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By
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This is to certify that this thesis titled “Clinical Profile Of Young Adult Patients With Acute Myeloid Leukemia: Impact Of Molecular Markers And Leukemic Stem Cells On Clinical Outcomes” is the bonafide work of the candidate Dr. Neeraj S during the period from August 2007 to August 2010 as part fulfillment towards the Degree of Doctor of Medicine (higher speciality) in Clinical Haematology towards the examinations to be conducted by the Dr. M.G.R. Medical University in August 2010.

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CONTENTS

CLINICAL PROFILE OF YOUNG ADULT PATIENTS WITH ACUTE MYELOID LEUKEMIA: IMPACT OF MOLECULAR MARKERS AND QUANTITATION OF LEUKEMIC STEM CELLS ON CLINICAL OUTCOMES

ABSTRACT

Abstract

Although the cytogenetic status of patients with AML is considered the single most important prognostic factor at diagnosis, additional markers are evolving which in conjunction with cytogenetics could help better define subsets at high risk for relapse and candidates for HSCT in CR1 or subsets of patients who may have a sufficiently favorable prognosis as to preclude a major benefit from HSCT in CR1. Between January 2003 and December 2009 a total of 192 patients who were diagnosed to have AML who met the inclusion criteria were retrospectively and prospectively analysed. Between October 2008 and December 2009, 41 patients with AML were analysed for the quantitaion of LSCs. All 192 patients underwent "3+7" standard induction therapy with Daunorubucin (DNR) 50mg/m^2 on days 1-3 and Cytosine Arabinoside 200mg/m^2 on days 1-7. A total of 117(60.9%) patients achieved complete remission(CR) after induction. There were 47 (24.5%) induction deaths. 118(61.5%) patients (112 in CR1, and 6 without achieving CR) received consolidation therapy. 40(20.8%) patients underwent allo SCT, 25 (13%) auto SCT, 39(20.3%)HIDAC, and 14(7.3%) patients received chemotherapy other than HIDAC. At a median follow up of two years the Kaplan- Meier estimate of OS, EFS and DFS was 47.30± 4.30%., 37.52± 4.20% and 54.49±5.38% respectively for the entire cohort. The difference in outcomes between the different cytogenetic and molecular subgroups well as for the different modalities of consolidation did not show statistical significance. The disease free survival curves for the two subgroups above and below the median value (8.12) of WBC index were significant(p=0.0225). Thus the CD34+ and CD34+ CD38- cells in the day 10 to day 14 marrow when quantified and analysed as a continuous variable was significant to predict events(p- 0.025 and 0.024 respectively).

INTRODUCTION AND

REVIEW OF LITERATURE

Introduction

Acute myeloid leukemia (AML) is a heterogeneous disorder resulting from various genetic and epigenetic alterations that, in turn, lead to abnormal differentiation and dysregulated proliferation of hematopoietic progenitors. The prevalence of AML is 3.8/100,000 persons under 65 years of age and 18/100,000 people 65 and older. The paradigm for predicting outcome following induction and consolidation therapy depends upon such factors as age, preexisting hematologic disorder, history of chemotherapy, white blood cell (WBC) count at diagnosis, and cytogenetics. The patient's cytogenetic profile is uniformly recognized today as the most important of these factors; karyotypic evaluation of leukemic blasts categorizes AML patients as having favorable, intermediate, or unfavorable risk disease. Patients diagnosed with AML at any age who have a normal karyotype are categorized as having intermediate-risk disease. However, this seemingly homogeneous karyotype conceals a wide variety of different molecular abnormalities, many of which confer distinct prognostic significance. These include internal tandem duplication (ITD) of the juxtamembrane domain in the FMS-like tyrosine kinase-3 gene*(FLT3),* mutations of the nucleophosmin, member 1 gene *(NPM1),* which results in localization of the gene product to the cytoplasm; abnormalities of the gene for CCAAT/enhancer-binding protein a *(CEBPA),* partial tandem duplication (PTD) of the myeloid/lymphoid or mixed-lineage leukemia gene *(MLL);* overexpression of the brain and acute leukemia gene *(BAALC);* and overexpression of the v-ets erythroblastosis virus E26 oncogene-like gene *(ERG).* The evidence for the existence of cancer stem

cells (CSCs) was first derived from the study of human AML, largely because of the availability of quantitative stem cell assays for the leukemic stem cell (LSC). These studies showed that only rare cells within the leukemic clone had the capacity to initiate AML growth after transplant into NOD/SCID mice, establishing the hierarchial organization of AML. In the present study we have tried to analyse the outcome in a young cohort of patients with AML We tried to find out the outcome differences in different cytogenetic and molecular subgroups. In a prospective subset of patients we quantified LSCs at diagnosis and in post induction marrow to assess its impact in predicting events.

Review of Literature

Significant advances have been made in the management of adult acute myeloid leukemia (AML) over the past several decades. However, most of these advances have been limited to young adults (< 55 years) in whom the average 5-year disease-free survival (DFS) rate in AML has improved from 11% to 37% between 1970 and 2000[1].Over a similar period in patients who were older than 55 years at the time of diagnosis, the 5-year DFS for AML has changed marginally (6%–12%)[1]. AML is a heterogeneous disease, thus options of therapy in first complete remission (CR1) depends on additional prognostic factors. With current induction regimens, 70% to 80% of patients with newly diagnosed AML achieve a complete remission; however, this is short-lived without consolidation therapy and most, if not all, of these patients will relapse and succumb to their illness[2,3]. Options for post remission induction therapy for AML in CR1 include intensive nonmyeloablative consolidative chemotherapy, autologous hematopoietic stem

cell transplantation (HSCT), or an allogeneic HSCT. Despite data consistently showing a significantly reduced risk of a relapse after an autologous or an allogeneic SCT, this has not translated to a significantly better DFS or overall survival (OS) because of the counter effect of the treatment-related mortality (TRM) associated with these approaches.

Although the cytogenetic status of patients with AML is considered the single most important prognostic factor at diagnosis, additional molecular markers like FLT3 and NPM1 are evolving which in conjunction with cytogenetics could help better define subsets at high risk for relapse and candidates for HSCT in CR1[4,5] or subsets of patients who may have a sufficiently favorable prognosis as to preclude a major benefit from HSCT in CR1. There have also been innovations and a steady improvement in the management of patients undergoing HSCT that has resulted in lower TRM and improved OS[6-10]. Since the discovery of LSCs it has been proved that AML stem cell frequency at diagnosis offers a new prognostic factor[71] which probably would complement the prognostication based on cytogenetics and molecular markers.

Risk Stratification of Patients with Acute Myeloid Leukemia in First Complete Remission:

Cytogenetics has been the cornerstone of risk stratification in AML[4]. The risk groups based on karyotyping as used by the cooperative groups (Cancer and Leukemia Group B, Southwest Oncology Group, and Eastern Cooperative Oncology Group) are illustrated in Table 2[11]. The good, intermediate, and unfavourable risk groups have 25%, 50%, and greater than 70% probability of

relapse and a 4-year probability of survival of greater than 70%, 40% to 50%, and less than 20%, respectively[12]. This applies to patients < 60 years of age. Additional parameters, such as age, white blood cell count at diagnosis, presence of certain gene mutations and response to induction chemotherapy can influence prognosis, while the type of consolidation therapy could potentially alter the predicted outcomes.

This risk stratification is particularly useful in making decisions regarding the type of consolidation chosen for a patient. In the good-risk group, an allogeneic HSCT with a TRM of 15% to 30% will not be the first option when 3 – 4 cycles of high-dose non-myeloablative consolidation chemotherapy has been reported to achieve long-term DFS as high as 70% with a less than 5% TRM. However, a more recent analysis involving a larger number of patients than had been previously analyzed for this group, suggests that the outcome in the good risk group is likely to be lower than that was previously reported, with a ten year overall survival of 44% (95% CI 39–50%)[13,14]. In the unfavorable group, with chemotherapy alone, only 10% to 20% of patients are likely to achieve long term DFS, thus an allogeneic SCT would be considered acceptable in an effort to improve the DFS[4]. In the intermediate risk group, which constitutes close to 40% to 50% of all patients with AML, the options in CR1 are less clearly defined. This group is heterogeneous in their response to therapy and most of them have a normal karyotype. New markers could help identify subsets at a high risk of relapse and candidates for a HSCT. Some such markers, whose role in the management of AML is still evolving, are summarized in table 3. Recently it was

reported that using a combination of two such gene mutations (NPM1 and FLT3-ITD), a subset defined as being NPM1+/FLT3-ITD- among patients with a normal karyotype had a good prognosis and would probably not benefit from an allogenic HSCT in CR1[15]. Although the data on some of these markers are still preliminary and remain to be validated in large clinical trials, they illustrate the potential of using these markers in risk stratification at diagnosis.

Induction Therapy

Modern induction therapy in AML began in the late 1960s, when both cytarabine and anthracyclines were shown to have significant activity as single agents. Series of classic experiments in the USA by the Cancer and Leukemia Group B (CALGB) some 25 years ago established what really became standard of care for at least two decades. Data from carefully controlled randomized studies established that continuous infusion cytarabine was most effective; 3 + 7 was more effective than 2 + 5; daunorubicin (DNR) was less toxic than adriamycin; and anythingless than 45 mg/m2 was significantly less effective. Furthermore, there was no advantage in giving 200 mg/m2 rather than 100 mg/m2 of cytarabine and 6-thioguanine did not improve the overall results of induction. The early 1990s ushered in a new era, during which newer anthracyclines were studied.Based on these studies, standard induction consisted of DNR 45 mg/m2 intravenously for 3 days and cytarabine 100 mg/m2 by continuous infusion for 7 days. This has been the standard against which most new regimens have been tested. With this regimen 60% to 80% of young adults and 40% to 60% of older adults can achieve a complete remission (CR).

Post-remission Therapy

Despite initial high CR rates, the overall survival (OS) of adults with AML is unsatisfactory, even in the most favorable cytogenetic groups, such as the core binding factor translocations. The need for any post-remission therapy was established in the landmark study conducted by the ECOG in 1983 which prospectively included an observation arm as part of the post-remission strategy. The options for post-remission treatment are broad and the ultimate choice of therapy is determined by the prognostic factors at diagnosis and beyond. Clearly, patient and physician biases also impact the therapy that is offered. A variety of strategies to prevent relapse have been explored. The following is the guideline for HLA matched sibling transplant in CR1 based on molecular markers and cytogenetics as published in ASH 2009.

Table 1: Guidelines for HLA matched sibling transplant in CR1 – ASH 2009

Cytogenetic risk factors	HLA-matched sibling
Favorable, all except c-kit	No Possible
Intermediate, all except NPM^+, $Flt3\text{-}ITD^-$	Yes Possible
Unfavorable	Yes

Autologous Hematopoietic Stem Cell Transplantation (Auto SCT)

The early phase II trials of autologous bone marrow transplantation in young adults with (< 60 years) with AML in CR1 showed an overall DFS ranging from 40% to 60%, relapse rates of 30% to 50%, and a TRM of 5% to 15%. Subsequent phase III trials confirmed a reduced relapse risk compared to intensive chemotherapy (summarized in Table 4)[11,16-18]. In two of these trials, this reduction translated into an improved DFS; however, there was not a significant difference in OS in any of the trials[16,21]. A recently published meta-analysis confirms these observations[19]. All these reported prospective trials had limitations, such as variable numbers of patients who actually received the assigned therapy and a high TRM (average of 12%). Most of these trials were initiated more than a decade ago and there has been significant interval improvement in the management of patients undergoing an autologous HSCT. Most large single center data are consistent with our own experience and demonstrate no significant difference in TRM compared to high-dose consolidation chemotherapy[3]. This reduction in TRM could potentially translate into reduced relapse risk and improved DFS and OS after an autologous SCT. It is unlikely that a prospective trial will clarify this in the near future since to show a 10% difference in survival (P = 0.05, with 90% power), more than 1000 patients would need to be enrolled[16]. From the available data, some generalizations can be made. Good-risk group patients would probably not benefit significantly from an autologous SCT in CR1[21]. In the unfavorable group, there are no data to suggest a benefit of an autologous SCT over chemotherapy, the outcomes after both these options appears dismal[4,20]. Patients in the intermediate-risk group are candidates

for an autologous SCT, especially subsets with a high risk of relapse as defined by additional parameters. However, this remains to be validated in large randomized clinical trials.

The conventional conditioning regimen prior to an autologous SCT has been a combination of Busulphan and Cyclophosphamide (BuCy)[22] or a modification of this with reduced dose of cyclophosphamide administered over two days (BuCy2)[23]. The role of Cyclophosphamide (predominantly immunosuppressive) in this setting has been questioned and more myeloid malignancy specific drugs in the conditioning regimen such as a combination of Idarubicin and Busulphan have been used, the preliminary data with this regimen is promising[24].

Role of purging the stem cell product before autologous hematopoietic stem cell transplantation: In an effort to reduce relapse, some investigators have purged stem cell products before infusion. The agents traditionally used in vitro for this purpose include mafosfamide and 4-hydroperoxy-cyclophosphamide (4-HC). There are retrospective data to suggest that purging is of benefit, whereas, in a large prospective trial, purging with 4-HC did not appear to be of significant benefit[11,25,26]. There is insufficient data to strongly recommend purging; however, the data is also inadequate to completely exclude a role for purging the stem cell product. Other experimental methods of purging the stem cell product including exposure to hyperthermia and immunologic purging by positive selection have been shown to have potential[27,28].

Role of consolidation therapy before autologous stem cell transplantation: After induction of CR1, additional consolidation chemotherapy before an autologous SCT appears to have a significant positive effect by reducing the relapse risk and improving the DFS[29,30]. A recent retrospective analysis of the Autologous Blood and Marrow Transplant Registry/International Bone Marrow Transplant Registry (ABMTR) database reached a similar conclusion[40]. The optimal consolidation regimen before an autologous SCT remains to be defined. Extrapolating from the Cancer and Leukemia Group B studies on the optimal consolidation chemotherapy for AML, it would appear that high-dose cytosine arabinoside (3 gm/m2 every 12 hours × six doses) administered for three or more courses would be ideal[2,14]. From the published data regarding autologous SCT in AML, two or more cycles of high-dose cytosine arabinoside-based regimen would appear to be adequate prior to the transplant[29] though the ABMTR data analysis did not show a significant difference when either standard dose cytosine arabinoside (<1gm/m2) or high dose cytosine arabinoside (1-3gm/m2) was used[31].

Source of stem cells for an autologous stem cell transplantation: Bone marrow versus peripheral blood: Retrospective data suggest that the use of cytokine-mobilized peripheral blood stem cells (PBSC) for an autologous transplant is associated with more rapid engraftment of neutrophils and platelets[32]. However, most studies do not show an improvement in relapse risk, TRM, DFS, or OS[33].

Allogeneic Hematopoietic Stem Cell Transplantation (Allo SCT)

Large prospective trials have consistently shown that an allogeneic SCT with standard myeloablative conditioning regimen is the most potent anti-leukemia treatment for AML in CR1 with a relapse risk of 24% to 36% compared to 46% to 61% with autologous SCT or chemotherapy (Table 4)[11, 16-18,34]. However, in none of these trials did this decreased relapse risk translate to a significantly improved OS. This was due to high TRM, which ranged from 10% to 25%. An allogeneic SCT is not an option to consider for patients in the good risk cytogenetic group in CR1 since (i) they have excellent response to therapy with high dose chemotherapy and (ii) even if they do relapse after consolidation chemotherapy, they still respond well to an allogeneic or autologous SCT in CR2[35,36,] . In the intermediate and unfavourable cytogenetic groups, the TRM associated with an allogeneic SCT may be acceptable in an effort to improve the DFS and OS. In the unfavorable group in CR1, an intergroup study showed a 5-year survival rate of 44% versus 15% with chemotherapy or an autologous SCT (Figure 1), whereas a similar but less dramatic difference was noted in the European Organization for Research and Treatment of Cancer/Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto AML-10 trial (EORTC/GIMEMA) and in the Dutch Belgian Hematolo-Oncology Cooperative Group / Swiss Group for Clinical Cancer Research (HOVON-SAKK) study[4,37,38]. Two other studies failed to show an advantage of an allogeneic SCT over chemotherapy or an autologous SCT in the unfavorable risk group[18,34]. In all of the studies, the

outcome in the unfavorable risk group with chemotherapy alone or with an autologous SCT was dismal. Based on the available data, if a donor is available, it is reasonable to proceed with an allogeneic-related HLA-identical SCT in CR1 with unfavourable cytogenetics. In the intermediate-risk group, the data from most large prospective clinical trials did not show an improved OS after allogeneic SCT in CR1. However, in one study, there was a significant improvement in the DFS[11,16-18,34]. The optimal therapeutic strategy in this group of patients in CR1 is still evolving. If a related HLA identical donor is available, other parameters could be used to aid in the decision-making process. Some of the factors that would favor an allogeneic SCT in CR1 include age of patient (< 40 years), high white blood cell count at diagnosis (> 30,000–40,000/mm3), requirement of more than one cycle of chemotherapy to achieve CR1, and the presence of additional molecular markers (Table 3) that predict a high risk of relapse[39-47]. While subsets such as those with NPM1+/FLT3-ITD- would probably not benefit from an allogeneic SCT in CR1[15], as mentioned earlier, the data on some of these markers are still preliminary and remain to be validated in large clinical trials.

Role of conditioning regimen on outcome: Two major myeloablative conditioning regimens (cyclophosphamide/total body irradiation [TBI] and cyclophosphamide/busulfan) have been used and studied in randomized trials. There are no strong data to suggest that one regimen is superior to the other, each regimen has its merits. In several retrospective studies, the use of a TBI-based regimen is associated with lower relapse rates and superior DFS[48]. In a

randomized single-center study, the use of fractionated TBI to a dose of 15.75 Gy in comparison to 12 Gy was associated with a lower risk of relapse, but it did not improve survival because of increased TRM and severe acute GVHD associated with the higher dose[49]. RIC regimens are increasingly being used in allogeneic SCT in an effort to decrease regimen-related toxicity and preserve the graft-versus-leukemia effect. There are limited data on the use of RIC regimens in young patients with AML in CR1 and should probably only be considered in the setting of a clinical trial. The Medical Research Council AML-15 trial intends to allow the possibility of a RIC regimen for patients 35 to 45 years who have a matched donor. Even in elderly patients the use of RIC regimens which showed promise by reducing the TRM has not translated to improved event free or overall survival due to the continued risk of relapse in these patients[50].

Role of consolidation chemotherapy before an allogeneic stem cell transplantation: Retrospective analysis of the IBMTR and European Group for Blood and Marrow Transplantation (EBMT) data suggest that consolidation chemotherapy before an allogeneic SCT does not benefit patients with AML in CR1[51,52]. Another retrospective analysis from a single center showed similar findings and suggested that multiple chemotherapy courses before an allogeneic SCT had a deleterious effect[53].

Bone marrow versus peripheral blood stem cells: Retrospective analysis of the EBMT and IBMTR database showed a benefit for use of PBSC in patients with advanced AML, but no benefit was shown in patients with AML in CR1, whereas another retrospective study showed a benefit for patients with AML in

CR1 who underwent a PBSCT[54,55 7]. A more recent retrospective analysis of the Acute Leukemia Working Party/EBMT registry suggests that there is improved outcome with the use of bone marrow versus PBSCT when the dose of bone marrow CD34+ cells exceeded 2.7×10^6/Kg[56]. The only prospective study addressing this issue demonstrated earlier engraftment, reduced TRM, and improved DFS with PBSCT, but there was no difference in OS[57].

AML in India

Treating AML in a developing country like India is a huge challenge. There is very limited availability of cytogenetics and molecular markers across the country. Moreover, there is very limited data on the treatment outcome with standard chemotherapeutic regimens. Also, there seems to be a higher rate of infections including fungal infection and treatment related mortality (TRM) when compared to the western data. Presently there are evolving new prognostic markers including molecular markers and multiparametric flow cytometry to document minimal residual disease. Most of these tests are expensive and are not available except in select research institutions. In this context, LSCs are definitely a new and exciting area of interest. Quantifying LSCs by a set of immunophenotypic markers at diagnosis and during assessment of response to therapy should be an inexpensive and easily reproducible test which could predict the outcome, resistance to chemotherapy and minimal residual disease.

Leukemic stem cell

The concept that only a subpopulation of rare cancer stem cells (CSCs) is responsible for maintenance of the neoplasm emerged nearly 50 years ago;

however, conclusive proof for the existence of a CSC was obtained only relatively recently. Two unique features characterize adult stem cells: the ability to generate new pleuripotent stem cells (to self-renew) and the ability to give rise to differentiated progeny that has lost its self-renewal capacity. Leukemia can be viewed as a newly formed, abnormal hematopoietic tissue initiated by a few leukemic stem cells (LSCs) that undergo an aberrant and poorly regulated process of organogenesis analogous to that of normal hematopoietic stem cells. A hallmark of all cancers is the capacity for unlimited self-renewal, which is also a defining characteristic of normal stem cells**.** Given this shared attribute, it has been proposed that leukemias may be initiated by transforming events that take place in hematopoietic stem cells. The cells from the hematopoietic system are continually generated from self renewing progenitors in the bone marrow called hematopoietic stem cells (HSCs), or blood-forming stem cells, which have been isolated in both humans and mice[59 60 61 62].The relative quiescence of LSC may be a major factor contributing to relapse. Consequently, defining unique properties of LSC remains a high priority in order to elucidate the molecular mechanisms driving stem cell transformation, and for developing therapeutic strategies that specifically target the LSC population[63].

Haematopoietic Stem Cells

HSCs can be divided into a long-term subset (LT-HSC), which are capable of indefinite self renewal, and a short-term subset (ST-HSC) that self-renew for a defined interval only. HSCs give rise to non self-renewing oligolineage progenitors, which in turn give rise to progeny that are more restricted in their

differentiation potential, and finally to functionally mature cells. Recent studies have demonstrated that normal stem cells and cancer cells share the ability to self-renew and that many pathways classically associated with cancer also regulate normal stem cell development. For most cancers, the target cell of the transformation events is unknown, but evidence indicates that certain types of leukemias arise from mutations that accumulate in HSCs. The first experimental evidence to indicate the existence of HSCs was the discovery in 1961 by Till and McCulloch [64]of a population of clonogenic bone marrow cells capable of generating myelo-erythroid colonies in the spleen of lethally irradiated hosts. Occasionally these colonies contained clonogenic cells that could be further retransplanted into secondary lethally irradiated hosts and reconstitute the immune system. These were proposed to be HSCs, i.e., progenitor cells with the essential characteristic of self-renewal and differentiation potential for all types of blood cells. The development of clonal assays for all major hematopoietic lineages together with the availability of multiparameter fluorescence-activated cell sorting (FACS) has enabled the prospective purification of HSCs from mice and to highly enrich for HSCs from humans according to the cell surface expression of specific molecules and their functional read-out *in vivo* and *in vitro* in stromal long-term colony initiating assays.

Leukemic Stem Cells in AML

Because the clonogenic read-out of the leukemic cells perfectly mirrored the difference in clonogenicity among the normal hematopoietic cells, the clonogenic leukemic cells were described as LSCs. However, not until 1997, in studies

published by Blair and colleagues [65]and Bonnet and Dick,[66]was there a clear demonstration that most of the leukemic cells were unable to proliferate extensively and only a small, defined subset of cells was consistently clonogenic. In these studies, LSCs for human AML were identified prospectively and purified as [Thy1-, CD34+, CD38-] cells from various patient samples. Although these cells represent a small and variable proportion of the totality of the AML cells (0.2–1% depending on the patient), they were the only cells capable of transferring AML from human patient to nonobese diabetic severe combined immunodeficient (NOD_SCID) mice and were referred as SCID leukemia-initiating cells or SL-IC.

In the studies by Bonnet and Dick using flow cytometry, despite the rarity of the CD34+CD38- fraction and the presence of high levels of AML-CFU in the CD34+CD38+ fraction, leukemic engraftment could only be initiated from CD34+D38- fractions. In keeping with a tradition of naming cells based on function, they termed this the SCID leukemia-initiating cell (SL-IC). The CD34+CD38- SL-IC generated large numbers of CD34+CD38-cells, AML-CFU, and mature blasts. This was proof that the cell population that initiated AML in SCID mice was distinct from AML-CFU; a new stem cell was thus identified in AML.[67]Although some exceptions were reported in subsequent years, a high proportion of AML patients show LSC activity in the CD34+CD38- fraction[68] [69] [70]. Recent studies have suggested that the ability to engraft NOD/SCID mice as well as increased frequencies of LSC in AML are prognostic for poor outcomes[71].

Collectively, these studies provide conclusive evidence for the hierarchy model in AML.

Phenotype of Leukemic Stem Cells

Subsequent studies showed that LSCs are also CD34+/HLA-DR-/CD71- and fail to express Thy1[72].[73]Although this phenotype is quite similar to that of normal primitive cells, it was hypothesized that malignant transformation would yield changes in LSCs that would allow them to be distinguished from normal stem cells. One potential difference between normal and leukemic cells lies in their response to hematopoietic growth factors. Numerous studies have examined the cytokine response of primary leukemia cells and demonstrated mitogenic activity for IL-3, G-CSF, GM-CSF, SCF, FL, TPO and other factors.[74] [75] [76]Although these studies have focused on bulk populations of leukemia cells, one can imagine that differential sensitivity to cytokines may also exist at the level of primitive AML cells.

Perhaps most central to the criteria by which stem cells are defined is the property of self-renewal. As mentioned above, studies by Bonnet and Dick demonstrated that human AML stem cells derived from multiple French-American-British (FAB) subtypes are able to undergo self-renewal[77]. Expression of the three antigens, CD90, CD117, and CD123, differ from normal HSC and thereby provide the means to separate normal from AML stem cells.

CD 123 is interleukin -3 receptor alpha chain. Total marrow generally has about 7% positive cells for CD123, but only about 1% of the population expresses the antigen at high levels. The CD34 □□population of normal marrow also has

readily evident CD123 expression, as would be expected for a population known to contain hematopoietic progenitors. The labeling profile shown is in good agreement with previous studies by Sato *et al* that have also examined IL-3Rα levels on human CD34□□ cells.[78] However, the more primitive CD34+/CD38- compartment shows no significant expression of CD123 (1%). In contrast, primary AML cells displayed high levels of CD123. In both the overall CD34□□+ population, as well as the more primitive CD34+□/CD38- compartment, greater than 99% of the cells were positive for CD123. CD123 is generally expressed at high levels and may be indicative of previously uncharacterized aspects of AML biology. Future studies will seek to define the biological role of CD123 in human AML and to exploit the presence of this antigen as a means to target AML stem cells for destruction.

Myeloid leukemias have been extensively characterized with regard to stem and progenitor cell involvement. Thus, as a focal point for both scientific and therapeutic endeavors, leukemic stem cells (LSC) represent a critical area of investigation. LSC appears to retain many characteristics of normal Hematopoietic Stem Cells (HSC) as evidenced by a hierarchial developmental pattern, a mostly quiescent cell cycle profile, and an immunophenotype very similar to HSC. These studies demonstrate that LSC retain key features of normal stem cells and are biologically distinct from the bulk of leukemia blast cells that do not self-renew. Our understanding of the mechanisms that determine whether, where and when a stem cell will self-renew or differentiate is still limited, but

recent advances have indicated that the stem cell microenvironment, or niche, provides essential cues that direct these cell fate decisions[79].

Stem Cell Niche

The existence of a stem cell niche, or physiological microenvironment, consisting of specialized cells that directly and indirectly participate in stem cell regulation has been verified for mammalian adult stem cells in the intestinal, neural, epidermal, and hematopoietic systems. In the light of these findings, it has been proposed that a "cancer stem cell niche" also exists and that interactions with this tumor niche may specify a self-renewing population of tumor cells.[80] The stem cell niche is an anatomical unit located in the endosteum within the bone marrow cavity that is composed of osteoblasts, osteoclasts and stromal fibroblasts. Interactions between stem cells and their surrounding microenvironment, or niche, are critical for the establishment and maintenance of stem-cell properties. The stem-cell niche has been studied in detail in the adult Drosophila testis. It has been shown that integrin-mediated adhesion is important for maintaining the correct position of embryonic hub cells during gonad morphogenesis. On the basis of these data, it has been proposed that integrins are required for the attachment of the hub cells to the ECM, which is essential for maintaining the stem-cell niche.[81]

Haematopoietic Microenvironment

The Haematopoietic Microenviornment (HM) in the bone marrow consists of a heterogeneous population of hematopoietic and non hematopoietic stromal cells, their extracellular biosynthetic products, and hematopoietic cytokines[82]. The mesenchymal stromal cells maintain bone remodeling and control the

differentiation of hemopoietic cells during the life span[83]. These cells include myofibroblasts, other fibroblastoid cells, endothelial cells, osteogenic precursors,adipocytes, and macrophages. These cells produce a complex array of extracellular matrix (ECM) molecules consisting of proteoglycans and their constituent sulfated glycosaminoglycans, chondroitin, heparan, and dermatan species as well as hyaluronic acid.(Table:5) In addition, they make a variety of interstitial and basal lamina collagens. Stromal cells also synthesize other matrix molecules,such as fibronectin, thrombospondin, hemonectin, sialoadhesin, laminin, and the tenascin glycoproteins [84] [85]

Interaction of Adhesion Molecules on Hematopoietic Precursors and Marrow Stroma

Hematopoietic progenitors express cell adhesion molecules (CAMs) that can be classified into six structurally distinct superfamilies: integrins; selectins; sialomucins,including CD34; immunoglobulins; CD44 cell surface proteoglycans; and cadherins.[86]

Integrins

Integrins are a family of glycoproteins consisting of non covalently linked heterodimers.[87] Integrins are expressed on many cells of the hematopoietic system and their functional importance has become apparent in recent years.[88] [89] [90]The beta 1 common chain (CD29) combines with different alpha chains to form a variety of VLA (very late activation antigen) molecules that mediate the adhesion of hematopoietic cells to ECM components and ligands on stromal and endothelial cells. Very late activation antigen (VLA) or beta 1 integrins are

primarily receptors for ECM proteins, with VLAs 1,2,3, and 6 being receptors for laminin; VLAs 1,2, and 3 for collagen; and VLAs 3, 4, and 5 for fibronectin.[91] [92] [93] [94] VL A-4 also binds the cellular receptor vascular cell adhesion molecule- 1 (VCAM- l), which is present on BM stroma[95] [96]. The α4β1 integrin Very Late activation Antigen-4 (VLA-4) is an α4 (CD49d)/β1 (CD29) heterodimer. It plays a key role in the adhesion of both hematopoietic progenitor cells and leukemic blast cells to bone marrow stromal cells which express the vascular cell adhesion molecule-1 (VCAM-1) or produce fibronectin. VLA-4 is widely distributed on hematopoietic cells, including thymocytes, peripheral blood lymphocytes, monocytes, activated T cells, T and B lymphoblastoid cell lines, and myeloid cell lines. However, VLA-4 is only weakly expressed on most adherent cell lines tested.[97]CD34 (+) cells express the integrin receptor VLA-4 (β4α1) (CD49d), whose ligand is VCAM-1 (vascular adhesion molecule-1) on marrow stromal cells and fibronectin in the ECM. VCAM-1 is variably expressed on marrow stromal and endothelial cells and can be upregulated by several cytokines, including interleukin-1 (IL-1). Because VLA-4 and its ligands are widely distributed, specificity is most likely conferred by the coexpression of other adhesion molecules and can be modulated by hematopoietic cytokines. The VLA-4/VCAM-1 interaction is a critical component of the complex process of stem cell homing.[98] The chemokine and chemoattractant stromal-derived factor 1 (SDF-1), another important element in the homing of hematopoietic stem cells to the bone marrow, is secreted by stromal cells and strongly upregulates the VLA-4-mediated adhesion of CD34(+) cells to stroma and ECM fibronectin.[99] Early

precursors also express VLA-5 (β5α1)(CD49e), which can bind to ECM fibronectin. Table 6 illustrates the various adhesion molecule interactions.

Haematopoietic Microenviornment, Tumor Cells and Drug Resistance

In AML, adhesion to stroma is mediated by the combined action of β1 (principally VLA-4) and β 2 integrins[100]. This rich environment serves as a safe haven for normal and malignant hematopoietic cells. This protection allows tumor cells to survive the insult of chemotherapy, leading to minimal residual disease, and thereby increases the probability for the development of acquired drug resistance. These complex interactions between leukemic blasts, cellular and matrix components of stroma, and cytotoxic lymphocytes, play a critical role in determining the fate of small numbers of leukemic cells surviving after cytotoxic chemotherapy.

Adhesion Molecule Expression on Leukemic Cells

AML blasts express many of the adhesion molecules identified on normal hematopoietic precursors. Studies by the Westmead group in Sydney confirm that adhesion of AML blasts, at least in part, is mediated by the interaction of VLA-5 with ECM fibronectin as well as via both β1 (VLA-4) and β2 (LFA-1) integrin interactions with stromal cells[101 102 103].

AML cells adhesion to ECM

The adhesion of AML cells to ECM elements may explain the tenacity with which residual leukemic blasts may persist in the marrow. As discussed above, AML blasts adhere to ECM fibronectin and laminin through the β1 (VLA-4, VLA5, VLA-6) and β2 (LFA-1) integrins found on the leukemic cells. Antibody-

blocking studies show that this only partially accounts for the adhesion and that other mechanisms, including CD44 binding, have been invoked.The following figure illustrates the interactions of normal and leukemic progenitors with the haematopoietic microenvironment.

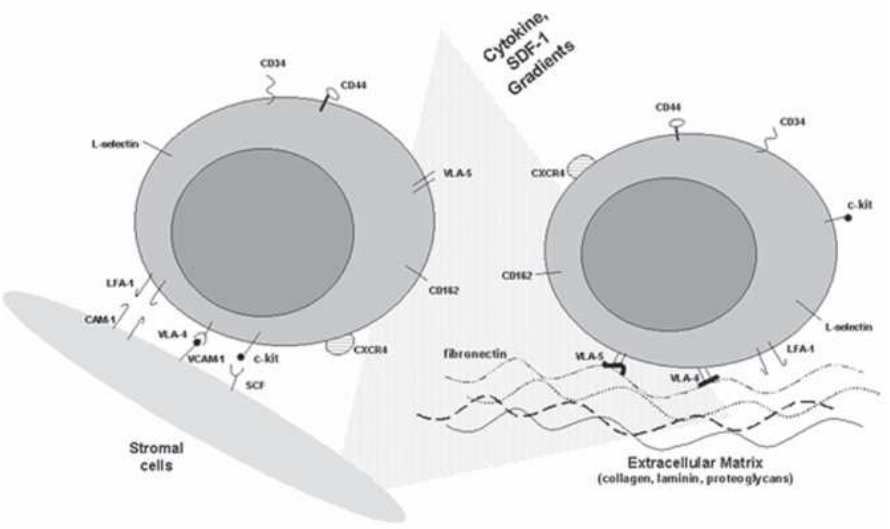


Figure 1: Interactions of normal and leukemic progenitors with the hematopoietic microenvironment (HM).

VLA 4

The structure-function relationship of the human integrin VLA-4 (alpha 4/beta 1; CD49d/CD29), has been studied in the human B-cell line Ramos by immunochemical and functional analysis[104]. The data reported indicate that VCAM-1 and integrin VLA-4 are obligatory adhesion proteins in the heterotypic adherence between human LL cells and Mesenchymal Stem Cells (MSC). The constitutive expression of VCAM-1 by MSC may be partially responsible for retention of leukemia cells in the bone marrow and metastasis of lymphomas to the bone marrow.[105] Cellular adhesion assays performed on non Hodgkin Lymphoma cell lines in the presence of either neutralizing antibodies to

VCAM or the alpha and beta subunit of VLA-4 resulted in inhibition of lymphoma cell line adhesion to the bone marrow stromal line[106]. Both VLA-4 and CXCR-4 have been proposed as mechanisms for retention of AML blasts in the marrow. Functional inhibition of either of these receptors has been shown to dislodge AML cells and enhance chemotherapy-induced apoptosis in vitro and improve eradication of leukemia in vivo.[107] Liesveld et al[108] have shown that human AML cells adhere to long-term BM stromal cultures. They showed that the binding of AML blasts to fibronectin is mediated by VLAs 4 and 5, with VLA-5 dependent adhesion predominating.

In patients with AML, VLA-4 expression has been associated with bone-marrow minimal residual disease, which causes relapse after chemotherapy[109]. Matsunaga et al[110]found that VLA-4-positive cells acquired resistance to anoikis (loss of anchorage) or drug-induced apoptosis through the phosphatidylinositol-3-kinase (PI-3K)/AKT/Bcl-2 signaling pathway, which is activated by the interaction of VLA-4 and fibronectin[110]. This resistance was negated by VLA-4-specific antibodies. In a mouse model of MRD, a 100% survival rate was achieved by combining VLA-4-specific antibodies and Cytosine Arabinoside (AraC), whereas AraC alone prolonged survival only slightly. In addition, overall survival at 5 years was 100% for 10 VLA-4 negative patients and 44.4% for 15 VLA-4 positive patients. Thus, the interaction between VLA-4 on leukemic cells and fibronectin on stromal cells may be crucial in bone marrow MRD and AML prognosis.

Matsunaga et al reported no deaths in 10 patients they classified as negative for VLA 4 expression (18.6%), who had median follow-up more than 3 years, whereas in a recently published study 13 of 23 patients with VLA 4 expression less than or equal to 18.6% died within 12 months. Potential differences between this study and the previously

reported study include heterogeneity of patient inclusion criteria, choice of chemotherapy regimens, and duration of treatment. In addition, the activation state of VLA 4 integrin, which could have influenced the outcome in a small group of patients, was not assessed in the previous study.

VLA-5

It has been demonstrated by van der Loo et al that VLA-5 is expressed on primitive mouse and human hematopoietic cells and they suggested that there may be significant cooperation between integrin receptors and proteoglycan molecules in the engraftment of bone marrow cells and hematopoietic cell adhesion in vivo[111]. Messinger et al. suggested that the expression of VLA-4 and VLA-5 on t(1;19) or t(4;11) leukemia cells likely determines their binding capacity to bone marrow stroma[112]. However there has been no reported literature on the adverse impact of expression of VLA-5 in blasts/leukemic stem cells on the outcome in AML.

Table 2: Risk group stratification based on cytogenetics at diagnosis

Good risk: (10-15%)	t(15;17) t(8;21) inv16, t(16;16)
Standard risk: (65-75%)	Normal karyotype del (9q) -y del 12 p Trisomy 8 t(9;11)
Poor risk: (15-20%)	Abnormal 5 or 7 inv3q del 20q del 21q t (9;22) t (6;9) non-t (9;11) 11q23 abnormalities with MLL rearrangements complex cytogenetics (3 or more clonal abnormalities)

Modified from Cassileth et al[11]

Table 3: Newly identified prognostic markers used in the management of AML.

Marker	Summary
FLT3-ITD[39,40]	Reported in 15 – 35% of cases with AML. Presence associated with an adverse outcome
BAALC gene over expression[41]	Brain and acute leukemia cytoplasmic (BAALC) gene over expression has been shown to predict poor survival in patients with AML and normal cytogenetics
bcl-2 and WT1[42]	Coexpression of apoptosis-related genes bcl-2 and WT1 has been associated with significantly inferior DFS and OS
Evi-1 mRNA[43]	Over expression of Evi-1 mRNA in patients with intermediate risk (by conventional cytogenetics), even in the absence of cytogenetic 3q26 abnormalities, identifies a subset with a worse prognosis
Partial tandem duplication of the	Partial tandem duplication of the MLL gene in one study was seen in 7.7% of patients with a normal karyotype and associated

MLL gene[44]	with a significantly shorter remission duration
FADD protein expression[45]	Absence of Fas-associated death domain (FADD) protein expression in AML has been associated with a worse outcome.
Mutations in CCAAT/enhancer-binding protein−α[46]	Several studies, most recently by Frohling et al[58], have demonstrated that mutations in the transcription factor CCAAT/enhancer-binding protein−α are associated with a good prognosis.
VEGFR-1 levels[47]	Plasma soluble vascular endothelial growth factor receptor-1 levels have been shown to have an inverse correlation with the attainment of CR after induction chemotherapy in AML.
NPM1 mutations[58]	The nucleophosmin (NPM1) gene mutations occur in 50% to 60% of adult AML with normal karyotype (AML-NK). NPM1 mutations in absence of FLT3-ITD identify a prognostically favorable subgroup in the heterogeneous AML-NK category.

Table 4: Relapse risk in phase III trials comparing nonmyeloablative chemotherapy, autologous SCT and allogeneic SCTs:

Study	**Allogeneic (%)**	**Autologous (%)**	**Chemotherapy (%)**
GIMMEMA[17]	24	40	57
GOELAM[18]	28	45	55
MRC[16]	19	35	53
ECOG/SWOG[11]	29	48	61

Table 5: Extracellular matrix constituents

Proteoglycans and constituent glycosaminoglycans
- Heparan sulphate
- Chondroitin sulfate
- Dermatan sulfate
- Hyaluronic acid

Collagen types I,III,IV,V,VI
Fibronectin
Thrombospondin
Sialoadhesin
Laminin
Tenascin

Table 6: Adhesion Molecule Interactions

Adhesion Molecule	Location	Ligand	Location
β1 integrins			
$\alpha_4\beta_1$ (VLA-4)	HP	Fibronectin (CS1 domain)	ECM
	AML cells	VCAM-1	MSC
$\alpha_5\beta_1$ (VLA-5)	HP	Fibronectin (RGD sequence)	ECM
β2 integrins			
LFA-1	HP, AML cells	ICAM-1	MSC, EC
Mac-1	DHC, AML cells		
Selectins			
E-selectin	EC	Sialylated sugar moieties; Lewis x; CD44	Myeloid cells, T cells
P-selectin	EC, platelets	P selectin, glycoprotein ligand 1 (CD162)	Myeloid cells, AML blasts
L-selectin	HP	Glycoprotein ligand	HP, AML blasts
CD44	HP	Hyaluronic acid	ECM
	AML blasts	Fibronectin	

Abbreviations: VLA, very late antigen; HP, hematopoietic progenitors; AML, acute myeloid leukemia; VCAM, vascular adhesion molecule; ECM, extracellular matrix; MSC, marrow stromal cells; LFA, lymphocyte function antigen; DHC, differentiated hematopoietic cells; ICAM, intercellular adhesion molecule; EC, endothelial cells.

OBJECTIVES AND METHODS

Objectives and aims of study

1. To study the clinical profile of newly diagnosed patients with AML undergoing standard induction and post remission therapies.

2. To study the incidence , impact and the clinical outcome of the molecular markers FLT3 ITD, FLT3 TKD and NPM1 mutations in the above cohort of patients.

3. To quantify the proportion of leukemic stem cells (LSC) by a set of immuno-phenotypic markers among the blast population in cases of AML at diagnosis and in a post induction bone marrow sample.

4. To identify and quantitate the expression of adhesion molecules VLA-4 andVLA-5 in this LSC population at the same time points.

5. To correlate the above parameters with the clinical outcomes such as achievement of complete remission (CR), induction death, relapse, event free survival, overall survival and disease free survival.

Hypotheses

1. The leukemia characteristics and the treatment outcome in Indian patients might be different from the data from the west.

2. Autologous SCT will be superior to high dose consolidation chemotherapy alone in the intermediate risk group without a donor in view of the markedly reduced transplant related mortality and allogenic SCT will be superior to the other two modalities of consolidation.

3. The prognostic value of the molecular markers FLT3 and NPM1 mutations in determining the treatment outcome in Indian population could be different from the Caucasian data.

4. The number of LSC in the blast population impacts the response to therapy and risk of relapse and the residual number of LSC at the end of induction chemotherapy will predict relapse.

5. The expression of adhesion molecules (VLA-4 and VLA-5) on the LSC aids in binding these cells to the bone marrow stroma which in turn protects it from the apoptotic action of chemotherapeutic agents and high levels expression of these adhesion molecules on LSC will predict response to therapy.

Inclusion Criteria (All)

1 .Patients with newly diagnosed non M3 AML >=15 years and <=60 years of age.

2. Those who have received standard "3+7 "(Daunorubicin and Cytosine Arabinoside) or equivalent induction therapy during the study period.

Exclusion Criteria (Any)

1. Patients outside the range of the age group mentioned above.

2. Those who have been treated with truncated "3+7" or other types of induction therapies

3. Patients previously treated in outside centres.

4. Patients with AML who presented to our centre at relapse or who were referred only for stem cell transplantation.

Study design

This was a descriptive study design. All newly diagnosed cases of Acute Myeloid Leukemia excluding APML in the age group 15-60 years in the hospital between January 2003 to December 2009 and who have been treated with standard induction therapy were

included. This included a retrospective analysis from January 2003 to December 2008 and a prospective cohort from January 2009 to December 2009.

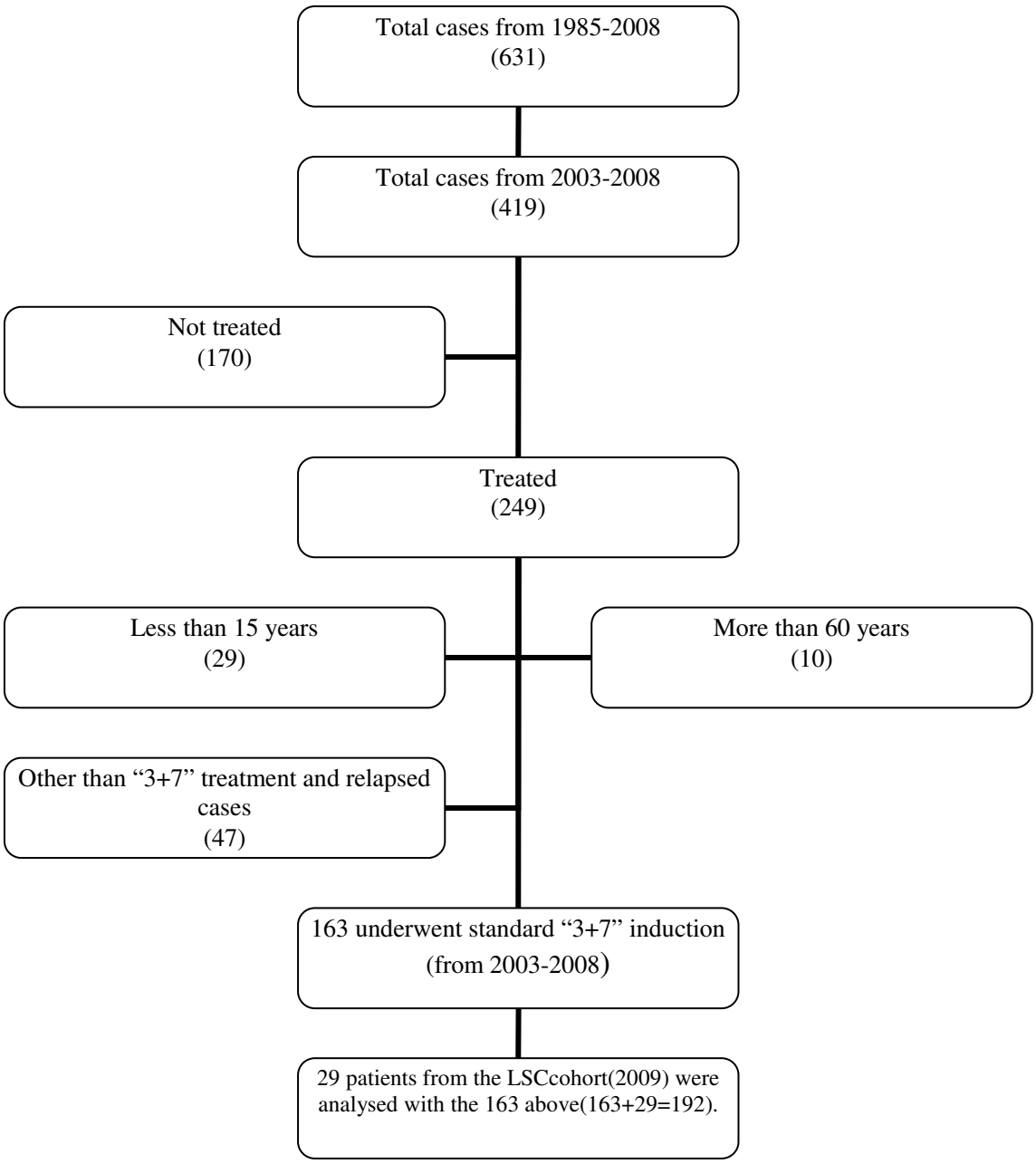
Patients

For the retrospective analysis patient records in the Haematology department database were searched and the data sheet of all patients who were admitted during the time period were collected. (n= 419 see the flow chart 1). Those patients who were discharged without induction chemotherapy were not included. Of those patients who underwent induction chemotherapy, those < 15 years, >60 years, upfront relapsed cases, those who had taken induction treatment from outside centres and those who received truncated induction therapy were excluded from the final analysis. Patients who were prospectively inducted also met the inclusion criteria. The patients had a complete blood count, liver and renal function tests, LDH, bone marrow aspirate, biopsy ,immunophenotyping and cytogenetics done at diagnosis.

Bone Marrow Aspirate

Bone marrow was aspirated and core biopsy taken at diagnosis. The bone marrow aspirate smears from patients with AML were stained with May Grunwald Geimsa , sudan black B , periodic acid schiff , chloroacetate esterase and alpha naphthyl acetyl esterase. The bone marrow blast percentage was quantified and a white blood cell index was calculated[*]. The Auer rods were documented if present. FAB classification was used to subtype.

[*] Ste´phanie Nguyen et al . Awhite blood cell index as the main prognostic factor in t(8;21) acute myeloidleukemia (AML): a survey of 161 cases from the French AML Intergroup. Blood. 2002; 99:(10) 3517-3523.



Flow chart(1) depicting the patient selection.

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Immunophenotyping

The bone marrow samples were also tested for immunophenotyping on a 4 colour FACS Calibur flow cytometer using mouse anti human monoclonal antibodies in the acute leukemia panel as described in the figure below. The fluorochrome isotypes used were IgG1 conjugated FITC, APC PE and PE-Cy5.We followed the EGIL scoring system in case of ambiguity in lineage. The percentage expression of the antigens in the gated population was quantified. The protocol we followed is briefly described below.

Protocol for sample preparation by Lyse-Wash Method for Immunophenotyping of hematolymphoid neoplasms (bone marrow and peripheral blood samples) using CD45 as a gating strategy

Heparin anticoagulated 500ul bone marrow apirate sample was mixed with 3ml of RBC-lysis solution (NH_4Cl based) and mixed thoroughly. The sample was then centrifuged at 800g for 3 minutes and supernatant was discarded One ml of phosphate buffered saline (PBS) buffer was added so as to make total volume of 2 ml and gently vortexed the pellet so as to break it. Then it was centrifuged at 800g for 3min. PBS was added so as to make 2 ml, pellet was broken and centrifuged once again, and final volume was adjusted so as to attain a cell count between 500,000-1,000,000 /mm3. An appropriate quantity of antibody from the cocktail mix (determined by antibody titration) was added to the cell suspension for the corresponding tube. Sample was incubated in dark for 20 minutes. Two ml of PBS was added and then washed and centrifuged at 800g for 3 min. PBS was added to make final volume of 200ul.The following table illustrates how the panel was designed.

Table 7:Design of the tubes for flow cytometry

	FITC	PE	Per-CP	APC
Tube 1	Isotype	Isotype	45	Isotype
Tube 2	4	8	45	19
Tube 3	7	34	45	117
Tube 4	19	10	45	20
Tube 5	HLA-DR	33	45	11b
Tube 6	64	56	45	13
Suspected Burkitt	λ	κ	45	20
Hematogones	19	10	45	20
	HLA-DR	34	45	38
T cell ALL	7	5	45	2
	1a	3	45	

Cytogenetics

Culture set up for bone marrow samples

Bone marrow aspirate samples were received in vacutainers with heparin as the anticoagulant. WBC diluting fluid was added and the cell count was taken in Neubauer chamber. Then appropriate volume of the sample was added to the RPMI culture medium. For one bone marrow sample two RPMI media were used. For one 5ml 100 microlitre of Colcimed was added and overnight incubation done. In the other 5 ml 250 microlitre of Colcimed was added and incubated for 30 minutes.

Harvest

This was done at 17-23 hours after culture setup. After adding Colcimed the sample is incubated, fixative was added and then centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in fixative.

Slide making

Glass slides were soaked in methanol in koplin jar. They were then wiped dry, dipped again in methanol and then swirled in milli Qwater. Cell suspension was then dropped on to the slide. Slides were then dried and examined under phase contrast microscope**.**

Banding

The slides were dipped in warmed trypsin solution. Then they were washed in ice cold water and stained with Leishman stain. Stain is blotted and examined under microscope. The metaphases (at least 20) were captured and analysed.

Molecular Markers

Screening for FLT3-ITD and NPM1 mutations

Mutation screening in these two genes were done using Multiplex-PCR followed by Gene Scanning. DNA was extracted from cytogenetic cell pellet. Cells (1 $x10^6$) were fixed in methonal-acetic acid(3:1), 70% ethanol,or 95% ethanol to a 1.5ml microcentrifuge tube and centrifuged at 13,000 for 2 mins to pellet cells. After removing the supernatant, 300ul cell lysis solution was added to the cell pellet and mixed. To this 2ul of Puregene Proteinase K (cat.no.158918) was added and the sample was incubated overnight .Then 100ul of Protein precipitation solution was added and centrifuged for 3-5 min at 13,000 rpm. The protein pellet was precipitated and if protein pellet was not

tight, the sample was incubated on ice for 5 mins and repeat centrifugation done. To the supernatant 300ul isoproponal was added and centrifuged for 1 min at 13,000-16,000g. The supernatant was then discarded and the DNA pellet was washed with 300microlitre of 70% alcohol. The sample was then centrifuged for 1 min at 13,000-16,000g.The supernatant was discarded and the pellet was air dried. 50ul of 1xTE buffer was added to dissolve the DNA. The sample was incubated at 37^0 C overnight.

Mutiplex-PCR amplification of genomic DNA using primers to exon 14 & exon 15 of FLT3 yielded a 330 bp from the wild type alleles and primers to exon 12 of NPM1 yielded a 169bp from the wild type alleles. HBG gene 469bp served as the internal control. FLT3/ITD mutation produced larger peaks with a range from 349 to 409, 21 to 81bp larger than the wild type, while NPM1 mutation produced a single peak at 171bp, 4bp larger than the wild type. HBG showed 469bp peak.

Treatment

Induction

All patients underwent standard "3+ 7" induction chemotherapy with Daunorubicin 50 mg/m2 on days 1-3 and Cytosine Arabinoside 200 mg/m2 as a continuous infusion on days 1- 7 .All patients were started on amphotericin prophylaxis 0.5 mg/kg /day on alternate days at the start of the chemotherapy and the dose increased to 1mg/kg/day at the onset of febrile neutropenia. This is based on the high incidence of fungal infections in our population. Early response assessment marrow was done between days 10-14, and if the same showed >20% blasts with cellularity>20%, a reinduction was considered. Persistence of disease documented by bone marrow after second course of induction was defined as primary refractory disease. Therapy of such patients was individualised. G

CSF was administered after early response assessment at the discretion of treating physician.

Consolidation

High dose cytosine arabinoside(HIDAC), autologous stem cell transplant (auto SCT) and allogentic stem cell transplant (allo SCT) were the principal options considered for patients in good general condition, without co morbidities and financial restrains. Patients with good risk cytogenetics who achieved CR were offered three doses of HIDAC. Patients with intermediate and poor risk cytogenetics were offered allo SCT if an HLA matched donor was available. For those with intermediate risk cytogenetics without a donor auto SCT was offered.

High Dose Cytosine Arabinoside (HIDAC)

Indications included 1)good risk AML and 2)Intermediate risk AML in whom an HLA matched donor was not available and where it was felt that the general clinical condition or co morbid conditions or financial restrains preclude the use of an autologous SCT. It consists of high dose cytosine 3gm/m2 q12h on days 1, 3 and 5 and was iniatiated one to two weeks after documentation of remission during induction chemotherapy. Each dose was diluted in 500ml normal saline and infused over three hours. Cerebellear signs were checked for prior to each dose and prednisolone eye drops were started 24 hours prior to onset of chemotherapy.

Intermediate Dose Cytosine Arabinoside

This was considered in the following situations- 1) Limited financial resources

2) Patients with co morbid conditions limiting the intensity of therapy.

Autologous Stem Cell Transplant (auto SCT)

Indications- Intermediate risk AML in whom an HLA matched donor was not available. Patients received 2-3 cycles of HIDAC prior to the mobilization of autologous SCT harvest. G CSF mobilized peripheral blood stem cells were harvested between 2-4 weeks after recovery from last cycle of HIDAC. Conditioning consisted of conventional Busulfan and Cyclophosphamide regimen. Minimum target cell dose to proceed with an autologous SCT was 2 x 10^8 MNCs/kg. Post autologous GCSF was started on day+7 till ANC>1500/mm3 on two successive days.

Allogenic Stem Cell Transplant (allo SCT)

Indication for allogenic SCT included all intermediate and high risk patients with AML who had an HLA matched sibling. Conditioning regimens included standard myeloablative Busulfan/Cyclophosphamide regimen and reduced intensity conditioning (RIC) regimens like Fludarabine Melphalan and FLAG IDA Melphalan. Currently for patients scheduled to receive an Allogenic SCT no consolidation is used post induction unless there is an anticipated delay in proceeding to transplant. All patients received a peripheral blood stem cell transplant. GVHD prophylaxis consisted of conventional Cyclosporine/Methotrexate regimen. In the absence of GVHD, Cyclosporine taper was initiated from day 60 and stopped by day 100. In the event of GVHD, the taper of immunosuppression was individualised.

LSC study

All newly diagnosed as well as relapsed cases of AML in the hospital between October 2008 to December 2009 who consented for the study and who have been

treated with standard induction therapy were included in the study for quantitation of LSCs. The study was reviewed and approved by the institution's research and ethics committee (IRB Min No.6661 dated17.09.2008). To be included in the analysis, the diagnosis had to be confirmed by bone marrow morphology with cytochemistry or immunophenotyping. A written informed consent was obtained from all patients in accordance with the Declaration of Helsinki. All the bone marrow aspirate samples(5 ml heparin anticoagulated) from patients with AML were processed for immunophenotyping (using antibodies against CD34, CD38 and CD123) to quantify the proportion of leukemic stem cells (LSC) among the blast population of newly diagnosed cases of acute myeloid leukemia (AML) at diagnosis and in a post induction(day 10-14) bone marrow sample. Second, we also identified and quantified the expression of adhesion molecules VLA-4 and VLA-5 in this LSC population. An absolute number of cells expressing these markers were quantified by dual platform method.(Bone marrow total nucleated cell count was measured in Sysmex cell counter).The impact on prognosis was assessed by the correlation with leukemic stem cell and adhesion molecule expression and effect on outcome as measured by Overall Survival, Event Free Survival and Disease Free Survival . The gating strategy we adopted is described below in figure 2.

The isotypes and the antibodies directed against specific antigens were run through titration experiments to find out the optimum volume. The fluorochrome conjugated antibodies were designed in three tubes as described in table 8. CD 44 conjugated with FITC did not yield optimum results with the titration and hence the results from the tube containing CD 44 was not considered for final analysis. PE Mouse Anti-Human

Gating Strategy for the LSC study.

LSCs were defined as CD 34+CD38-CD123+ cells. The gating strategy adopted is illustrated here.

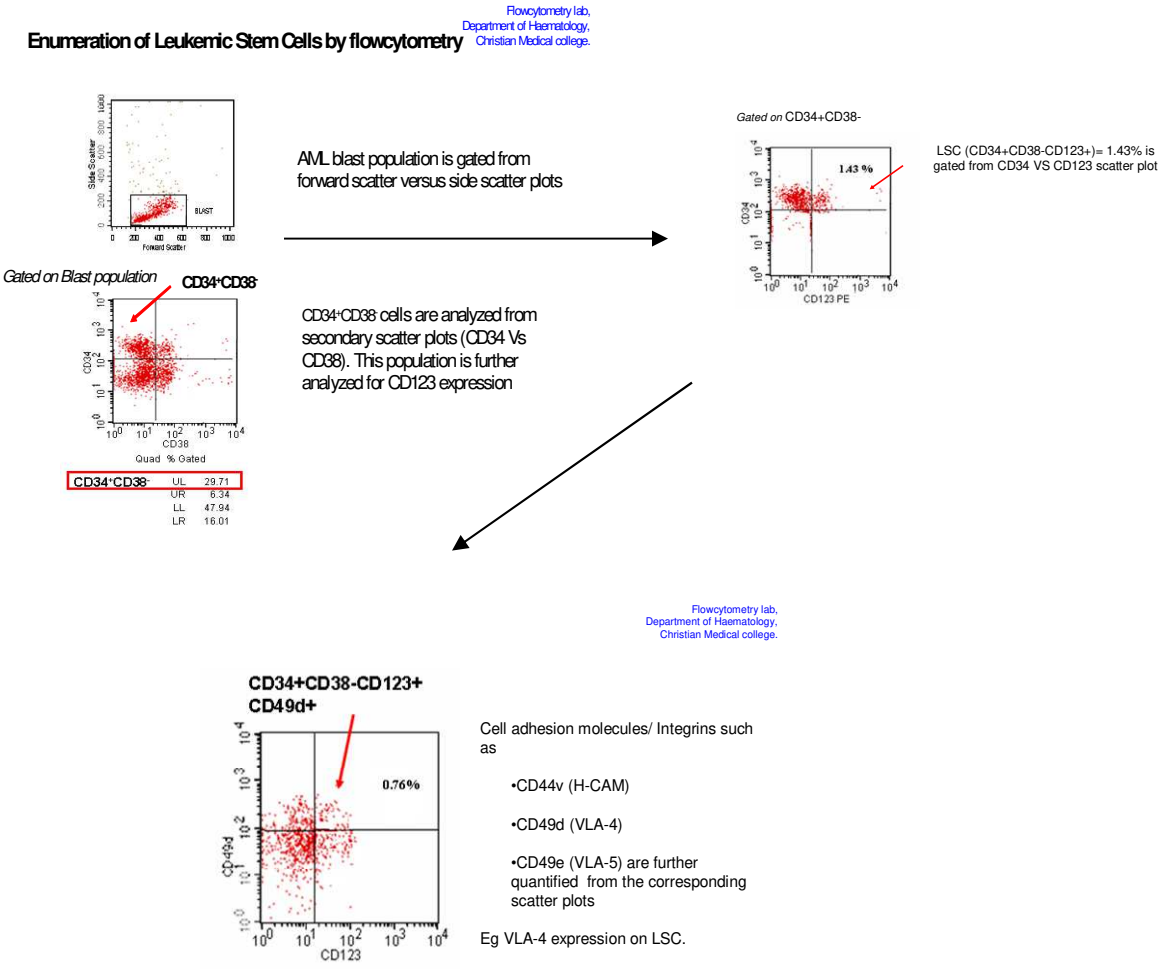


Figure 2: Gating strategy for the quantitation of LSCs

CD49dwhich reacts with the integrin alpha 4 chain expressed as a heterodimer with beta subunit1 to form VLA 4 and Phycoerythrin conjugated Mouse Anti Human CD49e which

reacts with the alpha 5 integrin that assoaciated with beta 1 integrin to form VLA5 were used to identify adhesion molecules. All the antibodies were bought from BD biosciences. The tube design is depicted in the following table.

Table 8:Design of tubes for flowcytometry for the LSC study

Tube 1(isotype controls)	Tube 2	Tube 3
IgG1 FITC 3 microlitre	CD 34 FITC 5 microlitre	CD 34 FITC 5 microlitre
IgG1PE 2 microlitre	CD 49e PE 5 microlitre	CD 123 PE 5 microlitre
IgG1PE-Cy5 3microlitre	CD 123 PE-Cy5 5 microlitre	CD 49d PE-Cy5 5 microlitre
IgG1 APC 2 microlitre	CD 38 APC 3 microlitre	CD 38 APC 3 microlitre

DEFINITIONS

Response criteria in AML

Complete remission (CR) Bone marrow blasts < 5%; absence of blasts with Auer rods; absence of extramedullary disease; absolute neutrophil count > 1.0 x 10^9/L (1000/_L); platelet count > 100 x10^9/L (100 000/_L); independence from red cell transfusion.

Induction Mortality: Death during standard induction treatment for AML.

Relapse: Bone marrow blasts > 5%; or reappearance of blasts in the blood; or development of extramedullary disease

Event: Death , relapse or persistent disease after two cycles of induction therapy were considered as event.

Outcome measures in AML

Category Definition

Overall survival (OS)

Defined for all patients. Measured from the date of diagnosis to the date of death from any cause; patients not known to have died at last follow-up are censored on the date they were last known to be alive.

Disease free survival (DFS)

Defined only for patients achieving CR ; measured from the date of achievement of a remission until the date of relapse or death from any cause; patients not known to have relapsed or died at last follow-up are censored on the date they were last examined

Event free survival (EFS)

Defined for all patients. Measured from the date of diagnosis to the date of induction treatment failure,or relapse from CR or death from any cause; patients not known to have any of these events are censored on the date they were last examined

Relapse free survival (RFS)

Defined for all patients and measured from the date of diagnosis until the date of relapse or induction failure; patients not known to have relapsed are censored on the date they were last examined.

Statistical analysis

The chi- square or Fisher exact test was used to compare differences between groups for response to therapy. The probability of survival was estimated with the use of the product-limit method of Kaplan and Meier for overall survival, event-free survival, relapse free survival and disease-free survival and compared by the log-rank test. All survival estimates are reported +/- 1 SE. The relationships of clinical features to outcome were analyzed by Cox proportional hazard model. All *P* values were 2-sided, with values of .05 or less indicating statistical significance. Statistical analysis was performed using SPSS 11.0 software (SPSS,Chicago, IL).

RESULTS

Between January 2003 and December 2009 a total of 192 patients who were diagnosed to have AML who met the inclusion criteria were retrospectively and prospectively analysed. Between October 2008 and December 2009, 41 patients with AML were analysed for the quantitation of LSCs.

Baseline Characteristics (Table 9)

Out of the 192 patients,120(62.5%) were males and 72(37.5%) were females .The median age was 36 years(range 15-60 years).Among the FAB subtypes, M2 was the most common with 95(49.5%) patients, followed by M1 with 39(20.3%) patients. The median WBC count at diagnosis was $12.6x10^9$/L ($0.4-326x10^9$/l). Median WBC index at diagnosis was 8.12(0.10-285). Cytogenetic data was available for 180 patients (93.8%) of which 21 (10.9%) were good risk, 121(63%)) were intermediate risk and 38(19.8%) were poor risk. Twelve cases had cytogenetic failure.

The immunophenotyping data was available for 152 patients, out of which CD13 was positive in 115(75.65%) patients, CD33 in 132(86.8%), CD34 was positive in 84(55.3%) and HLA DR in 126(82.9%)(figure 3). Bone marrow aspirate of 156(81.3%) patients stained positive for Sudan Black B and Auer rods were present in 36(18.8%).

Among the cohort of 41 AML patients who were analysed for the quantitaion of LSCs 25(61%) were males and 16(29%) were females. The median age was 34 years (range 5-59). In addition to the newly diagnosed AMLs 6 cases of relapsed AMLs were also included in the analysis.The baseline parameters of this cohort is summarized in table 9.

Table 9: Baseline characteristics

Variable	Median/n(range) /(%)	
	AML n = 192	**AML LSC analysis n=41**
Age	36(15-60)	34(5-59)
<15 years	0(0)	3(7.3)
15-30 years	66(34.5)	11(26.8)
31-40 years	52(27)	13(31.7)
41-50 years	45(23.4)	9(22)
51-60 years	29(15.1)	5(12.2)
Sex		
Male	120(62.5)	25(61)
Female	72(37.5)	16(39)
AML FAB subtype		
M0	15(7.8)	0(0)
M1	39(20.3)	12(29.3)
M2	95(49.5)	17(39)
M3	0(0)	0(0)
M4	18(9.4)	1(2.4)
M5	12(6.3)	3(7.3)
M6	6(3.1)	0(0)
M7	3(1.5)	2(4.9)
Secondary AML	4(2.1)	1(2.4)
Relapse	0	5(14.6)
Hb in gm/dl	8.8(3.8-14.0)	8.4(4.9-13.7)
WBC x 10^9/l	12.6(0.4-326)	26.4(1.9-152)
Platelet x 10^9/l	50(2.0-387)	39(5.0-249)
Peripheral blood blast%	44.5(0 -98)	69(1-95)
Bone marrow blast %©	70(21-100) *	76(20-100)
Cytogenetics		
Good	21(10.9)	03(7.3)
Intermediate	121(63)	31(75.6)
Poor	38(19.8)	07(17.1)
Not worked/not available	12(6.3)	0 (0)
LDH	766(113-15220)	1173(290-8374)
LFT		
Normal	176(91.7)	35(85.4)
abnormal	16(8.3)	06(14.6)
Creatinine	0.9(0.5-2.3)	0.9(0.4-1.9)
WBC index	8.12(0.1-285)	17.33(0.8-91.53)

© blast was defined as per WHO classification 2008

* information was available for 184 patients

Median values	62.5	73.3	38.9	74.6

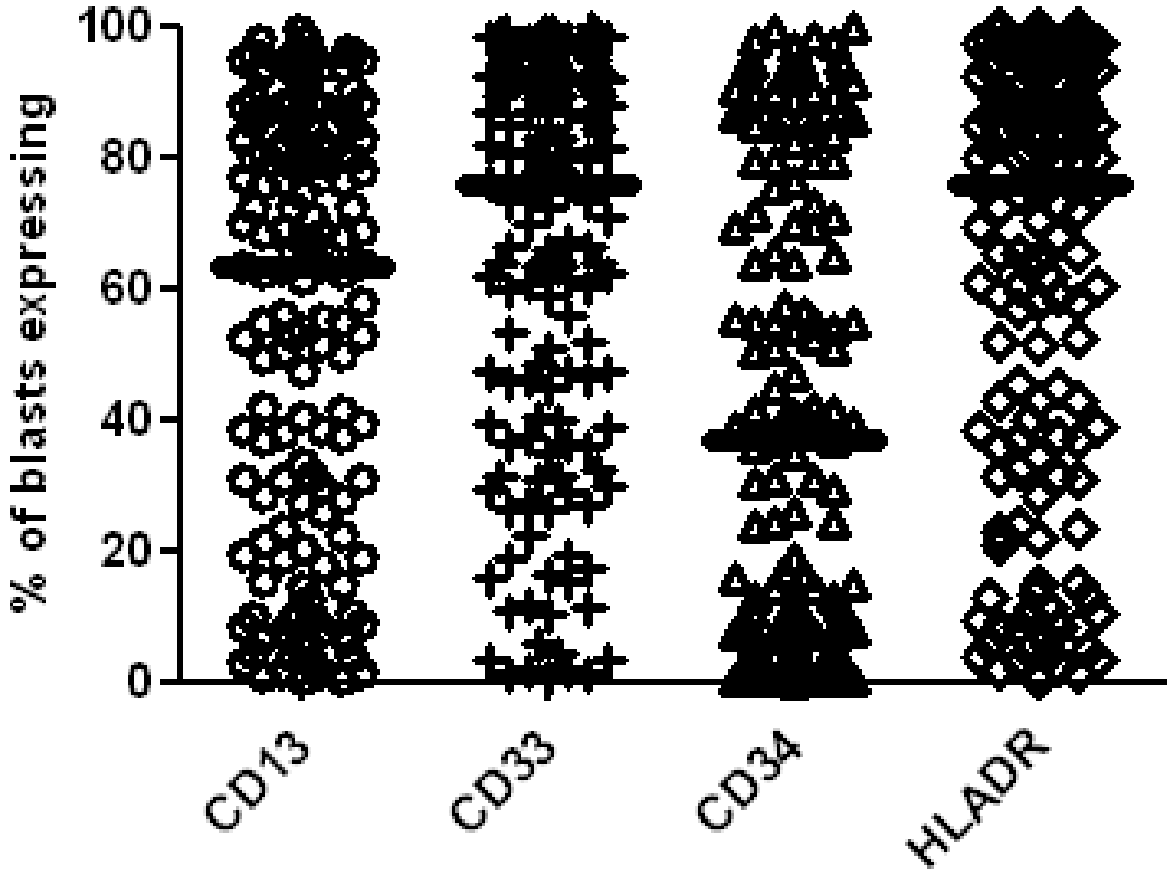


Figure 3: Immunophenotypic characteristics of patients. n=152.

Treatment related variables

Induction (Table 10)

All 192 patients underwent "3+7" standard induction therapy with Daunorubucin (DNR) 50mg/m^2 on days 1-3 and Cytosine Arabinoside 200mg/m^2 on days 1-7. Bone marrow aspirate and biopsy to assess aplasia done between day 10-14 showed residual disease in 33(17.2%) patients. Bone marrow assessment was not done in 10(5.2%) patients who died before day 10. A total of 117(60.9%) patients achieved complete remission (CR) after induction with "3+7" chemotherapy. There were 47 (24.5%) induction deaths. Out of the remaining (n=28), 19(10.4%) underwent second induction. Out of these, 12 achieved complete remission. The choice of chemotherapy for the second induction varied based on the discretion of the treating physician and included a repeat "3+7" chemotherapy, HIDAC, FLAG IDA or "3+3" chemotherapy with Idarubicin and high dose Cytosine. In one patient Gemtuzumab Ozagamicin was used as single agent for second induction. Thus the overall CR after induction therapy was 129(67.15%).The causes of induction deaths is enumerated in Table 12. Invasive fungal infection was documented in 33/47(70.2%) induction deaths. There were documented bacterial infection in 18(9.4%) out of which 14(7.3%) had gram negative and 4(2.1%) had gram positive infection. Invasive fungal infection was documented in 85(44.3%).According to the International Consensus criteria 81(42.2%) were classified as possible,1(0.5%) as probable and 3(1.6%) as definite fungal infection. HRCT was done in 32(16.7%) patients with probable fungal infection out of which 25 were reported as suggestive of fungal pneumonia.

Among the patients with AML for LSC analysis 28(68.3%) underwent standard "3+7 induction and 13(31.7%) had other induction which included truncated "7+3","3+3","5+5"® and standard pediatric BFM induction protocol. A total of 29 patients(70.7%) achieved CR after first induction .Two(4.9%) patients underwent second induction, out of which 1(2.4%) achieved CR. Eight (19.5%) patients died during induction.

Table 10:Treatment related variables- Induction

Variable	Median/n (range)/(%) **AMLn=192**	Median/n (range)/(%) **AML LSC n=41**
Induction		
CR after 1 cycle	117(60.9)	29(70.7)
CR after 2 cycles‡	12 (6.25)	01(2.4)
Overall CR	129(67.15)	30(73.1)
ANC < 0.5 x 10^9/L	22 (13-66)	22 (18-70)
Platelet< 20 x10^9/L	23 (18-61)	22(18-49)
Day 10- 14 (n=182) residual disease∗	33(17.2)	02(4.9)
Infection	110(57.3)	25 (61)
Fungal∗∗	85 (44.3)	18(43.9)
Documented bacterial	18(9.4)	06(14.6)
Induction death	47 (24.5)	08(19.5)

∗ was arbitrarily defined as > 20% cellularity with > 20% blasts.

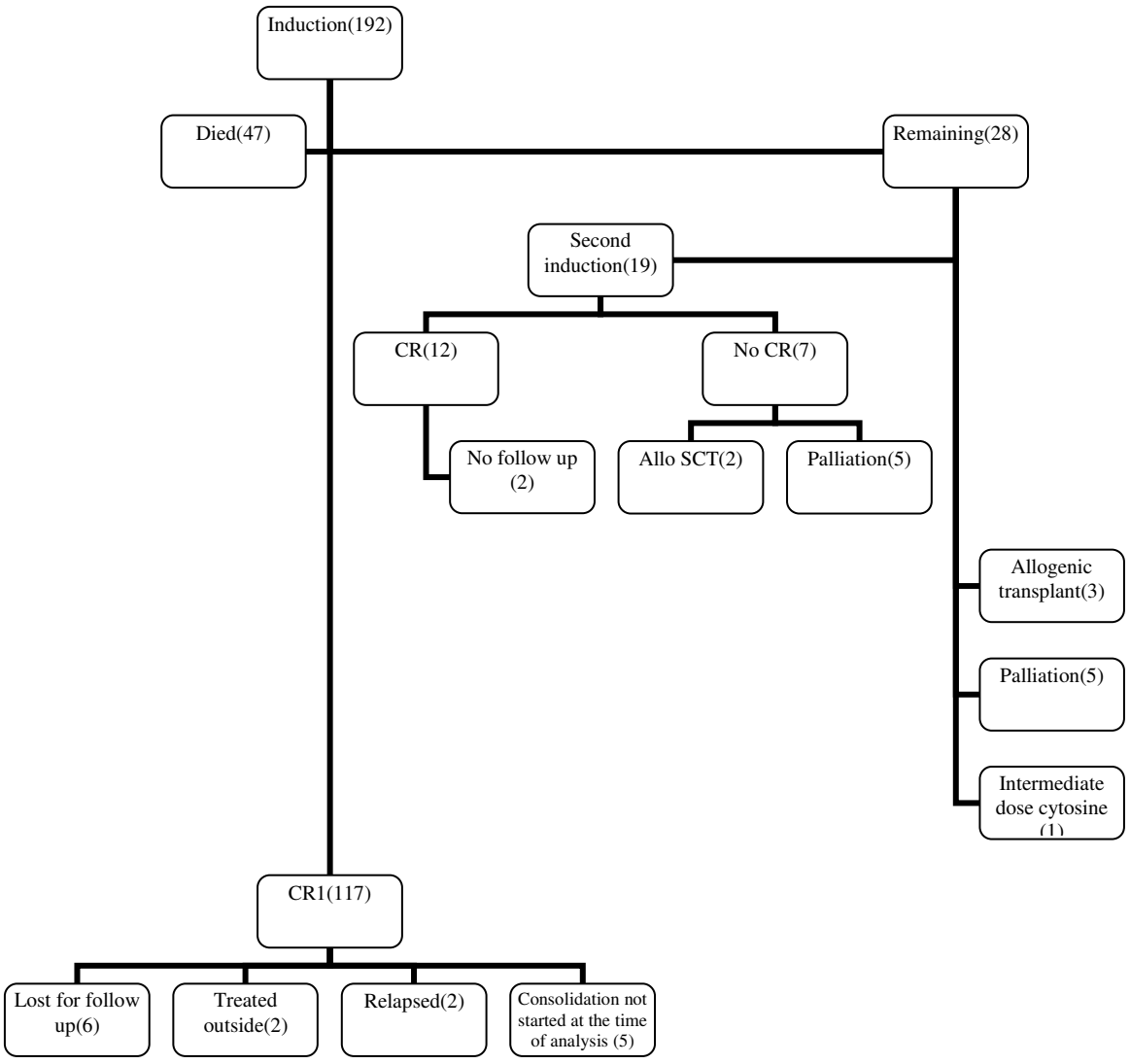
∗∗ was defined based on S. Ascioglu et al. Defining Opportunistic Invasive Fungal Infections in Immunocompromised Patients with Cancer and Hematopoietic Stem Cell Transplants: An International Consensus. Clinical Infectious Diseases 2002; 34:7–14.

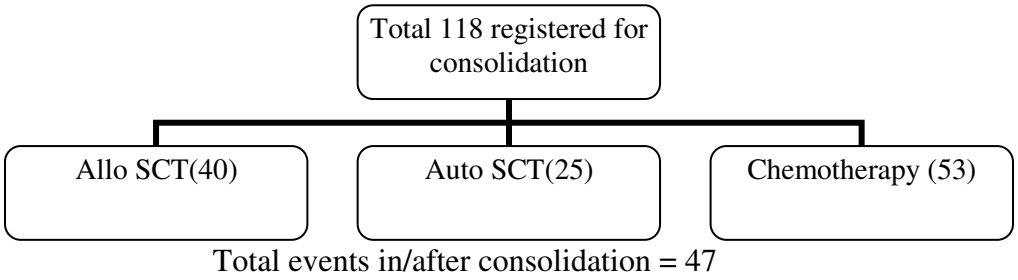
‡ 19 and 2 patients underwent 2nd induction in the AML and AML LSC cohort respectively.

®5+5 included Mitoxantrone and Etoposide for 5days

Consolidation (Table 10)

For the 9 (4.7%) patients who had not been in remission after first induction 3(1.5%) had an allogenic transplant, 5(2.6%) were discharged on palliation and 1(0.5%) patient was treated with intermediate dose cytosine. As mentioned before, 19 patients who were not in remission after first induction underwent a second induction. Seven (3.6%) patients did not achieve remission after 2 indcutions. Among these, 2(1%) underwent allo SCT and 5(2.6%) were treated with palliative therapy. Among the patients who achieved remission after the first induction 6(3.1%) were lost to follow up, 2 (1%) patients took further treatment at outside centre, 2 (1%) relapsed and were treated with palliative therapy. At the time of analysis 5(2.6%) patients had just completed induction and consolidation had not been started. Thus, 118(61.5%) patients (112 in CR1, and 6 without achieving CR) received consolidation therapy. Those who received allo BMT were analysed according to intention to treat basis. For example, a patient without good risk cytogenetics who had an HLA matched donor was analysed in the allogenic SCT group irrespective of whether he received his graft in CR1 or CR2. Similarly, a patient who received HIDAC only for one cycle also was analysed in the HIDAC group. Thus, 40(20.8%) patients underwent allo SCT, 25 (13%) auto SCT, 39(20.3%) HIDAC, and 14(7.3%) patients received chemotherapy other than HIDAC.(Table11 and flow chart 2)





Total events in/after consolidation = 47

Flow chart 2 describing the patients who were analysed in the study.

Allo SCT

Forty patients received allo SCT as consolidation (refer table 11).Of these 35(87.5%) had achieved CR and 5 patients had residual disease prior to conditioning. Of the patients who had achieved CR, 2 had a medullary relapse and one had a CNS relapse prior to the transplant. Graft source was G CSF mobilized peripheral blood stem cells (PBSC) in all. Thirty eight patients (95%) received graft from related donors and 2 received graft from unrelated donors. HLA of the donor was identical in 36(90%) patients, 5/6 match in 3(7.5%) patients and 8/10 match in 1(2.5%) patient. The median cell dose was 5.92(range 2.6 -10.8) x10 ^{8} mononuclear cells/kg and median CD34 dose was 13.5(range 5.76-33) x10^{6} cells /kg. Eighteen patients (45%)underwent allo SCT without taking any further chemotherapy after induction; 11 (27.5%) took " 5 +2" chemotherapy with daunorubicin and cytosine, 4 (10%)patients had "3+3" with Idarubicin and high dose Cytosine, one(2.5%) had Hyper C VAD and 5(12.5%) had one or more cycles of HIDAC. Overall 7(17.5%) patients died of non relapse causes of which 4(10%) were less than 100 days post transplant. Acute GVHD occurred in 18(45%) patients of which 14(35%) were grade 2-4. Five (12.5%) patients relapsed of whom two (5%) received second transplant from the same donor and subsequently are in remission.

Auto SCT

Twenty five (13%) patients underwent auto SCT. They received 1-3 cycles of high dose chemotherapy prior to auto SCT (refer table 11).All patients were in CR1 except one who was transplanted in CR2. Twenty three (92%) patients received atleast one dose of HIDAC. One patient received 2 cycles of "5+5" chemotherapy. One (4%) patient

received “3+3” followed by “5+5” chemotherapy. The conditioning regimen in all the patients was Busulfan/Cyclophosphamide. G CSF mobilized and cryopreserved peripheral blood stem cells was used as graft source. The median cell dose was 5.80(range3.28 -13.99) x 10^{8} mononuclear cells/kg and median CD34 dose of 4.4(range 1.37-22.6)x 10^{6} cells /kg. There was no treatment related mortality. The median time from CR to transplant was 146(range 76-249) days. Two (8%) patients in this cohort died in CR and 8(32%) patients relapsed. Of the patients who relapsed 2(8%) received allo SCT from matched unrelated donors and are in remission now.

HIDAC

Thirty nine (20.3% of the total) patients received HIDAC as consolidation. Of these only 33(84.6%) received the second and 25(64.1%) received the third cycle. Two (5.1%) patients developed cerebellitis in cycle 2 and one (2.5%) patient in cycle3. Three (7.6%) patients died during second cycle of consolidation (refer table 11).Of the remaining, 12 (30.4%) relapsed.

Other chemotherapy

Among the 14(7.29%) patients who were treated with chemotherapy other than HIDAC as consolidation, 10(71.4%) received intermediate dose cytosine, 3(21.4%) received “5+2”, and 1(7.14%) Gemtuzumab ozagamycin. One (7.14%) patient died of fungal pneumonia and 4 (28.56%) patients relapsed.

Table11:.Baseline characteristics and Treatment related variables- Consolidation AML

Variables	Median/n (range)/(%) **Allo SCT n=40**	Median/n (range)/(%) **Auto SCT n=25**	Median/n (range)/(%) **HIDAC chemo n=39**	Median/n (range)/(%) **Other chemo n=14**
Age in years	34(15-51)	25(15-60)	36(17-60)	47(22-57)
Sex male	27(67.5)	17(68)	23(59)	09(64.3)
female	13(32.5)	08(32)	16(41)	05(35.7)
Subtype				
M0	03(7.5)	03(12)	03(7.7)	03(21.4)
M1	10(25)	06(24)	09(23)	01(7.1)
M2	18(45)	10(40)	23(59)	05(35.8)
M4	05(12.5)	03(12)	02(5.1)	02(14.3)
M5	02(5)	02(8)	01(2.6)	02(14.3)
M6	02(5)	01(4)	01(2.6)	0(0)
M7	0(0)	0(0)	0(0)	0(0)
Secondary AML	0(0)	0(0)	0(0)	01(7.1)
WBC at diagnosis x 10^9/l	8.8(1.4-248.6)	10.6(0.6-123)	9.5(0.9-101.7)	46.5(1.9-167.9)
WBC index	7.1(0.5-159.1)	7.2(0.5-118.8)	6.0(0.4-91.53)	29.7(1.3-97)
Cytogenetic risk break up				
Not worked	01(2.5)	01(4)	01(2.6)	02(14.3)
Good	0(0)	01(4)	14(35.9)	02(14.3)
Intermediate	28(70)	20(80)	21(53.8)	07(50)
Poor	11(27.5)	03(12)	03(7.7)	03(21.4)
Conditioning regimen ‡				
Busulfan / Cyclophosphamide	15(37.5)	25(100)	n a	n a
Fludraine /Melphalan	15(37.5)			
Cyclophosphamide /TBI	3(7.5)			
FLAG IDA+/- Melphalan	7(17.5)			
ANC< 0.5x10^9/L(in days)	13(9-26)	10(8-14)	19(11-39)	20(14-24)
Death during consolidation	4(10)	0(0)	3(7.9)	1(7.7)
Number of cyclesof high dose chemo∗				
Three	n a	20(80)	24(63.2)	n a
Two		4(16)	8(21)	
One		1(4)	6(15.8)	

‡ applicable only for allo SCT and auto SCT patients

∗ applicable only for auto SCT and HIDAC patients. Refer text for the pre transplant chemotherapy details of Allo SCT group.

Table12:Treatment related mortality(calculated within100 days of starting treatment)

n (%)				
Induction n=47(24. 5%)	**Allo SCT n=4(10%)**	**Auto SCT n=0**	**HIDAC chemo n=3(7.7%)**	**Other chemo n=1(7.1%)**
Fungal pneumonia- 24(51) Intracranial bleed- 1(2.1) Sepsis- 9(19.2)* Hyper leucostasis- 1(2.1) Acute cardiotoxicity- 2(4.3) Acute renal failure- 1(2.1) Suspected pulmonary embolism- 1(2.1) Fungal pneumonia and sepsis- 8(17.1)**	Grd IV GVHD- 2(50) Hepatic failure-1(25) CMVcolitis- 1(25)	nil	Sepsis- 2(66.7) Fungal pneumonia 1(33.3)	Fungal pneumonia 1(100)

*5 cases had documented gram negative sepsis.
** 2cases had documented gram negative sepsis

Screening for NPM1 and FLT3 Mutations (Tables 13 and 14)

Out of the 83 samples in which DNA was extracted 18 had NPM1 mutations, 14 had FLT3 ITD mutations and 2 had FLT3 TKD mutation. Patients with ITD and TKD mutations were analysed as one group. The baseline characteristics and treatment related characteristics of these patients are depicted in the tables 12 and 13. All except 2 patients with NPM1 mutations had intermediate risk cytogenetics. No other variable showed significant difference between different subgroups.

Table 13:Baseline characteristics of patients with NPM1 and FLT3 mutations (analysed separately)

Variable n=82	NPM1 Mutated n=18 Median/n (range)/(%)	NPM1 not mutated n=64 Median/n (range)/(%)	P value	FLT3 mutated n=16 Median/n (range)/(%)	FLT3 not mutated n=66 Median/n (range)/(%)	P value
Age	34(22-58)	34.5(15-60)	0.449	34(17-58)	34.5(15-60)	0.829
Sex Male Female	 09(50) 09(50)	 41(64.1) 23(35.9)	0.290	 09(56.3) 07(43.8)	 41(62.1) 25(37.9)	0.777
WBC at diagnosis	32.2(1.4-300)	19.9(0.7-248)	0.216	25.6(2.0-248)	53.5(2-387)	0.139
WBC index	21.3(0.5-285)	10.3(0.32-159)	0.102	17.4(0.54-159)	10.3(0.32-285)	0.168
Cytogenetic Risk group Good Intermediate Poor	 0(0) 16(88.9) 2(11.1)	 13(21) 33(53.2) 16(25.8)	 **0.018**	 0(0) 13(81.3) 03(18.8)	 13(20.3) 36(56.3) 15(23.4)	 0.096
CR (percentage)	08(44.1)	41(64.1)	0.442	08(50)	41(62.1)	0.406
Induction death (percentage)	08(44.4)	15(23.4)	0.135	04(25)	19(28.8)	1.000
OS+/- 1 SE *	30.86+/-12.91	45.24+/-7.12	0.151	37.50+/-14.99	43.41+/-6.86	0.832
EFS+/- 1SE	27.01+/-11.86	37.25+/-6.82	0.155	25.00+/-13.40	37.77+/-6.59	0.347
DFS+/- 1SE	48.61+/-18.73	53.84+/-8.69	0.710	33.33+/-17.21	68.71+/-8.65	0.013
Relapses n (%)	2(11.1)	18(28.1)	0.215	04(25)	16(24.4)	1.000
Consolidation HIDAC Auto SCT Allo SCT Other Chemo	 02(25) 02(25) 03(37.5) 01(12.5)	 10(25) 07(17.5) 10(35) 09(22.5)	 0.912	 02(20) 02(20) 05(50) 01(10)	 10(26.3) 07(18.4) 12(31.6) 09(23.7)	 0.658

*Estimated Kaplan Mier Survival values at a median follow up of 2 years

Table 14: Baseline characteristics of patients with NPM1 and FLT3 mutations (analysed in combination)

Variable N=82	NPM1# mut£ FLT3 not n=11 Median/n(range)/(%)	NPM1 not FLT3 mut n= 09 Median/n(range)/(%)	NPM1 not FLT3 not n=55 Median/n(range)/(%)	NPM1 mut FLT3 mut n=07 Median/n(range)/(%)	P value
Age	34(24-58)	34(17-53)	35(15-60)	34(22-58)	0.803
Sex Male Female	 04(36.4) 07(63.6)	 04(44.4) 05(55.6)	 37(67.3) 18(32.7)	 05(71.4) 02(28.6)	 0.167
WBC at diagnosis	39.4(1.4-300)	40(8.4-248.6)	11.8(0.7-215)	22.2(2-119.9)	0.110
WBC index	27.4(0.5-285)	13.8(3.6-159)	6.5(0.32-142)	17.8(0.54-88)	0.140
Cytogenetic Risk group Good Intermediate Poor	 0(0) 10(90.9) 01(9.1)	 0(0) 07(77.8) 02(22.2)	 13(24.5) 26(49.1) 14(26.4)	 0(0) 06(85.7) 01(14.3)	 0.061
CR (percentage)	05(45.5)	05(55.6)	36(65.5)	03(42.9)	0.462
Induction death (percentage)	05(45.5)	01(11.1)	14(25.5)	03(42.9)	1.000
OS+/- 1 SE* EFS+/- 1SE DFS+/- 1SE	32.73+/-14.97 32.73+/-14.97 60.00+/-21.91	44.44+/-18.89 29.63+/-17.46 33.33+/-19.35	45.65+/-07.62 38.84+/-07.29 58.57+/-09.36	28.57+/-22.26 21.43+/-17.81 37.50+/-28.64	 0.489 0.084
Relapses n (%)	01(9.1)	03(33.3)	15(27.3)	01(14.3)	0.490
Consolidation HIDAC Other Chemo Auto SCT Allo SCT	 02(40) 01(20) 01(20) 01(20)	 02(28.6) 01(14.3) 01(14.3) 03(42.9)	 08(24.2) 08(24.2) 06(18.2) 11(33.3)	 00(0) 00(0) 01(33.3) 02(66.7)	 0.916

*Estimated Kaplan Mier survival values at a median follow up of 2 years .For the NPM1mut FLT3mut group only 1 year survival data was available

£'mut' denotes 'mutated' and 'not' denotes 'not mutated'

This group was considered as reference for cox regression analysis

Survival Data (Tables 15 and 16)

The overall survival (OS), event free survival (EFS) and disease free survival(DFS) were calculated for the whole cohort as well as for the different cytogenetic and molecular subgroups and the different modes of consolidation. The relationship between baseline characteristics and treatment related variables and the EFS was analysed using the logistic regression model. At a median follow up of two years the Kaplan- Meier estimate of OS, EFS and DFS was 47.30+/- 4.30%, 37.52+/- 4.20% and 54.49+/-5.38% respectively.(Figures 4 ,5 and 6).The difference in outcomes between the different cytogenetic and molecular subgroups as well as for the different modalities of consolidation did not show statistical significance(illustrated in figures 7and 8). However, when analysed using cox regression , age, WBC count at diagnosis(as a continuous variable), poor risk cytogenetics and WBC index at diagnosis (as a continuous variable) significantly correlated with event free survival(table 15). The disease free survival curves for the two subgroups above and below the median value (8.12) of WBC index were significant(p=0.0225).Similarly in the cohort for AML LSC analysis there was significant difference in the relapse free survival between the subgroups segregated according to stem cell frequency in post induction marrow sample. Multivariate analysis was done using cox regression-enter method where all the significant vaiables in predicting the EFS (age, WBC count, poor risk

cytogenetics and WBC index) were analysed together by enter method, age and poor risk cytogenetics retained significance. However, when analysed using cox regression forward stepwise method, where the most significant variable (WBC index) was analysed against other variables, WBC index was found to be the most significant variable.

Table 15: Baseline characteristics and impact on EFS

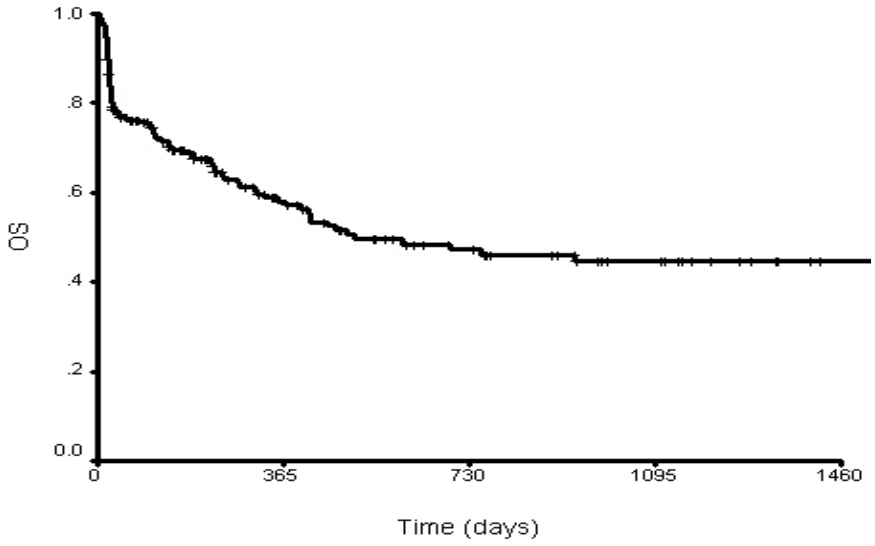
Variable n=192	**Median/n (range)/(%) Event n=104**	**Median/n (range)/(%) No Event n=88**	**RR (95% CI)**	**P value**
Age in years	37(16-60)	33(15-57)	1.0(1.00-1.03)	**0.045**
Sex				
Male*	64 (61.5)	56 (63.6)		0.446
female	40 (38.5)	32 (36.4)	0.8(0.58-1.27)	
AML Subtype				
M0	10 (9.6)	05 (33.3)	1.1(0.54-2.09)	0.868
M1	19 (18.3)	20 (22.7)	0.8(0.50-1.45)	0.549
M2*	50 (48.1)	45 (51.1)		
M4	10 (9.6)	08 (9.1)	0.9(0.46-1.83)	0.819
M5	05 (4.8)	07 (8.0)	0.7(0.29-1.86)	0.515
M6	04 (3.8)	02 (2.3)	1.2(0.44-3.38)	0.704
M7	02 (1.9)	01 (1.1)	2.6(0.64-11.03)	0.180
Secondary AML	04 (3.8)	0 (0)	2.5(0.89-6.87)	0.082
Hb(g/dl)	8.8(4.6-14)	8.65(3.8-13.1)	1.0(0.97-1.18)	0.176
WBC x 10^9/l	20.5(0.4-326)	9.6(0.6-167.9)	1.0(1.00-1.00)	**0.012**
Platelet x 10^9/l	52 (3-387)	47. 5(2-348)	1.0(0.99-1.00)	0.546
CTG				
Good risk*	07(7.2)	14(16.9)		
Intermediate risk	68(70.1)	53(63.9)	2.0(0.92-4.40)	0.079
Poor risk	22(22.7)	16(19.3)	2.4(1.04-5.78)	**0.040**
NPM mutated*	12(24.5)	06(18.2)		
Not mutated	37(75.5)	27(81.8)	0.6(0.32-1.02)	0.160
FLT3 mutated	10(20.4)	06(18.2)	1.4(0.69-2.85)	0.350
Not mutated*	39(79.6)	27(81.8)		
NPM mut FLT3 not*	07(14.3)	04(12.1)		
NPM mut FLT3mut	05(10.2)	02(6.1)	1.3 (0.40-4.07)	0.678
NPM not FLT3 not	32(65.3)	23(69.7)	0.7(0.294-1.52)	0.336
NPM not FLT3 mut	05(10.2)	04(12.1)	0.8 (0.26-2.60)	0.735
WBC Index at diagnosis	11.1 (0.1-285)	5.9(0.4-118.9)	1.0(1.00-1.01)	**0.002**

*These groups were considered as reference for cox regression analysis

Table 16: Treatment related parameters and impact on EFS

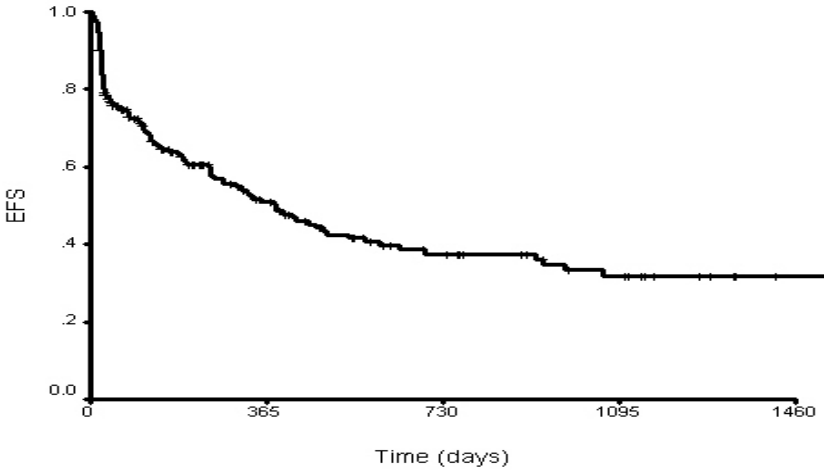
variable	Median/n(range)/(%) Event	No event	RR(95% CI)	P value
Induction				
CR after 1 cycle*	41 (89.1)	76 (91.6)		
CR after 2 cycles	05 (10.9)	07 (8.4)	0.8(0.33-2.16)	0.730
Consolidation				
HIAC	15 (32.6)	24 (33.3)	1.3(0.67-2.85)	0.368
Other Chemo	06 (13.0)	08 (11.1)	1.6(0.64-4.30)	0.298
Auto SCT	10 (21.7)	15 (20.8)	1.0(0.47-2.35)	0.895
Allo SCT *	15 (32.6)	25 (34.7)		

*These groups were considered as reference for cox regression analysis



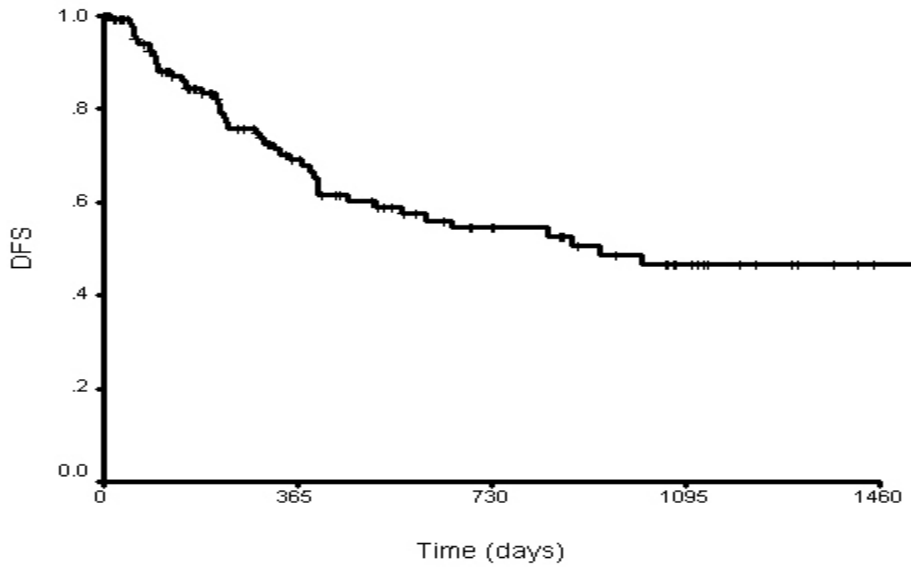
Overall Survival

Figure4: Kaplan Meier estimate of Overall Survival at a median follow up of 2 years was 47.30+/-04..30%.



Event Free Survival

Figure 5: Kaplan Meier estimate of Event Free Survival at a median follow up of 2 years was 37.52+/-04.20%.



Disease Fee Survival

Figure 6: Kaplan Meier estimate of Disease Free Survival at a median follow up of 2 years was 54.49+/-05.38%.

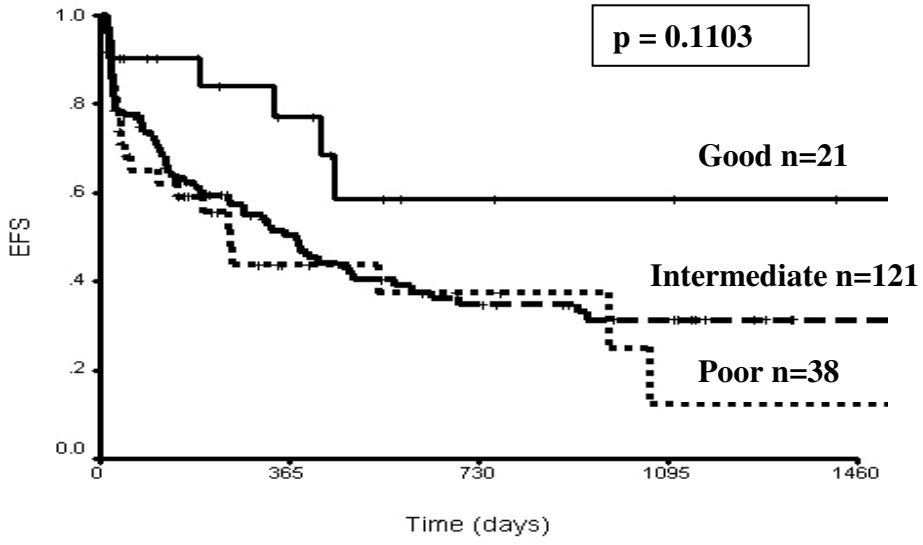


Figure 7: EFS in the different cytogenetic risk groups

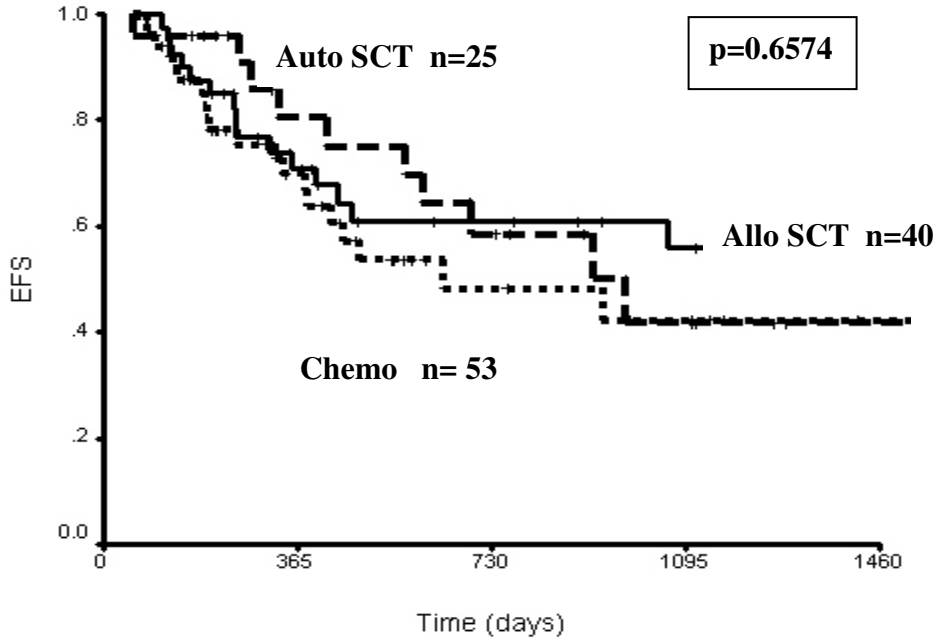


Figure 8: EFS in different modalities of consolidation.

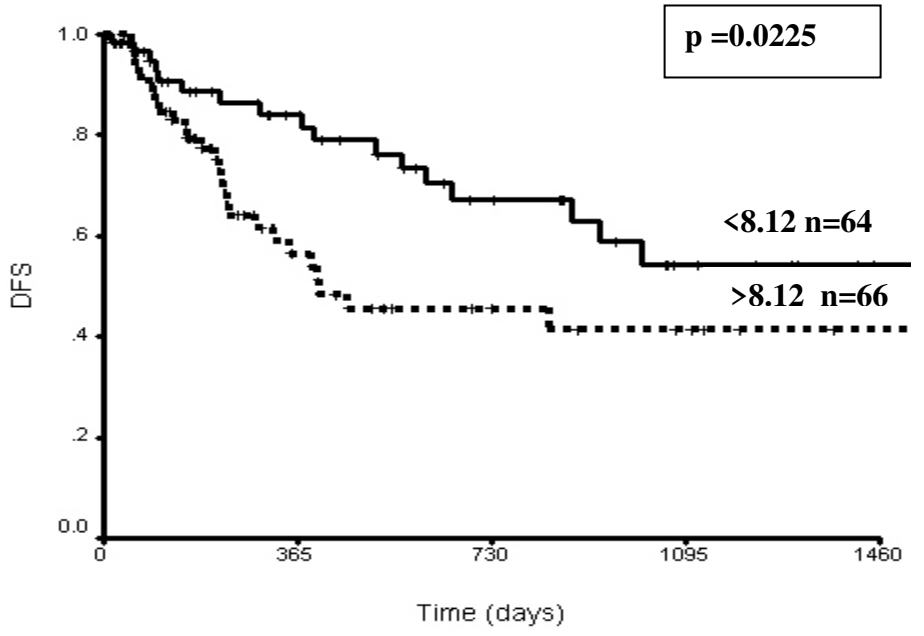


Figure 9: Disease Free Survival in subgroups according to WBC index

LSC analysis (Tables 17and 18)

Quantitation of LSCs in the bone aspirate samples at diagnosis and in the post induction marrow were done in 41 patients with AML. At diagnosis the CD34 + cells quantified as percentage of the gated population in forward scatter (FSC) vs side scatter (SSC) was 13.5 (range 0.02-81.6). Similarly CD34+CD38- and CD34+ CD38- CD123+ fractions were quantified (refer tables 17 and 18)).The expression of adhesion molecules VLA4 and VLA5 on the CD34+ CD38- CD123+ fraction as well as on the original gated population (i.e.FSC vs SSC) was also quantified. The post induction AML marrow sample and the APML samples were also analysed in a similar way. Thus the CD34+ and CD34+ CD38- in the day 10-14 marrow when quantified and analysed as a continuous variable was significant to predict events(p= 0.025 and 0.024 respectively).The CD34+CD38- cell compartment also seemed to predict relapse and refractory disease. However a median and quadrantile analysis were not significant. Hence we analysed survival against CD34+CD38- cell frequency above and below an arbitrary cut off of 0.2% of total gated population on FSC-SSC. This showed statistical significance with a p value of 0.031 (Figure 10).

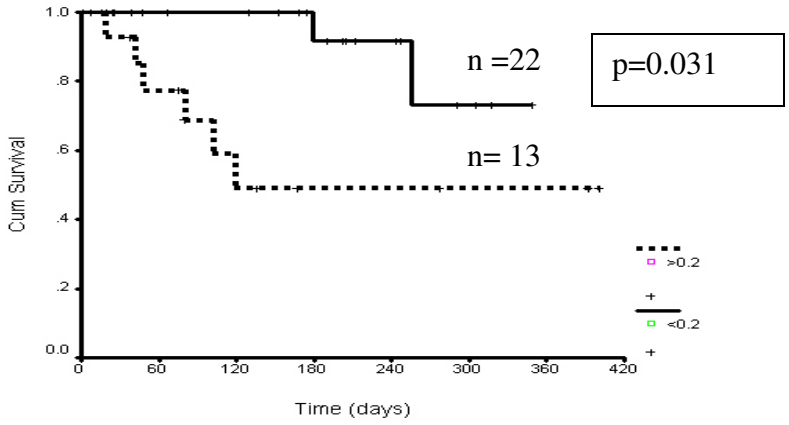


Figure 10:Relapse free survival of patients according to residual CD34+CD38- cell frequency in the post induction bone marrow

However when we analysed the expression of adhesion molecules on leukemic stem cells and their absolute expression on all blasts, none of the p values were significant when analysed against EFS.

Table 17: LSC analysis AML - Bone Marrow Flow Cytometric evaluation at diagnosis versus EFS

Variable n=41	Median/n (range)(%) Bone Marrow at diagnosis(AML)	RR(95%CI)	P value
CD 34 +ve as % of total gated population	13.5(0.02-81.6)	0.998(0.981-1.016)	0.828
CD34+CD38- as % of total gated population	0.095(0.00-49.00)	1.008(0.970-1.047)	0.683
CD34+CD38CD123+(LSC)as % of total gated population	0.02(0.00-37.5)	1.008(0.956-1.062)	0.773
Absolute count of CD34+CD38-CD123+	11.07(0.00-2447.5) *	1.000(1.000-1.001)	0.392
VLA4 expression on LSC as %of total gated population	0.01(0.00-6.57)	1.032(0.773-1.380)	0.829
VLA5 expression on LSC as % of total gated population	0.03(0.00-34.8)	1.011(0.958-1.067)	0.687
VLA4 expression on gated population of blasts	84.8 (3.83-99)	1.004(0.986-1.022)	0.652
VLA5 expression on gated population of blasts	81.25(2.0-97.2)	1.008(0.985-1.032)	0.478

* Bone marrow TC was available on 29 paitents with AML at diagnosis

Table 18:LSC analysis AML -Flow Cytometric evaluation of post induction Bone Marrow on Day10-14 versus EFS

Variable (n=35)	Median/n (range)(%)	RR(95%CI)	P value
CD 34 +ve as % of total gated population	0.38 (0.04-68.4)	1.038(1.005-1.071)	**0.025**
CD34+CD38- as % of total gated population	0.05 (0.00-5.11)	1.627(1.038-2.55)	**0.034**
CD34+CD38CD123+(LSC)as % of total gated population	0.02 (0.00-6.70)	1.286(0.898-1.840)	0.169
Absolute count of CD34+CD38-CD123+	0.20 (0.00-53.6)	1.036(0.990-1.084)	0.131
VLA4 expression on LSC as %of total gated population	0.02 (0.00-1.02)	5.185(0.616-43.642)	0.130
VLA5 expression on LSC as % of total gated population	0.02 (0.00-6.70)	0992(0.977-1.007)	0.303
VLA4 expression on gated population	82.3 (0.96-98.7)	0.996(0.977-1.016)	0.721
VLA5 expression on gated population	41.2 (2.64-78.3)	1.002(0.973-1.032)	0.878

DISCUSSION

AML is a heterogenous disease with various subgroups, be it morphological, cytogenetics vise or according to the molecular markers. There is very little data from the developing world regarding the clinical profile and treatment outcome in AML.We found a sex ratio (1.67). This could largely be attributable to the social situation in India, which will be reflected in any cohort of patients who seek medical attention. Among the FAB subgroup M2 was most common (49.5%) which was comparable to that of CALGB data (37%). The cytogenetic classification showed a predominance of the intermediate risk group which was comparable to that of the data from ECOG[4] and SWOG.

Induction Therapy

The overall CR rate after 1 or 2 cycles of induction was 67.15%.This is comparable to the data by Hugo.F.Fernandez et. al. published in NEJM which reported a CR of 57.3% in the group who received Daunorubucin 45mg/m^2.We had a high incidence of death rate (24.5%) during induction therapy. Equally alarming was the incidence of fungal infections (44.3%).This brings to light the immediate need for better policies in antifungal prophylaxis and treatment.

Consolidation Therapy

Of the 192 patients who underwent induction, 118 (61.45%) patients entered consolidation phase. The results of the different subgroups have to be interpreted with caution. Whereas the patient segregation between allo SCT group and the rest was based on an intention to treat basis, the same could not be applied to the subgroups within patients who did not have a matched donor (i.e. Auto SCT vs HIDAC).However this data provides us with a significant baseline on which we can build our future approaches towards consolidation therapy. Perhaps the most striking fact is the zero treatment related

mortality in the subgroup who received auto SCT. This is very reassuring, as the traditional argument against an auto SCT has been that the lesser relapse rates were offset by higher treatment related mortality.

Molecular markers FLT3 and NPM1 mutations

As the number of samples where the data on FLT3, NPM1 mutation status was known was small, no meaningful conclusions could be made regarding their impact on survival. However, this provides us a rough idea of the baseline characteristics across the different subgroups.

Survival

The Kaplan Meier survival curves were similar to the data from the different cooperative groups from the west. However our numbers were small and the median follow up was short. Long term follow up will provide us more information regarding late relapses and deaths. Although the cytogenetic subgroups did not show significant differences in the Kaplan Meier estimate of EFS, (this could be due to the small numbers), there was an evident trend towards better outcome in the good risk subgroups. Moreover, the poor risk cytogentic subgroup showed significantly bad outcome when analysed against EFS using a cox regression model. Similarly, age, WBC count and WBC index were found to be significant when analysed as a continuous variable against EFS. The ECOG/SWOG[4] data had also shown significance with age and WBC count at diagnosis.

WBC index

WBC index was initially used by the French group[113] to risk stratify their patients with t(8;21). We applied this to the whole AML cohort. When analysed as a continous

variable against EFS and DFS, the WBC index was found to be significant (p value-0.002).When analysed as two groups above and below the median value (8.12) it retained significance with DFS. This is a finding of great importance in our setting. This, unlike cytogenetics, is readily available at diagnosis and will help us in identifying a subset of patients with less risk of relapse. In a resource poor setting like ours, this can help us in offering cost effective treatment options to this subset of good risk patients.

Leukemic Stem cells

Rationale in quantifying LSCs by a set of immunophenotypic markers at diagnosis and in postinduction marrow was that LSCs are quiescent cells which are resistant to conventional chemotherapy and the residual LSCs in the post induction marrow sample would predict relapse. Thus the CD34+ and CD34+ CD38- in the day 10-14 marrow when quantified and analysed as a continuous variable was significant to predict events. A similar study was done by Anna van Rhenen[71]et al. in Netherlands. They quantified CD34+CD38- cells at diagnosis, at remission and after each consolidation. Ours is the first study to look at the CD34+CD38- cell frequency in a post induction marrow. This is a pilot study , the numbers were small and hence a multivariate analysis was not attempted, but it certainly seems to be worth pursuing this in a larger cohort of patients prospectively. If these results are indeed validated then it could translate into forming post induction treatment algorithms with better antileukemic potency for those patients with high residual stem cell frequency.

CONCLUSIONS

1. The male: female ratio was 1.67 and the median age at diagnosis was 34 years.

2. The most common FAB subtype was AML M2 (49.5%) followed by M1 (20.3%).

3. The median WBC count at diagnosis was 12.6 x 10^9 /l.

4. The majority of the patients belonged to intermediate risk cytogenetic subgroup (63%) followed by poor risk(19.8%) and good risk(10.9%).

5. One hundred and seventeen patients (60.9%) achieved remission (CR)after one cycle of induction and another 12 (6.25%) after two cycles.

6. Thirty three patients (17.2%) had residual disease in the day 10-14 bone marrow defined as >20 percent blasts with >20% cellularity.

7. Fourty seven (24.5%) patients died during induction, 33(70.2%) of them due to invasive fungal infections.

8. Eighty five (44.3%) patients had documented fungal infection(as defined by the international consensus criteria) during induction.

9. One hundred and eighteen (61.45%) patients took consolidation therapies; Out of these,40(33.9%) were treated with alloSCT, 25(21.2%) had auto SCT, 39(33%) had HIDAC and 14(11.9%) had other chemotherapies as consolidation.

10. The Kaplan Meier survival curves showed similar pattern as that of the western data; but the median followup was short.

11. The difference in overall and event free survival among different cytogenetic subgroups was not statistically significant. This might be due to the smaller numbers. The good risk cytogenetic subgroup did certainly show a trend towards better outcome.

12. Age, poor risk cytogenetics, WBC count and WBC index at diagnosis showed statistical significance against EFS using a cox regression model.

13. We found that the WBC index at diagnosis could predict the risk of relapse across the cytogenetic subgroups among the patients who achieved remission.

14. In the cohort of patients where LSC quantification was done, it was found that the frequency of CD34+CD38- cells remaining in a post induction marrow predicted early relapse and refractory disease.

15. This study justifies our treatment algorithm in young adults with AML as the results are comparable with the western data. It will provide a baseline from which future therapeutic modifications can be planned.

16. The WBC index could identify a subgroup of patients across the cytogenetic subgroups with a low risk of relapse. This needs to be validated in larger numbers.

17. Quantifying CD34+CD38- cells in the post induction bone marrow will identify a subset of patients with risk of early relapse. These patients might benefit from intensified and targeted post induction treatment algorithms.

18. This analysis also gives us an idea about the baseline characteristics of patients with NPM1 and FLT3 mutations, although a comparison between the different subgroups could not be meaningfully interpreted due to the small numbers.

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