell lineage.

***Biochemical characterization***

Various biochemical markers have been studied in ALL. Some have been found to be useful in the diagnosis and classification of the disease; others have been evaluated a potential avenues for selective therapy. Terminal deoxynucleotidal transferase (TdT) is an unusual DNA polymerising enzyme that catalyses the polymerisation of deoxynucleoside monophosphates into a single strand DNA primer without the need for template instruction. It is found in the nucleus and is thought to play a role in immunoglobulin and T-cell antigen receptor rearrangement, influencing the generation of immunologic diversity. Significant TdT activity is usually not present in normal lymphocytes but is detectable in normal cortical thymocytes and in leukemic lymphoblasts of T-cell and B-cell precursor lineage. Determination of TdT activity may be helpful in the diagnosis of ALL and in differentiating ALL from AML, in which TdT activity rarely occurs (Margolin and Poplack, 1997). Because TdT positive cells may present in increased numbers in patients recovering from chemotherapy or bone marrow transplantation, they cannot be used as a sole indicator of bone marrow relapse. Purine pathway enzymes play an important role in normal lymphocyte functions and this pathway has been extensively studied in ALL. A unique pattern of three enzymes, adenosine deaminase, 5’ nucleotidase and purine nucleotide phosphorylase has been observed in ALL. Elevated levels of LDH have been observed in ALL at diagnosis.

## PROGNOSTIC FACTORS

Prognosis of patients with ALL depends on several interrelated factors. These provide a potential means of delineating sub groups that predict relatively favourable or unfavourable clinical outcomes. It is a common practice for treatment centres to assign patients on the basis of prognostic factors into different risk groups and to tailor treatment accordingly. Prognostic characteristics of childhood ALL include age, initial leukocyte count, cytogenetic abnormalities, immunophenotype, CNS involvement, sex, race, extent of lymphadenopathy, presence of mediastinal mass, platelet count, serum immunoglobulin, expression of myeloid antigens, the length of time to attainment of remission, glucocorticoid receptor levels, nutritional status and response to therapy (Friedmann and Weinstein, 2000; Margolin and Poplack, 1997; Cortes and Kantarjian, 1995, Ribeiro and Pui, 1993) (Table 2).

### TABLE 2- PROGNOSTIC FACTORS IN PEDIATRIC ALL

| **Established factors associated with prognosis for patients with ALL** | |
|---|---|
| Initial white blood cell count | Serum immunoglobulin |
| Age at diagnosis | Rapidity of leukemic cell reduction |
| Sex | Myeloid antigen expression on leukemic cells |
| Cytogenetics/ploidy | Nutritional status |
| Immunophenotype | Remission status at day 28 |
| FAB morphology | Hemoglobin level |
| Mediastinal mass | Race |
| Organomegaly and Lymphadenopathy | Platelet count |

**Leukemic cell burden**

Leukemic cell burden can be assessed indirectly by evaluation of the extent of extramedullary disease. The degree of hepatosplenomegaly and lymphadenopathy has emerged in most univariate analysis as important prognostic variables (Margolin and Poplack, 1997).

**Myeloid antigen expression on leukemic cells**

Concomitant expression of myeloid antigen on ALL lymphoblasts of children is associated with poor prognosis (Uckun et al., 1997; Den Boer et al., 1999)

**Response to treatment**

Recently, response to treatment has emerged as an important indicator of prognosis (Schrappe et al., 1996; Arico and Masera, 1997; Lilleyman, 1998). Patients who do not achieve a complete remission within the usual 4-to 6-week/day 28-induction period have a high rate of relapse and shortened survival (Miller et al., 1989). Residual leukemia demonstrable in bone marrow on day 14 of induction is an important independent predictor of outcome (Steinherz et al., 1996). Patients with residual disease on day 14 have a lower rate of complete remission and a greater likelihood of early and late relapse. The day 7-marrow status has also been correlated with treatment outcome (Gaynon et al., 1990 and 1997). Another group reported that the failure to clear peripheral leukemic blasts by day 8 of therapy correlates significantly with a poorer 5-year event free survival (Gajjar et al., 1995).

## TREATMENT FOR ACUTE LYMPHOBLASTIC LEUKEMIA

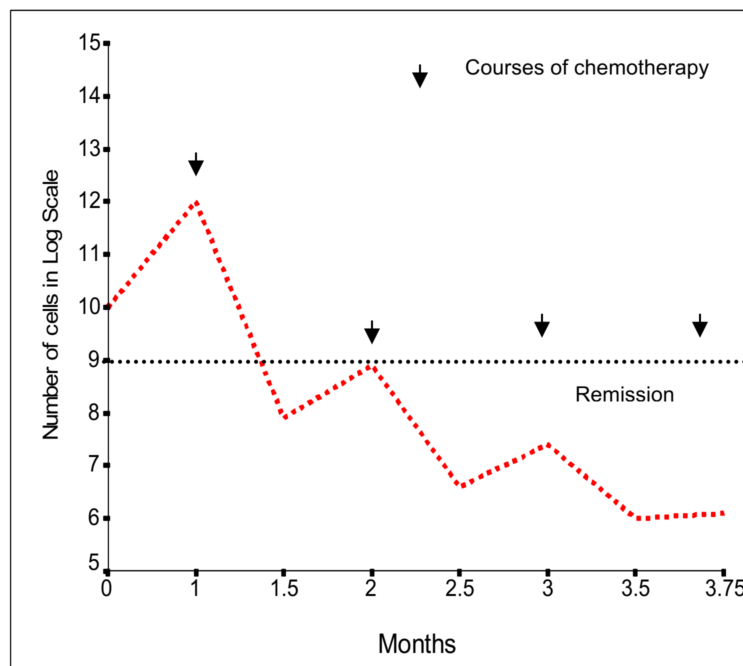
The recognition that ALL is a heterogeneous disease and that children can be stratified into various risk groups has profoundly influenced therapy. Although combination chemotherapy remains the primary therapeutic modality, it is no longer considered appropriate, in the context of current biologic knowledge, for all patients with ALL to be treated on a uniform or standard treatment regimen. Although the specific approaches to patients in various risk groups may be somewhat different, modern ALL treatment regimens divide therapy into four main treatment elements: Remission induction, Central Nervous System (CNS) preventive therapy, Consolidation and Maintenance therapy.

### Induction therapy

The initial aim of ALL treatment is induction of remission. By definition, patients in remission have no evidence of leukemia when evaluated by physical examination and hematologic assessment of bone marrow and peripheral blood after first induction (Margolin and Poplack, 1997). Peripheral blood values must be within the defined range of normality, and the bone marrow must be of normal cellularity, with fewer than 5% lymphoblasts. In clinically overt ALL, the leukemic cell burden is estimated to be approximately $10^{12}$ leukemic cells (Margolin and Poplack, 1997). To induce a complete remission, chemotherapy must reduce the total number of leukemic cells by 99%, leaving fewer than $10^{10}$ blasts. Although the basic two-drug combination of vincristine and prednisone induces remissions in approximately 85% of children with ALL, the addition of L-asparginase, and

anthracycline, or both improves the remission induction rate to approximately 95% (Ortega et al., 1977). The addition of a third agent to vincristine and prednisone also significantly prolonged remission. Whether the added leukemic cell killing theoretically achieved by including a fourth induction agent leads to improvement in remission duration is controversial (Aur et al., 1978). However, protocols using this four-drug induction combination with intensive consolidation and maintenance therapy, uniformly demonstrate improved overall remission duration even for high-risk patients (Reiter et al., 1994; Ritter et al., 1990).

**FIGURE 4- EFFECT OF CHEMOTHERAPY ON LEUKEMIC CELLS**




(*Chemotherapy treatment may be scheduled to exploit the slower regeneration time of leukemia cells compared with that of normal cells. Remission is achieved when there are normal number of blast cells in the bone marrow (<5%) and no leukemic cells are visible on light microscopy).*

### Consolidation and maintenance therapy

Elimination of 99% of leukemia cells is enough to achieve a remission. When diagnosed, if there are approximately one trillion (1,000,000,000,000) leukemia cells in the body, after elimination of 99% of cells by remission therapy, leaves about ten billion leukemia (10,000,000,000) cells. That is why an intensive 6-month program of consolidation treatment is needed after induction and why two years of maintenance chemotherapy is so important. This phase is important for eliminating leukemia from sites where the cells can hide, such as the central nervous system and testes. It is also important to reduce the number of leukemia cells still remaining in the body from ten billion to a much lower, more manageable number. Early studies demonstrated that without additional therapy, most patients relapse within a median of 1 to 2 months; the actual time of unmaintained remission varies with the intensity and duration of induction therapy. The optimal length of maintenance chemotherapy has not been established. Most centres treat patients for a total of approximately 2.5 to 3 years.

### Cancer chemotherapy for pediatric ALL in developing countries

#### *Multi Centre Protocol (MCP)-841 protocol*

This protocol is designed as subunits, which can be put together in different combinations for patients with different survival expectancies. This protocol emphasises on intensive induction and consolidation regimens.

Induction chemotherapy treatment as per the MCP-841 protocol consisted of Doxorubicin (30 mg/m$^2$ IV; days 8, 15, 29), Vincristine (1.4 mg/m$^2$ IV; days 1, 8, 15, 22, 29), Asparginase (6000 u/m$^2$ IM; days 2, 4, 6, 8, 10, 12, 14, 16, 18, 20), Prednisone (40 mg/m$^2$ p.o.; 28 days) and Methotrexate (6-12 mg IT; days 1, 8, 15, 22). Consolidation included Cyclophosphamide (750 mg/m$^2$ IV; days 1, 15), Vincristine (1.4 mg/m$^2$ IV; days 1, 15), Ara-C (70 mg/m$^2$ s.c; days 1-3 and 15-17) and 6-MP (75 mg/m$^2$ po; days 1-7 and 15-21) followed by Maintenance therapy consisting Vincristine (1.4 mg/m$^2$ IV; day 1, Doxorubicin (30 mg/m$^2$ IV; day 1), Prednisone (40 mg/m$^2$ po; daily days 1-7), Asparginase (6000 u/m$^2$ IM; days 1, 3, 5, 7), Methotrexate (15 mg/m$^2$ p.o once a week from day 15, missing every 4$^{th}$ week), 6 MP (75 mg/m$^2$ p.o daily from day 15, 3 weeks out of every 4 (i.e., one week gap every month).

**MOLECULAR PATHOGENESIS**

Improvement in cytogenetic and immunophenotyping techniques combined with advances in molecular biology has enabled the characterisation of leukemic lymphoblasts at molecular level. The molecular pathogenesis of ALL appears to be similar to that of most cancers (Rabbitts, 1994; Cline, 1994). The induction of malignancy may be the result of a single mutation, but in many instances, it may be attributed to the effects multiple mutational events (Cline, 1994; Frebourg and Friend, 1992). Altered patterns of oncogene expressions or direct mutations of normal genes (i.e., proto-oncogenes) can create oncogenes that induce malignancy. Similarly, mutations or losses of genes that prevent the formation of

cancer (tumor suppressor genes) can produce ALL. Oncogenes and tumor suppressor genes usually are derived from normal genes involved in cellular proliferation, differentiation or survival processes. Many of these genes code for signal transduction, transcription factors, and cell cycle regulating proteins. Multiple leukemia related antigens have been cloned after identification of individual genes involved in leukemic translocations (Margolin and Poplack, 1997). Other genetic events, such as multiple identified translocations involving the immunoglobulin and T cell Receptor (TCR) loci and various transcription factors, have also been useful in elucidating general mechanism of leukemogenesis. In addition to chromosomal translocations, there are a variety of genetic events that appear to be leukemogenic but not detectable with classical cytogenetic methods. These include small deletions and mutations of DNA that can inactivate tumor suppressor genes or activate oncogenes. Mutations of the genes encoding p53, p16 or p15, the interferon genes located on 9p, WT1 at 11p13 and the TEL and KIPI loci on 12p12-p13 have been described in fresh ALL samples and ALL derived cell lines (Felix et al., 1994; Cheng et al., 1990; Stegmaier et al., 1995; Mensen et al., 1995; Hebert et al., 1994; Delmer et al., 1995). Although the abnormalities detected in most of these genes may not be definitively leukemogenic as the bcr-abl and other fusion gene mutations, they are commonly observed in pediatric ALL and appear to contribute to the development of leukemia.

The expansion of the cell population is the net result of cell birth by mitosis and cell loss by cell death and /or by differentiation. Because differentiation is blocked in ALL, cell loss is based mainly on cell death occurring in the form of programmed cell death (PCD) or apoptosis (Hirt et al., 1997). Defects in the processes controlling PCD can extend cell life span, contributing to neoplastic cell expansion independently of cell division. Therefore, another potential mechanism in the development of ALL involves mutational events that prevent apoptosis. Moreover, failures in normal apoptosis pathways contribute to carcinogenesis by creating a permissive environment for genetic instability and accumulation of gene mutations, promoting resistance to immune based destruction, allowing disobeyance of cell cycle check points that would normally induce apoptosis, facilitating growth factor independent cell survival, reducing dependence on oxygen and nutrients, and conferring resistance to cytotoxic anticancer drugs and radiation (Reed, 1999).

# CHAPTER 2

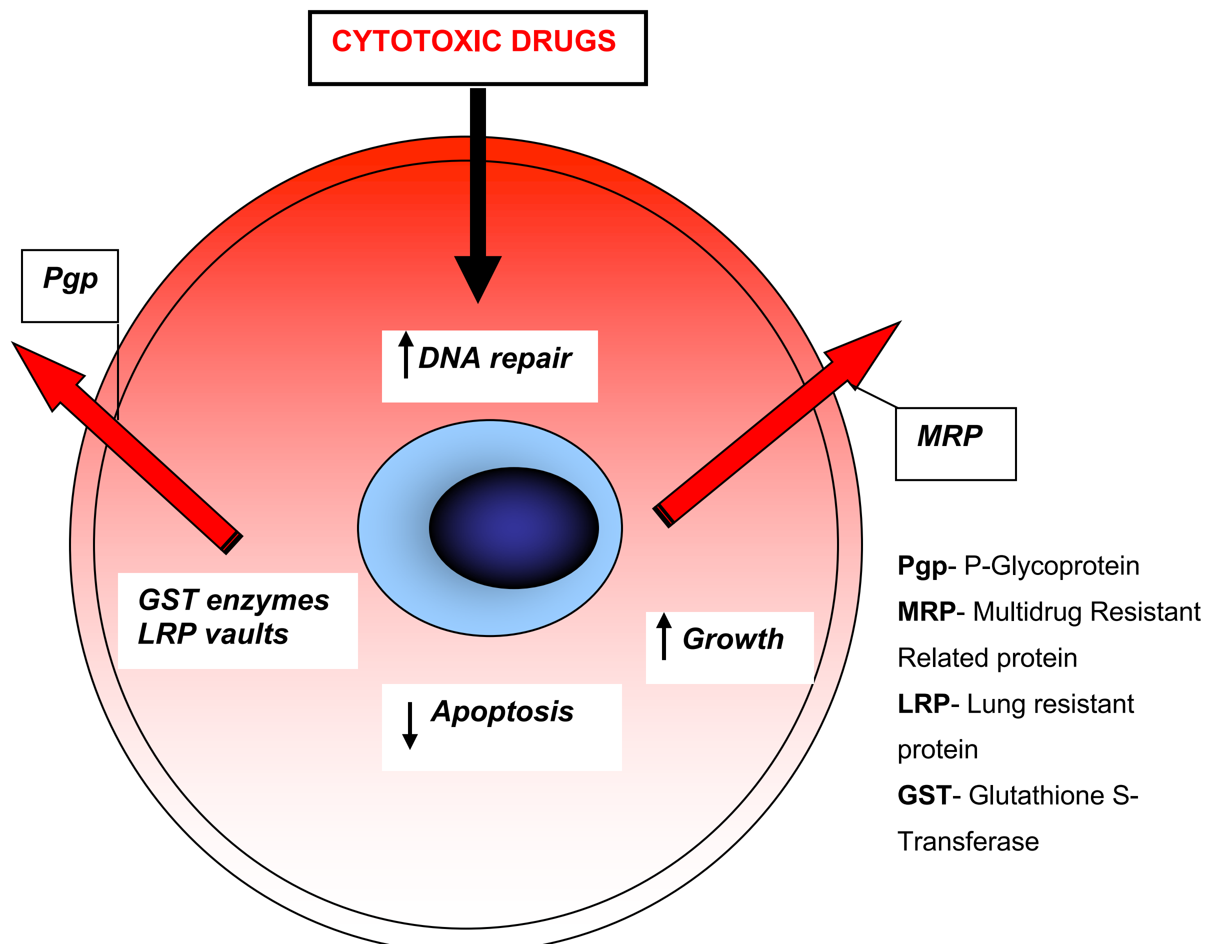
## *OVERALL RESEARCH DESIGN*

### NEED FOR PROGNOSTICATION OF DISEASE

Twenty-five years ago, the prognosis of children suffering from ALL was very poor, and most of them survived only 2 or 3 months after diagnosis (Margolin and Poplack, 1997). Over the past few years, cure rate has dramatically improved for ALL, mainly as a result of the identification of better prognostic indicators and newer therapeutic modalities. Long term event free survival (EFS), only 5% in 1965, has now increased to 70% (Pui, 1994 and 1995). However, current protocols fail in about 30% of children with newly diagnosed ALL; bone marrow relapses representing the most common treatment failures (Pui, 1994). Clinical markers have been used to define high, low and intermediate risk groups. However, these have limitations because some patients with no adverse clinical or chromosomal markers do not respond to therapy. Therefore, identification of newer clinical and biological features that accurately predict patient response to therapy has been a continuing goal of leukemia specialists. The combination of such biologic parameters will allow better identification of standard and high-risk groups, the latter being characterized by less remission and lowest survival rates. Cellular drug resistance is related to a high risk of treatment failure in childhood leukemia. It is well known from early single agent studies during the development of ALL treatment, that a proportion of children with initial ALL were not

responsive to a single drug (Kaspers et al., 1994). Primary resistance to chemotherapeutic agents may be an important reason for relapses in ALL. A variety of reasons have been postulated to be the reason fior the development of drug resistance exhibited by leukemic cells (Figure 5) (Ivy et al., 1996; Gottesman et al., 1993).

**FIGURE 5- SCHEMATIC DIAGRAM REPRESENTING DRUG RESISTANCE MECHANISMS IN LEUKEMIA CELLS**




*(Pgp and MRP can effectively efflux cytotoxic drugs from the cell. GST enzymes can detoxify drugs in the cytoplasm and LRP vaults may be involved in the re distribution of drugs away from the nucleus. Increased DNA repair, and decreased susceptibility to apoptosis can also confer a drug resistant phenotype).*

The fact that tumors may develop resistance to multiple drugs even before treatment has major implications for cancer therapy (Pieters et al., 1991) Earlier studies have shown clinical importance of de novo in vitro drug resistance. When children with ALL at diagnosis were divided into a resistant and a sensitive group by the median $LC_{50}$ value of specific drugs, the risk of relapse was significantly higher in the resistant group (Hongo et al., 1997). Clinical resistance can also be due to the reduced efficacy of the apoptotic machinery.

It has now been established that most (if not all) of the cytotoxic agents used in treatment protocols for leukemia kill cells by inducing apoptosis (Ling et al., 1993; Tosi et al., 1994; Begleiter et al., 1994). Expression of the cell death controlling genes has been shown to affect chemosensitivity. Thus preliminary studies have suggested that the nature of genes that define the capability of cells to undergo apoptosis after drug treatment may be an important arbiter of therapeutic response in leukemia.

We have in the present study looked for associations between in vitro drug sensitivity as detected by MTT assay in a group of pediatric patients with ALL. The results of this study will delineate those patients in whom therapeutic strategies should be altered so as to achieve maximum clinical outcome with reduced deaths due to toxic effects of drugs.

## RATIONALE FOR THE STUDY

### Need for individualization of treatment: role of drug sensitivity evaluation

Chemotherapy is the mainstream treatment protocol for children with ALL. At most centers in India, pediatric ALL patients are treated according to the M C P - 841 regimen which includes induction, consolidation and maintenance phases consisting of Daunorubicin (30 $mg/m^2$ IV; days 8, 15, 29), Vincristine (1.4 $mg/m^2$ IV; days 1, 8, 15, 22, 29), Asparginase (6000 $u/m^2$ IM; days 2, 4, 6, 8, 10, 12, 14, 16, 18, 20), Prednisone (40 $mg/m^2$ p.o.; 28 days) and Methotrexate (6-12 mg IT; days 1, 8, 15, 22). Most of the children achieve complete remission after induction treatment while a small percentage of group tend for induction failure and further relapse. Another good percentage of patients further enters into complications due to the drug toxicity which some times proves fatal.

Glucocorticoids are highly cytotoxic to lymphocytes and thus have been a key component of treatment regimen for ALL; Prednisone being the most commonly used of these compounds in ALL therapy. However, toxicity often causes complex problems including peptic ulcer, obesity, diabetes, osteoporosis and phsysocis. Anthracyclins such as Daunomycin and Doxorubicin are currently used in combination with several other classes of drugs in treatment for ALL. However, clinical use is limited by cardio toxicity and development of drug resistance. Effects caused by other agents have been summarised in Table 3.

**TABLE 3-CHEMOTHERAPEUTIC AGENTS USED AND ITS DOCUMENTED TOXIC EFFECTS**

| DRUG USED | TOXICITY |
|---|---|
| **CORTICOSTEROIDS** | Peptic ulcer, Obesity, Diabetes, Osteoporosis, Psychosis |
| **ANTHRACYCLINS** | Cardiac Toxicity, Myelosuppression, Phlebitis, Mucositis |
| **VINCRISTINE** | Neuropathy, Hair loss, Alopecia, Paresthesias |
| **L- ASPARGINASE** | Hypersensitivity, Low albumin and Coagulation factors, Pancreatitis, CNS toxicity |
| **METHOTREXATE** | Mouth ulcers, Gut toxicity, Hepatotoxicity, |
| **6- MERCAPTO PURINE** | Jaundice, GI Ulceration, Anorexia |
| **ARA-C** | Gut toxicity, Hemolytic Anemia, Stomatitis |
| **HYDROXY UREA** | Gut toxicity, Atrophy |
| **ADRIAMYCIN** | Loss of hair, dose related Cardiac toxicity |
| **CYCLOPHOSHMAMIDE, CHLORAMBUL, BULSULFAN** | Cardiomyopathy, Loss of hair, Marrow Aplasia, Pulmonary Fibrosis, Hyper Pigmentation |

Thus with the current method of treatment, some patients do develop resistance to chemotherapy or relapse at a later stage. Standard therapy dose may not be therefore sufficient enough in some group of patients to completely eradicate tumor cells, while in others the drug concentration may be in excess of optimum higher enough to exert adverse effects. This difference arises due to the changes in the tumor cellular characteristics manifested in these patients. It is therefore vital to develop effective therapy for children with ALL in whom no remission occurs or later suffer relapse with current protocols. It is also important to protect

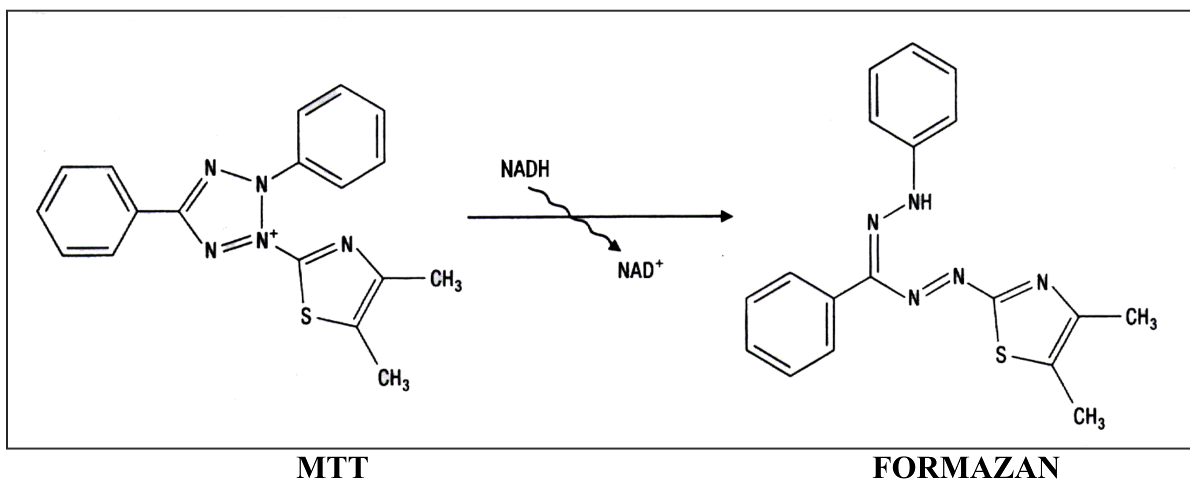
the patient from toxicity due to over dosage. It is also an accepted fact that overdosage of drugs leads to the further complications during the treatment period. Therefore, monitoring of individual sensitivity of these drugs becomes mandatory in these patients undergoing treatment. Advance prognostic information can also stimulate clinical trials to reduce toxic effects or can indicate the need of more intense treatment.

**IMPORTANCE OF IN VITRO ASSAYS FOR DRUG SENSITIVITY**

In vitro cell culture drug sensitivity assays have a number of potentially valuable applications in leukemia as well as in other malignancies. Recently, a number of studies have reported the clinical relevance of in vitro drug sensitivity assays in childhood leukemia (Hongo et al., 1997; Kaspers et al., 1997). Although they are already being used for drug screening, there is a reluctance to use these assays for selection of patients for risk stratified therapy and individualised tailored therapy. Earlier studies show that children with ALL with in vitro drug resistant leukemic cells have a poorer prognosis compared to patients with relatively sensitive cells at initial diagnosis (Hongo et al., 1997). The poor proliferative capacity of ALL cells in vitro, limits the use of long-term clonogenic assays. Recently there has been an increase in reports of a short term culture drug resistance assay using MTT (3 - [4 ,5- dimethyl thiazol - 2 - yl ] - 2, 5 - diphenyl tetrazolium) dye (Hongo et al., 1997; Carmichael et al., 1987)(Figure 6). These assays are based on measuring total cell kill of both proliferating and non-

proliferating cells and in fact, measures the end effect of actual resistance mechanisms.

**FIGURE 6- MOLECULAR STRUCTURE OF MTT AND ITS CORRESPONDING REACTION PRODUCT**




**MTT** **FORMAZAN**

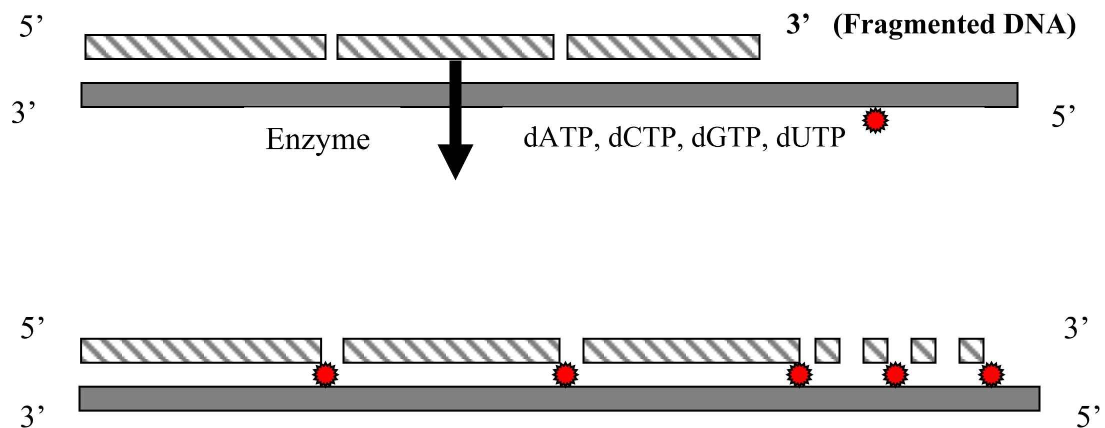
(3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl
-2H-tetrazolium bromide)

This assay first described by Black and Speer in 1954 and revised by Mossmann in 1983 (Mosmann, 1983) is now being adapted for testing ALL cells. This assay can be applied clinically to select effective drugs as it can be performed in a 96 well plate, and results can be analyzed using a scanning multiwell spectrophotometer. Thus the 4-day semi automated MTT assay is an efficient tool for large scale drug resistance testing. A precise identification of those children with poor prognostic features will greatly increase the probability of applying more effective therapeutic approaches. Individual tumors, even those of the same histologic type, show sensitivity to a specific cytostatic agent. This

variation in sensitivity can help attempt to develop an individualized chemotherapy programme and thus sensitivity testing serves as an orientational aid in planning chemotherapy.

**Determination of apoptosis by TUNEL assay**: Terminal deoxynucleotidyl transferase mediated biotin dUTP Nick End Labeling (TUNEL) assay is a non-radioactive system designed to provide simple, accurate and rapid detection of apoptotic cells *in situ* at the single cell level (Gorzycza et al., 1993). The system can be used to assay apoptotic cell death in both tissue sections and cultured cells. This method measures nuclear DNA fragmentation, an important biochemical indicator of apoptosis in many cell types (Figure 7).

**FIGURE 7 - PRINCIPLE OF *IN SITU* END LABELING TECHNIQUE**




*(At the top of the figure, fragmented DNA, which contains free 3'OH group, acts as a substrate for the action of DNA polymerase. In the presence of a labeled nucleotide (dUTP*), the label is incorporated into the DNA breaks)*

## WORKING HYPOTHESIS

- Cellular drug resistance may play an important role in induction failure and relapse.
- Clinical outcome is often highly altered by the process of drug resistance.
- In vivo drug resistance/sensitivity may be effectively predicted by performing a dye based in vitro drug resistance assay.
- In vitro drug resistance may be related to apoptotic process.
- The above data of drug sensitivity may have clinical application, to either reduce toxic doses or to initiate more intensive treatment.

## SPECIFIC OBJECTIVES

- Evaluation of in vitro drug sensitivity of individual drugs by MTT assay in pediatric ALL.
- Correlation of in vitro drug sensitivity and apoptosis induction to in vivo response of the individual patient.
- Correlation of the drug resistance assay to final clinical outcome and prognosis.

# CHAPTER 3

## *METHODOLOGY*

### PATIENT SAMPLES

A total of 72 cases of confirmed pediatric acute lymphoblastic leukemia in whom the diagnosis had been made by standard clinical and hematological criteria (4) were included in the present study. The study population included patients from all parts of Kerala, India and were aged between 1-12 yrs. The lineage of the leukemic cells were analysed using CD2, CD3, CD10 monoclonal antibodies (Dako, Denmark). Myeloid leukemias were excluded by characteristic morphologic features and further histochemically by myeloperoxidase and Sudan black staining. Ethics and Human Subject clearance was obtained from the Institutional Review Board and Ethical Committee of the Regional Cancer Centre. All patients underwent induction chemotherapy treatment as per the MCP-841 protocol, consisting of Doxorubicin/Daunorubicin (30 mg/m$^2$ IV; days 8, 15, 29), Vincristine (1.4 mg/m$^2$ IV; days 1, 8, 15, 22, 29), Asparginase (6000 u/m$^2$ IM; days 2, 4, 6, 8, 10, 12, 14, 16, 18, 20), Prednisone (40 mg/m$^2$ p.o.; 28 days) and Methotrexate (6-12 mg IT; days 1, 8, 15, 22). Follow up of the patients were done by regular check up of patient/ disease status (bone marrow status) at definite intervals. The median follow up time was seventeen months with the minimum being one month and the maximum being sixty-eight months. All clinical and laboratory details were collected from individual medical records. Complete remission was defined as less than 5% of leukemic blasts in representative bone

marrow containing megakaryocytes and granulocytic precursors with some degree of maturation, and no manifestation of leukemia elsewhere after the completion of induction therapy.

Five ml of blood was collected by venipuncture into sterile heparinized tubes, before the commencement of chemotherapy. In most cases, the mononuclear cell compartment was flooded with lymphoblasts. Mononuclear lymphoid cells were isolated by density gradient centrifugation using the method described by Boyum (1968). Cells were washed twice in PBS and resuspended in RPMI 1640 (Gibco BRL) containing 10% fetal calf serum (FCS). The mean percentage of lymphoblasts from these patients was 90 ± 2.7% (range: 80 to 95%). Leukemic lymphoblasts thus obtained were checked for viability by Trypan Blue dye exclusion assay and were then considered for the study.

**IN VITRO SENSITIVITY ASSAY**

The *in vitro* sensitivity to drugs was tested by a methyl-thiazol-tetrazolium (MTT) assay with drugs in six serial fold dilutions (Klumper et al., 1995). The used drug concentration range covered the clinical plasma concentrations (Klumper et al., 1995). Briefly, 96 well micro culture plates contained 100 µL cell suspensions with 6 concentrations of each drug (in quadruplicate samples). Six wells contained leukemic cells in drug free medium to determine the control cell survival and the percentage of leukemic cells after culture. The following drugs and range of concentration were tested: Doxorubicin (0.008 to 8 µg/mL),

Prednisone (0.08 to 250 µg/mL), Vincristine (0.05 to 50 µg/mL), L-Asparginase (0.003 to 10 IU/mL), 6-MP (15.6 to 500 µg/ mL), Cytarabine (0.002 to 2.5 µg/mL) and Dexamethsone (0.0002 to 8 µg/mL). All drugs were obtained from Sigma (MO, USA). Drugs were freshly prepared in RPMI-1640 and stored at -70$^{o}$C. After 4 days of incubation of cultures in 5% carbon dioxide at 37$^{0}$ C in a humidified incubator, 10 µL of MTT was added to each well and subsequently incubated for another 6 h. The yellowish tetrazolium salt MTT is reduced to dark coloured formazan by viable cells only. Formazan crystals were dissolved in acidified isopropanol. The optical density (OD) was measured at 570 nm with a Multiskan MS ELISA Reader (Labsystems, Helsinki, Finland). The leukemic cell survival (LCS) was calculated as follows:

$$LCS = (OD_{\text{drug exposed}} / \text{mean } OD_{\text{control}}) \times 100\%.$$

The drug concentration lethal to 50% of the leukemic cells (the $LD_{50}$) calculated from the individual dose response curves was used as a measure of resistance. For each single drug, patients were classified into two groups, either as sensitive (S) (whose $LD_{50}$ values were lower than median $LD_{50}$) or resistant (R) (whose $LD_{50}$ values were equal to median or higher than the median $LD_{50}$). Patients were also classified into three categories, super sensitive (SS), intermediate sensitivity (IS) and relative resistance (RR) by sensitivity to the combination of four drugs (DAVP; Doxorubicin, Asparginase, Vincristine, Prednisone). SS was defined as S to all four drugs, IS as S to two or three drugs, and RR as S to no drugs or to one

drug. At Regional Cancer Centre, Daunorubicin/Doxorubicin is used in the induction therapy regimen and hence is considered important for the above four-drug classification.

## DETERMINATION OF APOPTOSIS

Apoptosis was evaluated in all samples by **T**erminal deoxynucleotidyl mediated Biotin d**U**TP **N**ick **E**nd **L**abeling (TUNEL) assay standardized by us before and explained in detail elsewhere (Srinivas et al., 2000). Briefly, cell smears were incubated with 50 µl of labelling mixture under a coverslip for 1h at $37^0$C. The labelling mixture consisted of 0.01mM biotin-dUTP, dCTP, dGTP and dTTP (Perkin Elmer, USA) in 50 mM Tris HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.005% bovine serum albumin and 5 units of Klenow enzyme (Boehringer Mannheim, Germany). Endogenous peroxidase activity was blocked by incubation in $H_2O_2$/methanol for 20 min. After washing, the smears were subjected to the Avidin-Biotin Peroxidase procedure and colour developed by using Diamino Benzidine (DAB) as chromogen. Smears were counterstained by haematoxylin and mounted with DPX. Negative control was run by adding PBS instead of Klenow enzyme. At least 500 tumor cells were counted and results expressed as percent positive cells. All the blood samples were processed with utmost care with respect to lymphocyte isolation procedure, preparation of cell smear and cell fixation to avoid errors during sample preparations. All the TUNEL reactions were done within 2-3 days of preparation of slides. Apoptotic index/PCD index was calculated by the following formula.

$$\text{Apoptotic Index} = \frac{\text{Number of TUNEL positive cells}}{\text{Total number of cells counted}} \times 100$$

**STATISTICAL ANALYSIS**

Data analysis was done using the Statistical Software for Social Sciences (SPSS) statistical package (Windows version 9). The Kaplan Meier method was used for estimation of overall survival and log rank test was conducted for assessing the difference between curves. Overall survival (OS) duration measured from the date of ALL diagnosis was established to the date of death or (in censored cases) the date of last follow up. A five-year follow up period was taken for the calculation of OS. In case of induction failure OS was taken as 30 days.

# CHAPTER 4

## *RESULTS*

We analysed seventy-two samples during this pilot study. Basic clinical data of the patients included in this study are listed in Table 1.

**TABLE 4 – CHARACTERISTICS OF 72 CHILDHOOD ALL PATIENTS**

| Feature | Category | Number |
|---|---|---|
| Age | ≤ 1year<br>2-9 year<br>≥ 10 year | 1<br>59<br>12 |
| Leukocyte count | <50 x$10^3$/L<br>≥50 x$10^3$/L | 38<br>34 |
| FAB Morphology | L1<br>L2 | 12<br>60 |
| Lymphadenopathy | No<br>Yes | 29<br>43 |
| Hepatosplenomegaly | No<br>Yes | 19<br>53 |
| Mediastinal Mass | No<br>Yes | 65<br>7 |
| Sex | Male<br>Female | 48<br>24 |
| Serum LDH | < 500<br>≥ 500 | 56<br>16 |
| Platelet count | < 50 x$10^3$/L<br>≥ 50 x$10^3$/L | 41<br>31 |
| Hb level | <7.5<br>≥7.5 | 48<br>24 |
| Remission Status | Complete<br>No | 67<br>5 |

*De novo* apoptosis as detected by TUNEL assay and morphological criteria was detected in 68% of the cases (49/72). A positive TUNEL reactivity was observed

as intense brown nuclear staining (Figure 8). The extent of apoptosis varied from 0 to 30 (mean apoptotic index = 7.4 ± 1.05%). The median apoptotic index was 4. We therefore classified patients into two groups, i.e., one with apoptotic index >4 and those with apoptotic index <4.

$LD_{50}$ values for the drugs used were determined from individual dose response curves. $LD_{50}$ values varied markedly between the patient samples for all drugs analysed and the median values ($LD_{50}$) of each drug are given in Table 5.

**TABLE 5 – MEDIAN CONCENTRATION OF $LD_{50}$ AND RANGE OF EACH DRUG TESTED**

| Abbreviation (Drug Name) | Median Concentration (µg/mL) of $LD_{50}$ | Range (µg/mL) Minimum- Maximum |
|---|---|---|
| Doxorubicin (DOX) | 0.307 | 0.005-10.322 |
| Vincristine (VCR) | 1.692 | 0.008-53.657 |
| L- Asparginase (L-ASP) | 0.249# | 0.02-8.470# |
| Prednisone (Pred) | 5.025 | 0.015-339.96 |
| 6- Mercapto Purine (6 MP) | 71.51 | 9.35-523.64 |
| Cytarabine (Ara-C) | 0.053 | 0.02-1.824 |
| Dexamethasone (DEX) | 0.097 | 0.001-0.979 |

## CORRELATION OF APOPTOSIS AND IN VITRO SENSITIVITY ASSAY WITH CLINICAL PARAMETERS

There was no correlation between DPAV sensitivity and any of the clinical parameters analyzed except for CR status ($P$ < 002). Apoptotic index was correlated with the CR status and organ involvement. Higher the apoptotic index better the CR and lesser the chance of organaomegaly. There was also a positive correlation with serum uric acid levels and higher apoptotic index.

## CORRELATION OF APOPTOSIS WITH IN VITRO SENSITIVITY ASSAY

It is now understood that anticancer drugs act through induction of apoptotic program. Therefore, it was possible that there could be an association between the resistant pattern exhibited by patients with regard to DPAV sensitivity and propensity for apoptosis. In order to investigate whether any such correlation exist between drug sensitivity and apoptosis, Spearman correlation analysis was done. Patients with a lower apoptotic index exhibited a RR pattern for DPAV sensitivity (r value = -0.3325; p = 0.036). There was also a correlation between the patients in the different groups (SS, IS, RR) and the apoptotic index calculated. The patients in the SS group had an apoptotic index of 8.89 ± 1.6, in the IS group, 7.13 ±1.2 and those in the resistant group was 6.2 ± 2%. These results further substantiate that defective apoptosis could be the cause for the patients being resistant to front line drugs.

## CLINICAL OUTCOME

In vitro drug sensitivity was correlated with both short and long term clinical outcome. CR was defined as less than 5% leukemic blasts in representative BM containing megakaryocytes and granulocytic precursors with some degree of maturation, and no manifestation of leukemia elsewhere. Status after five-year follow up was used to find out overall survival (OS).

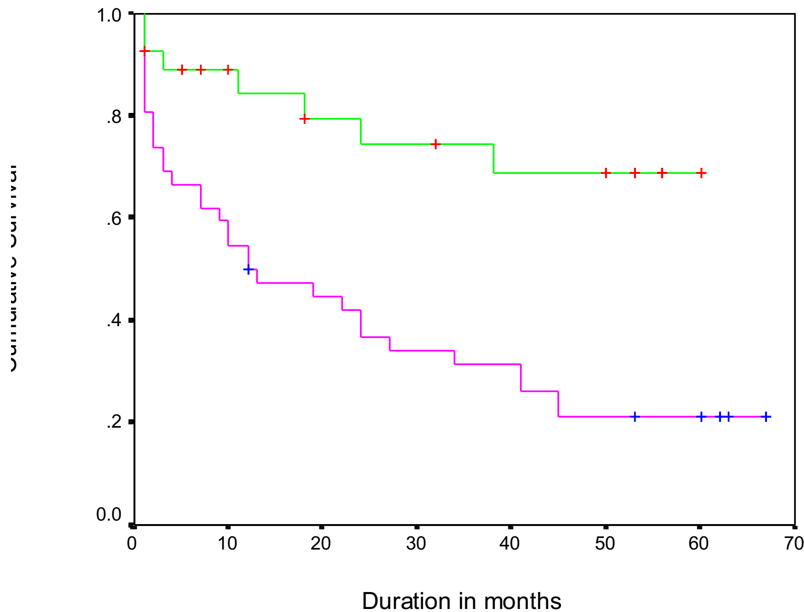
## DRUG SENSITIVITY TESTING AND PROGNOSIS

We then analysed for survival according to patients who had shown $LD_{50}$ value less than and greater than the median values, sensitivity to Prednisone, Asparinase, Vincristine and 6-mercapto purine proved to be of prognostic importance (log rank test, $P < 0.005$). More specifically, patients sensitive to Prednisone, Asparginase, Vincristine and 6-MP had higher OS compared to patients whose blast cells were resistant to these drugs. For the other drugs tested (Doxorubicin, Dexamethasone and Cytarbine) OS did not vary from that of the resistant patients (Table 6)(Figure 9-15).

## TABLE 6 – OVERALL SURVIVAL OF THE PATIENTS SENSITIVE FOR DRUGS

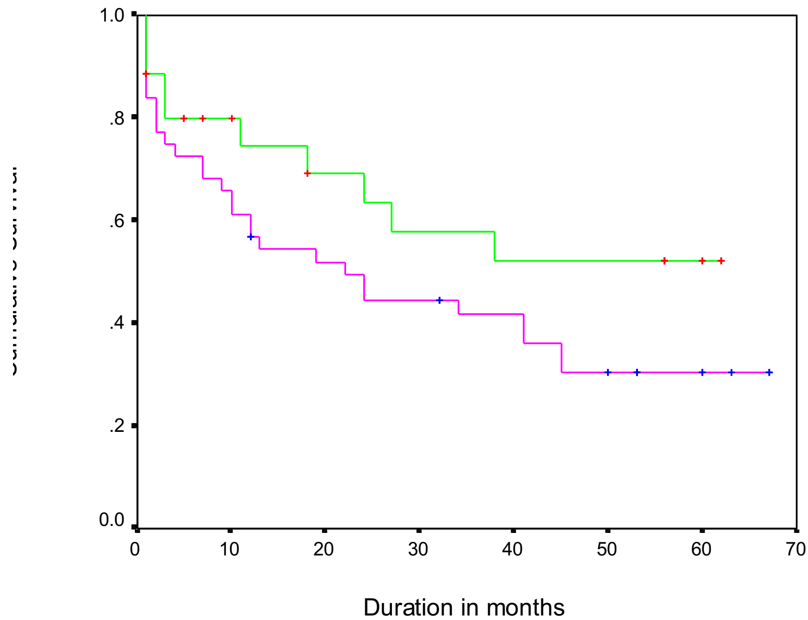
| Drug | Cumulative survival (sensitive vs resistant) | Log rank P value |
|---|---|---|
| Prednisone | 69, 21 | 0.005 |
| Vincristine | 66,14 | 0.002 |
| L- Asparginase | 74, 13 | 0.0001 |
| Doxorubicin | 52, 30 | 0.13 |
| 6- Mercapto Purine | 70, 14 | 0.005 |
| Cytarabine | 50,30 | 0.75 |
| Dexamethasone | 40,35 | 0.51 |

## FIGURE 9 - SURVIVAL CURVE FOR PATIENTS WITH REGARD TO PREDNISONE SENSITIVITY



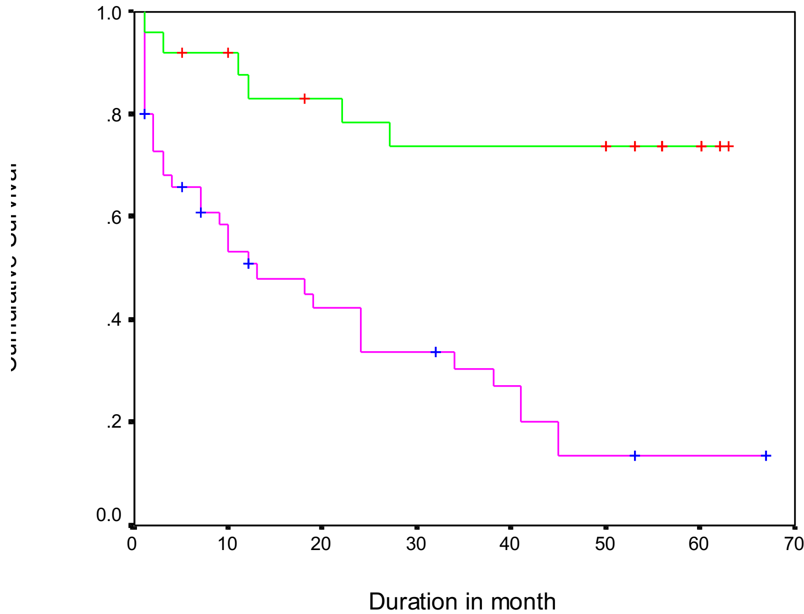

*When analyzed for survival according to patients who had shown $LD_{50}$ value less than and greater than the median values, prednisone sensitivity proved to be of prognostic importance(69 % versus 21%; log rank test, p value = 0.006)*

## FIGURE 10 - SURVIVAL CURVE FOR PATIENTS WITH REGARD TO DOXORUBICIN SENSITIVITY



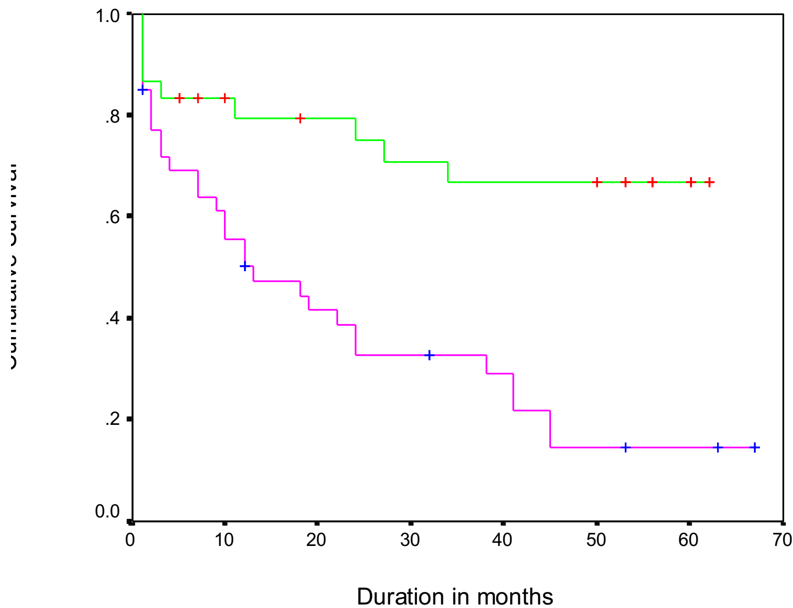

*When analyzed for survival according to patients who had shown $LD_{50}$ value less than and greater than the median values, doxorubicin sensitivity proved not to be of prognostic importance. (52 % versus 30%; log rank test, p value = 0.13)*

## FIGURE 11 - SURVIVAL CURVE FOR PATIENTS WITH REGARD TO ASPARGINASE SENSITIVITY



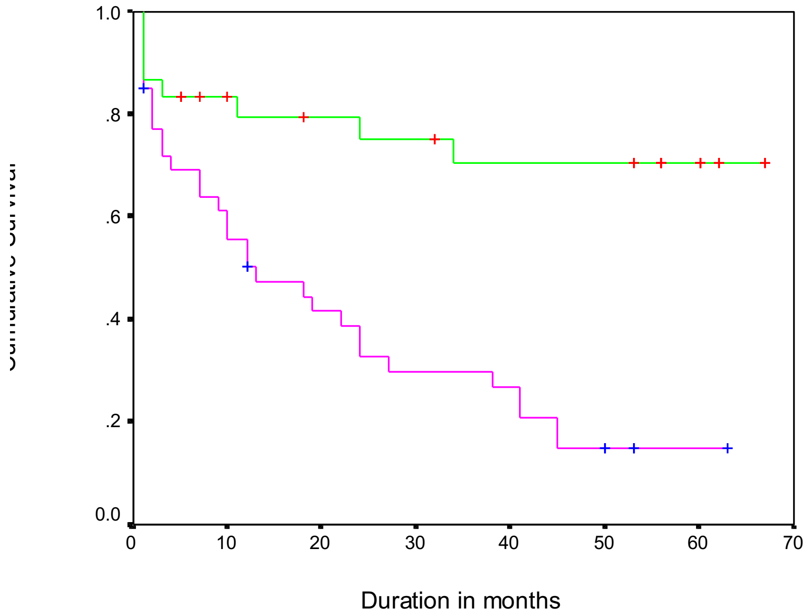

*When analyzed for survival according to patients who had shown $LD_{50}$ value less than and greater than the median values, asparginase sensitivity proved to be of prognostic importance. (74 % versus 13%; log rank test, p value = 0.0001)*

## FIGURE 12 - SURVIVAL CURVE FOR PATIENTS WITH REGARD TO VINCRISTINE SENSITIVITY



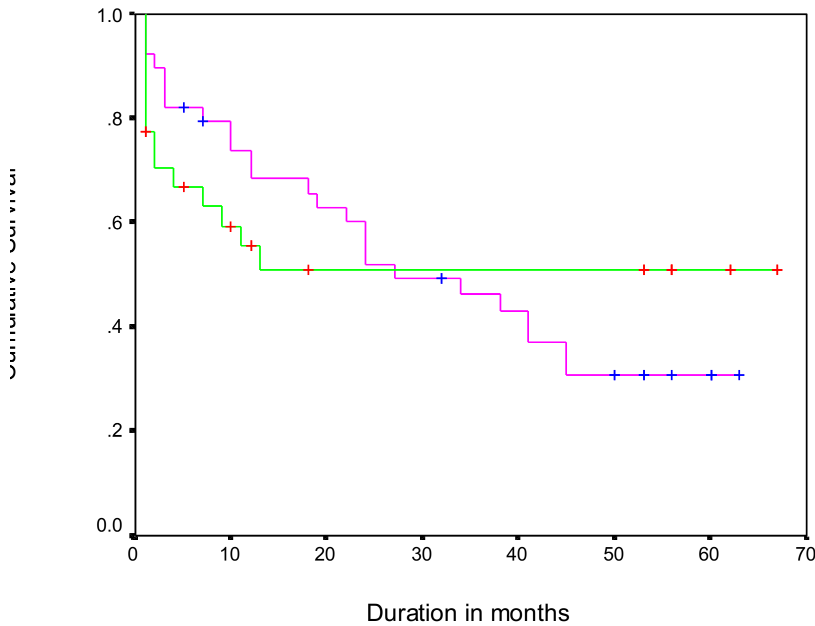

*When analyzed for survival according to patients who had shown $LD_{50}$ value less than and greater than the median values, vincristine sensitivity proved to be of prognostic importance. (66 % versus 14%; log rank test, p value = 0.002)*

**FIGURE 13 - SURVIVAL CURVE FOR PATIENTS WITH REGARD TO 6-MERCAPTOPURINE SENSITIVITY**



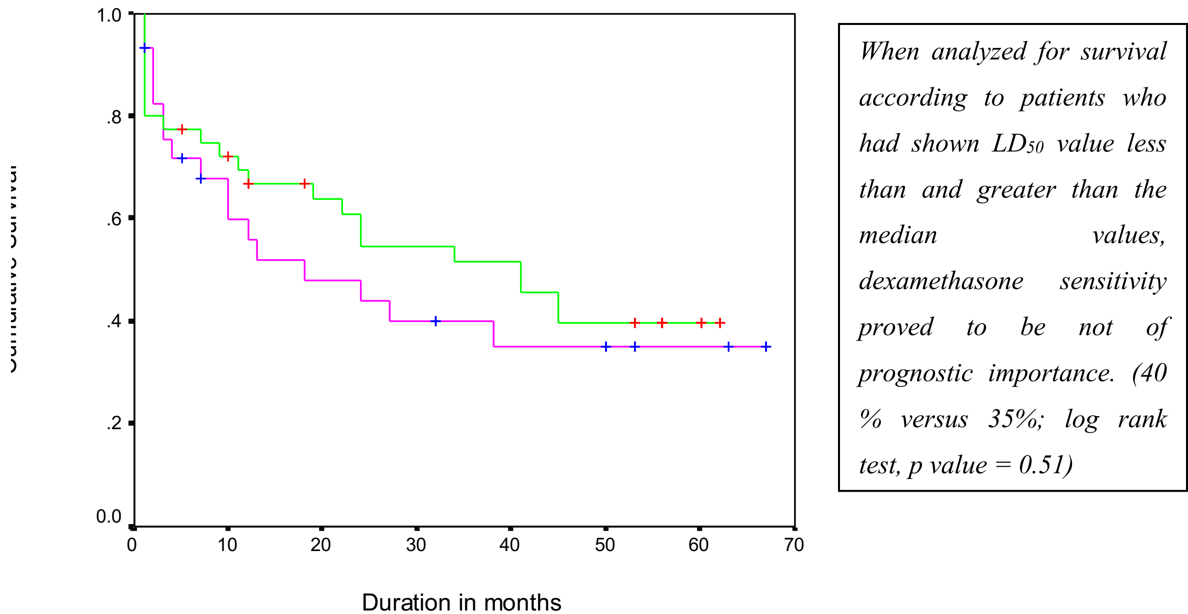

*When analyzed for survival according to patients who had shown $LD_{50}$ value less than and greater than the median values, 6-mercapto purine sensitivity proved to be of prognostic importance. (70 % versus 14%; log rank test, p value = 0.005)*

**FIGURE 14 - SURVIVAL CURVE FOR PATIENTS WITH REGARD TO ARA-C SENSITIVITY**




*When analyzed for survival according to patients who had shown $LD_{50}$ value less than and greater than the median values, ara-C sensitivity proved not to be of prognostic importance. (50 % versus 30%; log rank test, p value = 0.75)*

**FIGURE 15 - SURVIVAL CURVE FOR PATIENTS WITH REGARD T O DEXAMETHOSONE SENSITIVITY**




*When analyzed for survival according to patients who had shown $LD_{50}$ value less than and greater than the median values, dexamethasone sensitivity proved to be not of prognostic importance. (40 % versus 35%; log rank test, p value = 0.51)*

Patients were then classified into three groups according to their DAVP combination sensitivity. DAVP sensitivity was strongly associated with overall survival (log rank test, *P*= 0.0001) (Table 7, Figure 16).

**FIGURE 16 – KAPLAN MEIER SURVIVAL GRAPH FOR PATIENTS CLASSIFIED INTO SS, IS AND RR GROUPS BY SENSITIVITY TO FOUR DRUG COMBINATION**

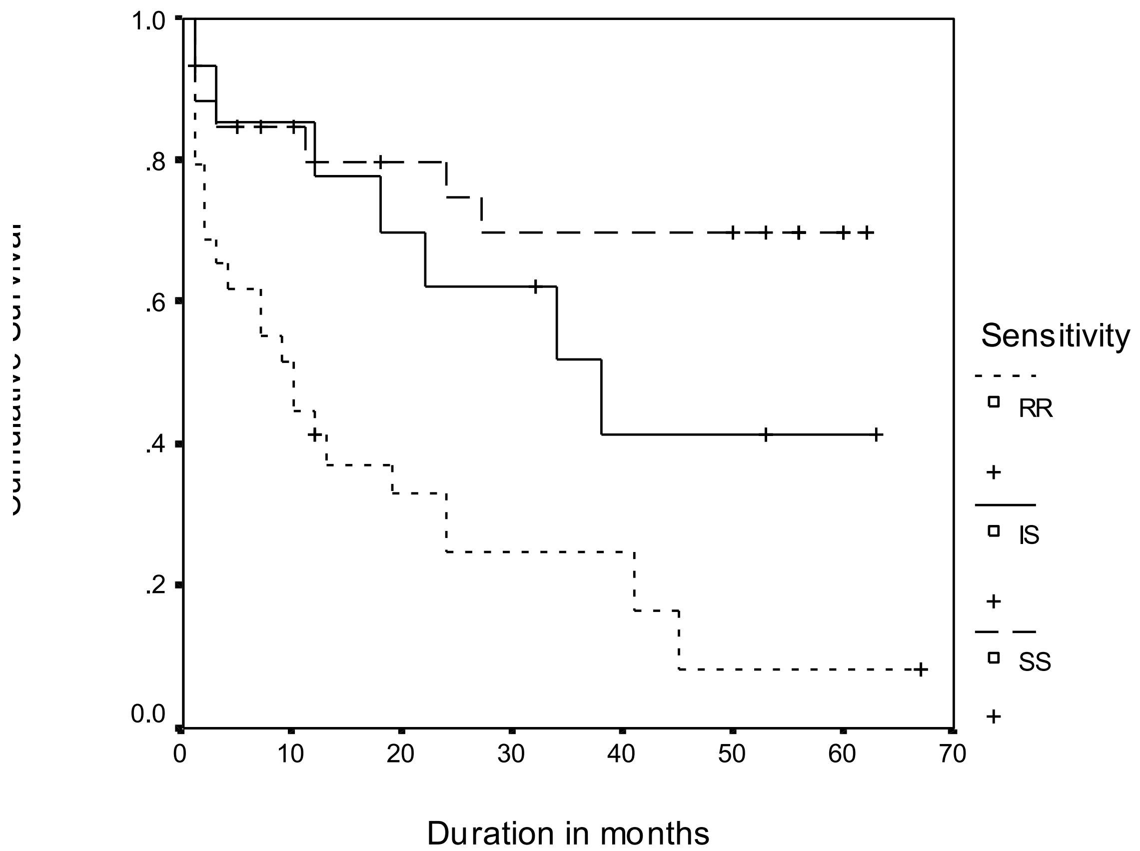



**TABLE 7- THE RELATIONSHIP BETWEEN DPAV SENSITVITY AND SURVIVAL IN CHILDHOOD ALL PATIENTS**

| DAVP | Cumulative survival | Duration Mean ±SE | Confidence interval | Percentage censored | P value |
|---|---|---|---|---|---|
| **SS** | 69.9% | 40 ±7 | (26-54) | 62.5% | 0.0001 |
| **IS** | 41.4% | 32 ± 8 | (16-47) | 50% | |
| **RR** | 8.2% | 16 ± 6 | (5-27) | - | |

*(SS – super sensitivity, IS- Intermediate sensitivity, RR- relative resistance)*

Five year overall survival of SS group (n= 17) was 60%, that of IS (n=8) was 39% and that of RR (n=15) was ~8.5% (log rank test, p value = 0.001; Breslow, p value = 0.0560, Table 8). For DPAV sensitivity, there was a significant worsening of prognosis from the extremely sensitive patients through an intermediate sensitive group to the most resistant group. Moreover, the RR patient group relapsed earlier than the other groups.

**TABLE 8 – THE RELATIONSHIP BETWEEN DAVP SENSITIVITY AND SURVIVAL IN CHILDHOOD ALL PATIENTS**

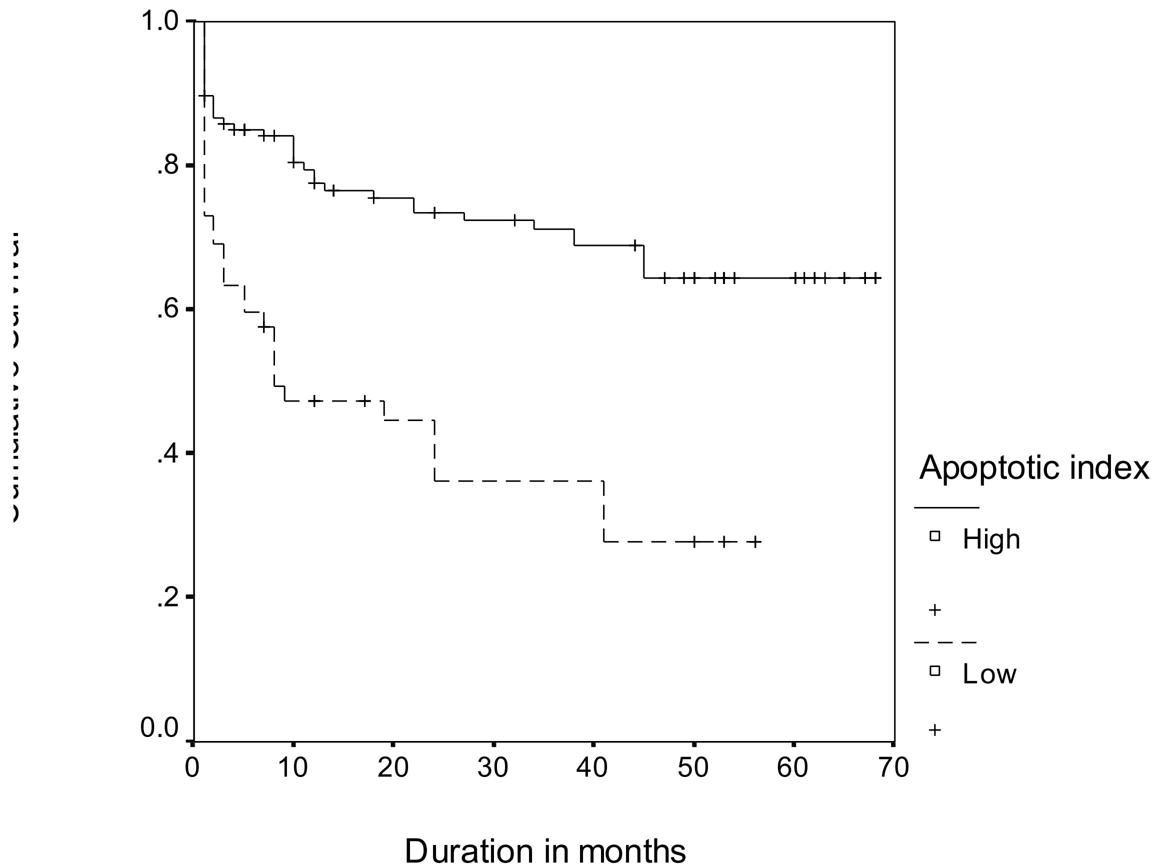
| DAVP sensitivity | Cumulative survival | Number of Patients | P value |
|---|---|---|---|
| **SS** | 69.9% | 29 | 0.0001 |
| **IS** | 41.4% | 15 | |
| **RR** | 8.2% | 28 | |

*(SS – super sensitivity, IS- Intermediate sensitivity, RR- relative resistance)*

## APOPTOSIS AND PROGNOSIS

Kaplan Meier analysis of these patients according to the apoptotic index showed that patients with lower AI had lower survival rates than patients with high AI (*P* <0.0001) (Figure 17).

**FIGURE 17 – KAPLAN MEIER SURVIVAL GRAPH FOR PATIENTS WITH LOW AND HIGH APOPTOTIC INDEX**




# CHAPTER 4

## *DISCUSSION*

It is an accepted fact that standard therapy dose may not be sufficient in some group of patients to completely eradicate tumor cells, while in others the drug concentration may be in excess of optimum and higher enough to exert adverse effects. This difference arises due to the changes in the tumor cellular

characteristics manifested in these patients. It is therefore vital to develop effective therapy for children with ALL in whom no remission occurs or later suffer relapse with current protocols. It is deemed important to protect the patient from toxicity complications due to over dosage during the treatment period. Therefore, monitoring of individual sensitivity of these drugs becomes mandatory in these patients undergoing treatment.

A number of recent studies have by indirect means suggested that the survival capacity of neoplastic cells may be of significance to clinical drug resistance (Krajewski et al., 1995; Wattel et al., 1994; Campos et al., 1993; Miyashita and Reed, 1993). However, studies that directly correlate *in vitro* survival capacity of the neoplastic cells to overall survival is small. In this context, it is reasonable to point out that although it has during recent years been generally assumed that a link exists between the capacity of neoplastic cells to survive *per se* and clinical drug resistance, only few studies have documented such a link. Reports have over the years focussed on the link between the *in vitro* spontaneous tendency of the neoplastic cells to undergo cell death and the tendency to undergo drug or irradiation induced cell death (Pieters et al., 1991; Norgaard et al., 1999). From these studies it has been almost unequivocally suggested that spontaneous ability of the neoplastic cells to survive under in vitro circumstances is associated with low tendency to undergo drug induced cell death.

Predictive value of this assay has been validated recently in a number of studies (Hongo et al., Salamons et al., 1999; Mihal et al., 1999). Previous studies that suggest overlapping of $LD_{50}$ values for samples with initial and relapsed ALL

shows the possibility of a group of patients being already drug resistant at initial diagnosis (4). At present there are no published reports regarding the in vitro drug sensitivity in Indian children.

Our results indicate that there exist considerable variations in the drug sensitivity pattern for patients from Kerala. The overall survival was significantly lower in patients with resistant cells than those with sensitive cells for single drugs such as Predinisone, Vincristine, Asparginase and 6-MP (Table 6). De novo resistance to specific classes of drugs can be overcome by combination chemotherapy. Hence we classified patients into three categories (SS, IS and RR) by sensitivity to DAVP combination (these are the widely used drugs for induction therapy at Regional Cancer Centre). Children with DAVP sensitivity had good clinical outcomes, whereas children with DAVP resistant leukemia underwent induction failure when treated with the same drug combination ($P$ = 0.0001) (Table 7).

The *in vitro* drug sensitivity was correlated with the pretreatment apoptotic ability of these cells, i.e., those cells showing high apoptotic index had lower $LD_{50}$ values compared to those patients showing minimal/nil apoptosis ($P$ <0.05).

Our results suggest that patients can be stratified into different groups according to their sensitivity to the four front line drugs (DAVP). Furthermore, patients who show resistance could be additionally supplied with higher concentrations of these drugs or other drugs to which their cells are more sensitive. But since this is a preliminary observation with limited sample population, only further studies on a larger sample size will reveal the actual implications of this investigation.

Nevertheless, the present study shows that *in vitro* drug sensitivity testing provides significant prognostic information in childhood ALL.

The most important finding of the present study appears to be that we have in a cohort of newly diagnosed ALL patients been able to confirm the clinical relevance of in vitro leukemia cells survival to the short and long term clinical outcome. However, one possible exception from this apparent general rule may well be in the case of drugs requiring cell proliferation for excretion of the cytotoxic drug effect.

Resistance to prednisone monotherapy has been considered as an important factor in the failure of chemotherapy in childhood ALL (Pieters et al., 1991). In our study it was shown that additionally, resistance of Vincristine, Asparginase and 6 MP too was related to prognosis. When children with ALL at initial diagnosis were divided into a resistant and a sensitive group by median $LD_{50}$ value of specific drugs, the risk of relapse was significantly higher in the resistant group.

From our results, the probability of continuous complete remission (CCR) was significantly lower in patients with resistant cells than those with sensitive cells for single drugs such as Prednisone ($p < 0.006$) Vincristine ($p < 0.01$), Asparginase ($p < 0.002$) and 6 MP ($p < 0.005$). De novo resistance to specific classes of drugs can be overcome by combination chemotherapy. Thus we classified patients into three categories (SS, IS and RR) by sensitivity to DPAV combination (this is the widely used drugs for the treatment of induction therapy). Five year overall

survival of SS group (n= 17) was 60%, that of IS (n=8) was 39% and that of RR (n=15) was ~8.5% (log rank test, p value = 0.0205). Children with DPAV sensitivity had good clinical outcomes, whereas children with DPAV resistant leukemia underwent induction failure and or faster relapse when treated with the same drug combination.

The present result clearly agrees with that of Kaspers et al., (1997), where they had classified patients according to the three groups as sensitive (33% lowest $LD_{50}$ values), intermediate sensitive (33% intermediate $LD_{50}$ values), or resistant (33% highest $LD_{50}$ values). When Kaplan Meier curves were plotted, resistance to Prednisolone, L - Asparginase and Vincristine were found to be prognostic significance.

The standard way to validate an in vitro drug sensitivity assay is to demonstrate such a study in a prospective study. A comparison between responders and non-responders in terns of achieving a complete remission is hard to make because the group of non-responders is too small in children with newly diagnosed ALL. Predictive value of this assay has been validated recently in a number of studies (Norgaard et al., 1999; Kaspers et al., 1997; Hongo et al., 1997). Clinical relevant data, assessed with the MTT assay, have emerged to be of prognostic significance in newly diagnosed childhood ALL (Pieters et al., 1991; Kaspers et al., 1994). Previous studies suggest an overlapping of $LD_{50}$ values for samples with initial and relapsed ALL shows the possibility of a group of patients being already drug resistant at initial diagnosis (Klumper et al., 1995).

The present study clearly shows that in vitro drug sensitivity testing provides significant prognostic information in childhood ALL at the time chemotherapy commences and that early detection of drug resistance may provide a successful strategy for individualizing treatment. Relapsed leukemia requires the development of a method for the rapid and accurate prediction of clinical response to specific chemotherapeutic agents. Thus this study could be further continued by evaluating the drug resistance pattern at initial diagnosis and at relapse.

An assay in which large number of drugs can be tested is likely to be more beneficial to the patient, especially when the disease has become resistant to first line chemotherapy. In such a situation there is often a feeling of urgency that time not wasted on a trial with a compound that will not be effective, and it is helpful to select an effective drug and to eliminate those drugs that will have little or no antileukemic effect. In future, patients may be treated selectively only with those drugs to which their leukemic cells are sensitive, avoiding unnecessary exposure to ineffective and potentially toxic agents.

# CONCLUSIONS

This study looked for any associations between *in vitro* drug sensitivity and clinical outcome in pediatric acute lymphoblastic leukemia (ALL) with the standard drugs used for leukemia therapy. A total of seventy-two samples were analyzed. *In vitro* sensitivity to drugs was tested by a methyl-thiazol-tetrazolium

(MTT) assay in six serial fold dilutions. Apoptosis was determined by TUNEL assay and apoptotic index was calculated for each sample. Patients sensitive to Prednisone, Asparginase, Vincristine and 6-Mercapto Purine (6-MP) had higher overall survival compared to patients whose tumor cells were resistant to these drugs ($P$ < 0.01). For the other drugs tested, overall survival (OS) did not vary from that of the resistant patients. For Doxorubicin, Asparginase, Vincristine, Prednisone combination (DAVP) sensitivity, there was a significant worsening of prognosis from the extremely sensitive patients through an intermediate sensitive group to a most resistant group. The present study thus shows that *in vitro* drug sensitivity testing provides significant prognostic information in childhood ALL. However, the small sample population of the present project warrants more detailed study for obtaining more representative data.

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