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IP Journal of Diagnostic Pathology and Oncology

Journal homepage: https://jdpo.org/

Original Research Article

Mixed phenotypic acute leukemia, diagnostic challenges and significance of various prognostic markers - A one year follow up a retrospective cohort study

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Abstract

Aim: To analyze the clinical and immunophenotypic characteristics of patients diagnosed with Acute Leukemia at the Department of Hematology, Mazumdar Shaw Medical Centre, Narayana Hrudayalaya, Bengaluru, between January 1, 2017, and July 31, 2022, and to determine the prevalence of Mixed Phenotypic Acute Leukemia (MPAL) or Acute Leukemia of Ambiguous Lineage based on WHO and EGIL criteria.

Materials and Methods: A retrospective cohort study was conducted on 542 patients presenting with suspected Acute Leukemia. Flow cytometric analysis using the Acute Leukemia Flow Cytometric Diagnostic panel (ALCD) was performed to assess the immunophenotype features. 88 patients were excluded based on exclusion criteria. The remaining 454 patients were analyzed for demographic characteristics, immunophenotypic features, and survival outcomes.

Results: 1. A total of 454 patients were included in the study: 266 males and 188 females, with a median age of 27 years (range: 0.33 to 80 years); 2. Of the total, 369 patients were newly diagnosed; 3. 21 cases (5.69%) were identified as having features of Acute Leukemia of Ambiguous Lineage (EGIL criteria) or Mixed Phenotypic Acute Leukemia (MPAL) according to WHO criteria; 4. 15 of these cases (71.4%) met the WHO criteria for MPAL; 5. The most common immunophenotype was T/Myeloid (47.6%, n=10); 6. The overall survival (OS) rates were as follows: ● Day 30: 90.5%; ● Day 60: 85.7%; ● Day 90: 85.7%; ● 6 months: ● 71.4%; ● 1 year: 46.8%; 7. The median OS between EGIL-defined and EGIL-WHO populations was comparable.

Conclusion: The study highlights the prevalence of Mixed Phenotypic Acute Leukemia and Acute Leukemia of Ambiguous Lineage in the study population. The overall survival rates indicate a substantial decline over time, and there was no significant difference in survival between patients categorized by EGIL and EGIL-WHO criteria. These findings suggest the importance of early diagnosis and further research into the management and prognosis of ambiguous acute leukemias.

Keywords: Leukemia, Immunophenotyping, Mixed phenotypic acute leukemia, Biphenotypic acute leukemia, Leukemia.

Received: 18-09-2025; **Accepted:** 16-10-2025; **Available Online**: 29-10-2025

1. Introduction

Patients diagnosed with acute leukemia (20% blasts in blood or marrow, or fewer in the case of certain chromosomal translocations or an extramedullary presentation) can generally be classified as having either myeloid lineage–derived disease (AML) or lymphoid lineage– derived disease (ALL). Sometimes the immature cells display cyto-chemical and/or immunophenotypic features of both lineages (biphenotypic) or there are different populations of leukemia cells (bilineal). The distinction between bilineal and biphenotypic leukemias is often blurred, especially because 2 "populations" of cells perhaps represent subclones derived from a unique stem cell. Accordingly, this distinction does not generally affect our diagnostic or therapeutic approach.[1]

Mixed-phenotype acute leukemia (MPAL) is a rare disease and comprises 1.5% to 5% of all acute leukemia.[2,3] Shi and Munker analyzed data from the Surveillance, Epidemiology and End Results (SEER) registry and identified 313 reported cases of MPAL as compared with 14739 acute lymphoblastic leukemia (ALL) cases and 34326 acute myeloid leukemia (AML) cases of all ages over a period of 10 year. (3) The incidence of MPAL was calculated as 0.35/1000000 person-years. A bimodal age distribution

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https://doi.org/10.18231/j.jdpo.47397.1761717592

was observed with peaks at age 19 and 60 years of age or older.[3]

Leukemias with multilineage protein expression often respond poorly to chemotherapy. The proposed reasons that mixed phenotype may protend a worse prognosis include: primitive multipotent progenitors being chemoresistant owing to slow replication, mixed-phenotype blasts ability to adapt to therapy by switching phenotype and epression of high level of multidrug resistance proteins.[4]

Given its rarity, there are few actively enrolling clinical trials or randomized controlled trials from which to guide management. Treatments are largely extrapolated from ALL and AML.[5]

1.1. Lacunae in literature

Wolach et al, have noted the absence of essentially any useful prospectively collected data on how to treat mixed-phenotypic acute leukemia in adults.[1,6] Differences in prognosis for adult and children with MPAL have been noted. However, there are not enough studies describing the same in the adult population.[2,5] Reported survival rates for MPAL continue to vary with changes to the WHO classification.[2,3] The role of immunophenotypic and genetic markers in guiding chemotherapy choice and post remission strategy, as well as the utility of targeted therapies in non–Ph-positive MPALs is scarce.[4,7] Better understanding of the biology of MPAL is therefore essential to appropriately classify these rare leukemias. Further research is needed to develop optimal therapy. Further prospective validation of MRD is essential to refine risk-stratified therapy for pediatric MPAL. Optimal salvage for those who fail to achieve remission with ALL chemotherapy is unknown and requires further study.[1,7]

1.1.1. Justification for the study

The difficulties in classifying this leukemia, the lack of prospectively collected data concerning therapeutic outcomes, and rare incidence result in much uncertainty as to the best approach for patients with MPAL. Hence MPAL needs a systematic approach to collection of data of such rare cases. [1,7]

In this study we will try to pool all data from patients with MPAL at our institution. We will also analyze the cases among which, coming to the diagnosis of acute leukemia of either type was difficult due to various causes. We will describe the various treatment challenges faced. We hope that this data will be useful in the future to approach such cases with confidence and better understanding

1.1.2. Research question

What are the diagnostic challenges in recognizing the Mixed Phenotypic Leukemia based on WHO and EGIL classification, with focus on prognostic significance of same?

2. Aims

The aim of this study is to assess and compare the proportion of Mixed Phenotypic Acute Leukemia diagnosed using EGIL, WHO 2017 criteria and to describe the importance of recognizing Mixed Phenotypic Acute Leukemia as a distinct entity in the treatment of hematological malignancies.

2.1. Primary objective

To assess and compare the proportion of Mixed Phenotypic Acute Leukemia diagnosed using EGIL, WHO criteria against the total acute leukemia cohort during this period.

2.2. Secondary objectives

1. Identify the challenges in diagnosis of Mixed Phenotypic Acute leukemia based on EGIL vs WHO 2017 criteria (as listed in methodology).
2. To assess the disease-free survival, EFS and mortality at Day 30, Day 100, 6 months and one year with focus on the MRD status and type of induction protocol and other prognostic markers (if any).

3. Materials and Methods

3.1. Study area

All acute leukemia cases diagnosed at our hospital, and analyzed with a multicolor flow cytometry report were screened.

3.2. Study duration/ period

1 Jan 2017 to 31 July 2022.

3.3. Study design

Retrospective Cohort Study

3.4. Study site

Department of Hematology, Mazumdar Shaw Medical Centre, Narayana Hrudayalaya, Bengaluru

3.5. Study population

Patients presenting to the Clinical Hematology (both Pediatric Haemato-oncology and Adult Haemato-oncology) with diagnosis of acute leukemia.

3.6. Inclusion criteria

1. Patients meeting the EGIL criteria for MPAL and / or
2. Patients meeting the WHO criteria for MPAL.

3.7. Exclusion criteria: (1)

1. Secondary leukemias (arising after prior cancer therapy or myelodysplasia)
2. Leukemias with FGFR1 mutations that have features of both T-lymphoid and myeloid differentiation, and
3. Chronic myeloid leukemia (CML) in blast crisis, which can present with a variety of lineages.

3.8. Data collection method

1. Laboratory Data was collected from the records of**:** Department of Hematopathology, Mazumdar Shaw Medical Centre, Narayana Hrudayalaya (Laboratory information system)
2. Clinical history, course, medications, Transplant details (if done) and complications was collected from. Hospital information system, case summaries and Medical Record documents

3.9. Outcome measure

3.9.1. Primary

Proportion of patients with MPAL assessed using EGIL and WHO criteria compared with AML and ALL.

3.9.2. Secondary

Disease-Free Survival/ Progression Free Survival and Overall Survival / mortality at Day 30, Day 60, Day 90, 6 months and one year or at the time of last collection of data, whichever is earlier.

Comparison was done with past data for AML and ALL from our centre, Mazumdar Shah Medical Centre, Narayana Hrudayalaya, Bommasandra

3.9.3. Sample size

500 acute leukemia cases were screened.

3.10. Formula and calculation:

Sample size was calculated using OpenEpi version 3.01 based on the expected incidence (1, 2, 4) of Mixed Phenotypic Acute Leukemia as 1.5% among the patient presenting as Acute Leukemia (expected number 200), with confidence interval 5% to 95%, 5% as precision/ confidence limits. The minimum sample size was calculated as 21 (for secondary objectives to have significance).

3.10.1. Calculation of screening sample size (Based on primary objective)

Each year approximately 100 AL cases are reported, of which the proportion of mixed phenotypic acute leukaemia varies from 1% – 5%.(2,3) Assuming average of 4 cases of mixed phenotypic acute leukaemia per year, to get 21 cases, we will have to screen AL cases for 5 years, which will be 500 AL cases. Hence, A total of 500 Acute leukaemia cases needed to be screened to reach these 21 mixed phenotypic acute leukaemia cases.

3.11. Methodology

3.11.1. Step 1

Bone marrow aspiration and biopsy register from 1 Jan 2017 to 31st Sept 2022 was screened for cases diagnosed as Acute Leukemia (with a target of about 500 cases).

3.11.2. Step 2

All acute leukemia cases were selected. Patient (if adult) or parents were contacted (in the OPD or telephonically) for consent and any missing data in our records. Medical records like (i) BMA/biopsy requisition form (ii) Data from clinical information software (CIS) (iii) Laboratory information software (LIS) were screened.

3.11.3. Step 3

Newly diagnosed Cases and Relapsed cases were separated. Baseline clinicopathological data such as age, sex, blood counts, blast % in peripheral blood (PB), bone marrow (BM) morphology, immunophenotyping at diagnosis and follow up were recorded from the Medical Record Documents.

3.12. Flow cytometric (immunophenotypic) analysis and diagnostic criteria

Bone marrow or peripheral blood samples were collected in EDTA and heparin. Flow cytometric analysis (8-color) was performed on blast cell populations identified by CD45 versus light side-scatter properties, using Becton Dickinson FACS Calibur instrument (8-color 3laser BD FACS CANTO) and standard staining and analytic methods. All cases were characterized with a panel of antibodies to according to the institutions SOP for ALCD panel, Stain – Lyse – Wash Method.

The panels of monoclonal antibodies used in flow cytometric immunophenotyping to detect B-cell, T-cell, and myeloid antigens were as follows: myeloid lineage (anti-MPO, CD13, CD14, CD33, CD64, and CD117), megakaryocytes (CD41 and CD61), natural killer cells (CD56), lymphoid lineage (CD10 and nTdT), T- lymphoid lineage (CD2, surface CD3, cytoplasmic CD3, CD4, CD8, CD5, and CD7), B-lymphoid lineage (CD19, CD20, and cytoplasmic CD22), and hematopoietic precursor (CD34 and HLA-DR). Additional antigen markers like CD11c, CD36, CD15, CD1a, CD79a, CD38, Cd66c, CD123 and CD58 were used pro re nata. Few markers like NSE, LYZ, CDw65, IgM, CD24 are not routinely used in our lab. A marker was considered positive by this method when 20% or more. (>10% for MPO) of the blasts reacted with antibodies to that marker with a definite intensity shift greater than a corresponding negative control.

FACS Diva software v8.0.1 was used for analysis of about 50,000 events recorded on BD FACS CANTOII.

3.12.1. Step 4

For both newly diagnosed and relapsed cases Flow cytometric immunophenotyping reports (8 color flow reports) were screened and recorded.

1. EGIL and WHO scores were assigned.
2. BAL based on EGIL and MPAL based on WHO were selected and proportion was calculated.

3.12.2. Step 5

For cases identified in Step 4 (MPAL / BAL), (i) Treatment methods and outcome data regarding the induction chemotherapy regimen, response to chemotherapy, use of HSCT, relapse, and death as recorded in medical record documents were collected (ii) Cytogenetics (iii) Molecular reports – were recorded from CIS/LIS. This study is retrospective cohort study. All therapies administered were according to the current guidelines.

3.12.3. Step 6

Diagnostic challenges are addressed (in discussion section).

3.13. Statistical analysis plan

Data was analyzed using SPSS version 25.0. Continuous variables were expressed as mean ± standard deviations; categorical variables were expressed as frequency and percentages. Outcome at day 30, day 60, Day 90, 6 months and one year is reported for patients meeting either EGIL or WHO criteria.

Probabilities of survival was estimated using the Kaplan – Meier (K-M) estimation method. P value less than 5% will be considered as statistically significant.

3.14. Ethical considerations

1. The study was reviewed and approved by Institutional Ethics Committee and Scientific Research Committee. Data collection was done after thesis protocol approval.
2. Written Informed Consent was obtained from patients who are followed up in OPD. For patients whose records are collected retrospectively; telephonic verbal consent was obtained.
3. Confidentiality of patient details will be maintained throughout the study and in the future also.
4. Management of these patients was be along the standard international guidelines. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.
5. As the study did not involve any extra procedure, no compensation was offered during and after the study.

4. Observations and Results

During the study period 1 Jan 2017 to 31 July 2022, Acute Leukemia cases presenting at our institute were screened from bone marrow aspiration and biopsy registers. A total of 542 cases presenting to the Department of Hematology were analysed by flow cytometric technique for Immunophenotype features by Acute Leukemia flow Cytometric Diagnostic panel (ALCD). After careful scrutinization of history, physical examination findings and morphology of bone marrow aspirate and biopsy, 88 reports were excluded based on exclusion criteria.

4.1. Demographic data

Age and Gender distribution- There were a total 454 patients of acute leukemia in our study. Out of which 266 patients were male and 188 patients were female, with a median age of presentation at 27 years (9 - 48). Range (0.33 – 80years).

369 were newly diagnosed acute leukemia and 85 relapsed cases during the study period.

Out of the 369 newly diagnosed cases, 21 patients had features of either Mixed Phenotypic Acute Leukemia (defined by WHO 2008) or Acute Leukemia of Ambiguous lineage based on EGIL criteria.

4.1.1. Age distribution

Mixed Phenotypic Acute Leukemia / Acute Leukemia of Ambiguous Lineage was predominantly seen among children and AYA population in our study, although few scattered cases presented upto the age of 55 years of age.

4.2. Biphenotypic acute leukemia defined by egil and mixed phenotypic acute leukemia defined by WHO

EGIL: A total of 21 cases (4.62%) were identified to have features of Acute Leukemia of Ambiguous Lineage by EGIL criteria (n=20/454) or MPAL by WHO criteria. Of these 14 cases (67.7%) were fulfilling WHO criteria of Mixed Phenotypic Acute Leukemia. Three Acute Undifferentiated Leukemias were included under EGIL category. The number of cases fulfilling EGIL criteria were 20(4.41%).

B / Myeloid: Six cases were B / Myeloid by both WHO and EGIL criteria. An additional 1 cases was identified by EGIL scoring.

T / Myeloid: Seven cases were T / Myeloid by both criteria. One case could be defined as T/ Myeloid Mixed Phenotypic Acute Leukemia by WHO criteria alone. An additional 2 cases were identified by EGIL.

The below table gives details of the EGIL score and WHO diagnosis of patients in this study.

A diagnosis of T / Myeloid BAL was made in 10 cases. Eight identified by WHO criteria and ten by EGIL scoring. One case was diagnosed only by WHO because of the blast percentage cut – off (as described in discussion).

A diagnosis of B / Myeloid BAL was made in 7 cases. Five by WHO criteria and seven by EGIL scoring.

One case of MPAL (B/T) was identified during the study period. The patient was 59year old male.

Three cases had features of Acute Undifferentiated Leukemia and were included in the EGIL category.

Four Bilineal MPAL were diagnosed in our study. Blasts %, hemoglobin, WBC count and platelet count were higher in the cases diagnosed by EGIL criteria only.

Cytogenetic evaluation was possible in 10/21 patients (47.6%). Normal karyotype, Ph positive with additional cytogenetic changes, Hyperdiploidy and Complex karyotype were some of the findings.

Seven patients received AML like induction including one patient who received Azacytidine+Venetoclax. Ten patients received ALL like chemotherapy. One patient refused treatment and started on alternative medicine. Three patients opted for palliative care.

Assessment of Morphological Response to induction was possible in fifteen patients. MRD assessment was possible in twelve patients. MRD assessment was not possible in 29.4% patients overall because of financial issues.

Post Induction and consolidation four patients had relapse of disease and underwent salvage chemotherapy. Patients with targetable mutations and those on ALL type protocols had maintenance therapy given. One pediatric patient received radiotherapy.

Five patients underwent transplant.

4.3. Survival analysis

Overall Survival of all cases identified in this study: For our study population (n=21) irrespective of diagnostic criteria (EGIL/WHO) and treatment opted, the probabilities of survival at various time points were estimated using Kaplan – Meier method and were compared using log – rank test.

Overall survival at various time points:

1. 1 month - 90.5%
2. 3 months – 86.0%
3. 4 months – 81.0%
4. 6 months – 71.0%
5. 11 months – 52.0%

Survival based on diagnostic criteria: (WHO / EGIL / BOTH) Overall Survival of BAL defined by EGIL only was compared with OS of cases fulfilling both EGIL-WHO criteria irrespective of treatment opted. The probabilities of survival at various time points were estimated using Kaplan – Meier method and were compared using log – rank test.

Hence the difference in survival based on diagnostic criteria was not statistically significant.

A log-rank test was calculated to see if there was a difference between groups 2(bothWHO/EGIL), 1(EGIL only) and 0(WHO only) in terms of the distribution of time to event occurrence. For the present data, the log-rank test showed that there is no difference between the groups in terms of the distribution of time until the event occurs, p=.546. The null hypothesis is thus not rejected.

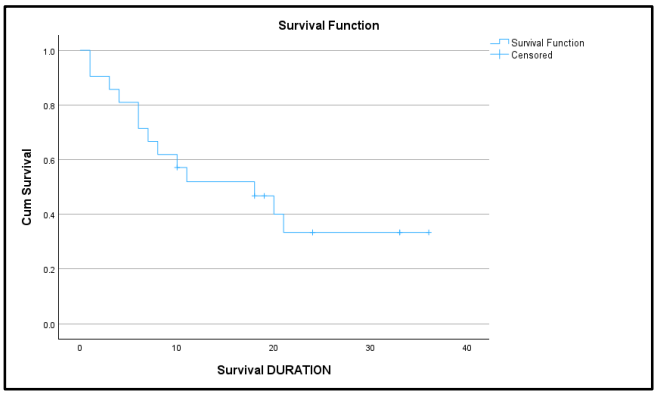

Figure 1: Overall survival of 21 BAL/MPAL cases in our study

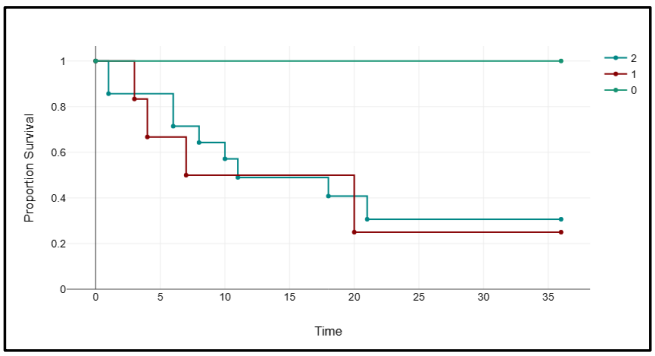

Figure 2: Overall survival of BAL defined by EGIL only (red line) vs MPAL fulfilling both EGIL – WHO criteria (green line)

Table 1: Comparison of EGIL Score and WHO Diagnosis. (Cases are colour coded according to the criteria met: green – both criteria; yellow – EGIL only; blue – WHO only.)

Case	EGIL Score	WHO Diagnosis	Lineage according to
UPN1	M3T5B0	MPAL - T(mature/post thymic ALL)/ Myeloid (AML	M/T
UPN2	M0T0B0	AUL	UNDIFF
UPN3	M0T3B3	MPAL (B/T type)	B/T
UPN4	M3T5B1	MPAL(Myeloid/T)	M/T
UPN5	M4T1B4	MPAL (Myeloid/ B)	B/M
UPN6	M5T1B4	MPAL (Myeloid/ B)	B/M
UPN7	M2T3B0	MPAL	M/T
UPN8	M0T0B0	AUL	UNDIFF
UPN9	M4T1B4	MPAL (B/ MYELOID)	B/M
UPN10	M4.5T1.5B5.5	Mixed Phenotypic Acute leukemia B / Myeloid	B/M

UPN11	M4T0.5B3	B ALL SWITCHED TO AML with aberrant CD19,	B/M
UPN12	M5T4B1	PH POSITIVE MPAL T/M	M/T
UPN13	M5T3B2.5	MPAL: T/ Myeloid	M/T
UPN14	M2.5T2.5B0	ETP ALL with aberrant CD10 expression	M/T
UPN15	M3.5T3B0	ETP ALL	M/T
UPN16	M4T0B3	Ph POS MPAL (B/Myeloid)	B/M
UPN17	M2T7B0	T ALL / Lymphoma possibility of MPAL cannot be	M/T
UPN18	OUTSIDE	MPAL (T / MYELOID)	M/T
UPN19	M3B0T0	CD56+ AUL	M/U
UPN20	M5.5T4B0	MPAL	M/T
UPN21	M4T1B3	MPAL	B/M

A diagnosis of T / Myeloid BAL was made in 10 cases. Eight identified by WHO criteria and ten by EGIL scoring. One case was diagnosed only by WHO because of the blast percentage cut – off (as described in discussion).

Table 2: Markers expressed in T / Myeloid MPAL

Case	Age/ Gender	Myeloid	T lineage	EGIL Score	WHO Diagnosis	Lineage according to WHO/EGIL
UPN1	53/M	CD117	CD3, cCD3, CD2, CD5, CD4, CD8	M3T5B0	MPAL - T (mature/post thymic ALL)/ Myeloid (AML M1)	M/T
UPN4	25/M	MPO, CD13, CD117, CD15	cCD3, CD2, CD7	M3T5B1	MPAL (Myeloid/T)	M/T
UPN7	39/M	MPO, CD64, CD33	cCD3, CD5, CD7	M2T3B0	MPAL (Myeloid/T)	M/T
UPN12	42/M	MPO, CD13, CD33, CD117, CD15	cCD3, CD2, CD5, CD7	M5T4B1	Ph positive MPAL (Myeloid/T)	M/T
UPN13	20/M	MPO, CD13, CD33, CD117, CD15	cCD3, CD2, CD5, CD7	M5T3B2.5	MPAL (Myeloid/T)	M/T
UPN14	1/F	CD13, CD117, CD15	cCD3, CD7	M2.5T2.5B0	Early T Precursor ALL with aberrant CD10 expression	M/T
UPN15	30/F	MPO, CD13, CD117, CD15	cCD3, CD2, CD7	M3.5T3.5B0	ETP ALL	M/T
UPN17	14/M	cMPO(subset) – strong; CD33(Subset)	cCD3, CD2, CD5, CD7	M2T7B0	T ALL / Lymphoma, possibly T/ Myeloid MPAL	M/T
UPN18	15/M			Outside diagnosed	MPAL (Myeloid/T)	M/T
UPN20	52/F	MPO, CD13, CD33, CD117, CD15	cCD3, CD2, CD5, CD7	M5.5T4B0	MPAL (Myeloid/T)	M/T

A diagnosis of B / Myeloid BAL was made in 7 cases. Five by WHO criteria and seven by EGIL scoring.

Table 3: Markers expressed in B / Myeloid MPAL

Case	Age/ Gender	Myeloid	B Lineage	EGIL Score	WHO Diagnosis	Lineage according to WHO
UPN5	7/M	MPO, CD11c, CD14, CD64, CD13, CD33, CD117, CD15,	CD19, CD20, CD22, cCD22	M4T1B4	MPAL (B/Myeloid)	B/M

UPN6	10/F	MPO, CD13, CD33,	CD19, cCD79a, cCD22,	M5T1B4	MPAL (B/Myeloid)	B/M
UPN10	14/F	MPO, CD11c, CD64, CD13, CD15.	CD19, cCD22, CD20	M4.5T1.5B 5.5	MPAL (B/Myeloid)	B/M
UPN11	3/M	MPO, cCD11, CD14, CD64, CD13, CD33, CD15	CD19, cCD79a,	M4T0.5B3	CD10 NEGATIVE B ALL WITH ABERRANT CD56 SWITCHED TO AML with aberrant CD19, CD7, CD56 and CD79a	B/M
UPN16	18/M	MPO, CD13, CD33, CD117, CD15	CD19, cCD79a, cCD22,	M4T0B3	Ph POS MPAL (B/Myeloid)	B/M
UPN21	17/M	CD14, CD36, CD64, CD33	CD19, cCD79a, cCD22,	M4T1B3	MPAL (B/Myeloid)	B/M

One case of MPAL (B/T) was identified during the study period. The patient was 59year old male.

Table 4: Markers expressed in B/T MPAL

Case	Age/ Gender	T lineage	B Lineage	EGIL Score	WHO Diagnosis	Lineage according to WHO
UPN3	59/M	cCD3, CD2, CD5, CD7	CD19, Ccd22, cCD79a	M0T2.5B2; M0T3B3	MPAL (B/T type)	B/T

Three cases had features of Acute Undifferentiated Leukemia and were included in the EGIL category.

Table 5: Markers expressed in AUL

Case	Age / Gender	Hematopoietic	Myeloid	Lymphoid	T lineage	B Lineage	EGIL	EGIL Score	WHO	WHO Diagnosis	Lineage according to WHO
UPN2	1/M	None	None	None	None	None	Y	M0T0B0	N	AML M0	UNDIFF
UPN8	26/M	None	None	None	None	None	Y	M0T0B0	N	AUL	UNDIFF
UPN19	37/M	None	None	None	None	None	Y	M3B0T0	N	AML CD56+ AUL	M

Four Bilineal MPAL were diagnosed in our study. Blasts %, hemoglobin, WBC count and platelet count were higher in the cases diagnosed by EGIL criteria only.

Table 6: Comparison of Clinical Characteristics and laboratory parameters at presentation

	EGIL (N=6)	both (n=14)	Total (n=21)
Age	14 (3, 26)	22 (16, 45)	22 (14, 40)
Gender (F:M)	2:4	3:11	6:16
Hepatomegaly	2	6	8
Splenomegaly	3	7	11
Lymphadenopathy	1	3	4
Cns involvement	0	1	1
Testicular involvement	none	none	None
Blasts_no.			
Biphenotypic	6 (100%)	10 (71%)	17 (81%)

Bilineal	0 (0%)	4 (29%)	4 (19%)
Blasts % (on FCM)	58 (25, 84)	34 (26, 44)	35 (25, 58)
Baseline hemoglobin (GMS%)	11.10 (9.20, 11.80)	6.50 (6.00, 7.55)	7.20 (6.43, 8.62)
Baseline platelet (cells/dl)	130,000 (74,500, 170,000)	30,000 (21,000, 58,500)	31,000 (20,500, 74,750)
Initial wbc count(cells/dl)	57,070 (30,860, 77,745)	6,800 (3,475, 51,050)	10,900 (4,000, 70,750)
Initial peripheral blood blast %	26 (20, 31)	50 (22, 67)	32 (18, 55)
Initial bone marrow blast %	55 (52, 58)	44 (30, 72)	48 (33, 65)

Cytogenetic evaluation was possible in 10/21 patients (47.6%). Normal karyotype, Ph positive with additional cytogenetic changes, Hyperdiploidy and Complex karyotype were some of the findings.

Table 7: Comparison of treatment of induction chemotherapy of choice at presentation

Induction chemotherapy of choice	**Defined by egil only (n=6)**	**Defined by both criteria (n=14)**	**Total (n=21)**
2 + 5	0	2	2
3 + 7	3	1	4
AZA + VEN	0	1	1
All BFM	2	7	9
Uk all	0	1	1
Refused treatment or alternative medicine	1	3	4
Total	6	14	21
Induction type			
AML type	3	4	7
All type	2	8	10

Assessment of Morphological Response to induction was possible in fifteen patients. MRD assessment was possible in twelve patients. MRD assessment was not possible in 29.4% patients overall because of financial issues.

Table 8: Response to Induction Therapy in the study population.

Induction therapy of choice	**AML type (n=7)**	**All type (n=10)**	**Total (n=17)**
Bone marrow Response			
Day 28 - 33			
Response not assessed	-	20%	11.8%
Morphological persistent disease	28.6%	40%	35.3%
Morphological remission (cmr)	71.4%	40%	52.9%
MRD positive	42.85%	50%	47.1%
mrd negative	14.3%	30%	23.5%
mrd not available	42.85%	20%	29.4%

Table 9: Comparison of Overall Survival of BAL defined by EGIL only vs MPAL fulfilling both EGIL - WHO criteria.

Time from presentation	**BAL (defined by EGIL) n=6**	**MPAL (defined by BOTH) WHO/EGIL n=14**
1 month	100% (n=6)	86%
3 months	83%	
4 months	67%	
6 months		71%
7 months	50%	64%
8 months		
10 months		57%
11 months		49%
18 months		41%
20 months	25%	

5. Discussion

In this study we retrospectively analysed the immunophenotyping data from 542 cases during the period 1 Jan 2017 to 31 July 2022. Based on exclusion criteria 88 cases were removed. Among 454 cases of Acute Leukaemia, 369 were newly diagnosed. Of these 369 patients, total of 21 cases were recognised as either BAL by EGIL or MPAL by WHO criteria accounting for 5.69%. Previous studies have reported 2.2% (8), 3.2% (9), 4.1%.[10]

A total of 20 cases (5.42%) were identified to have features of Acute Leukemia of Ambiguous Lineage by EGIL criteria (n=20/454). Further, 15 cases (71.4%) were fulfilling WHO criteria of Mixed Phenotypic Acute Leukemia. All - inclusive 17 cases were biphenotypic and 4 cases were bilineal. A previous study at our centre, Mazumdar Shah Medical Centre, Narayana Hrudayalaya, Bangalore, between Apr 2014 and Apr 2016 showed the prevalence of MPAL as 3.4%(n=5) among 151 Acute Leukemia cases (unpublished,thesis).

In this study we have compared the clinical characteristics, laboratory parameters treatment and outcomes among patients fulfilling EGIL exclusively versus both WHO and EGIL. We could not compare with cases who fulfilled diagnosis of MPAL only as there was only one such case in our study. Huang J et al have also called attention to previous EGIL criteria eliminating a subset of patients from ALAL. In their study they have questioned whether diagnosis based on WHO and treatment accordingly for this subset of patients are appropriate. Our study is the first such study from India looking at the outcome of EGIL only cases versus cases diagnosed by WHO – EGIL.[11]

5.1. Age and gender

Mixed Phenotypic Acute Leukemia / Acute Leukemia of Ambiguous Lineage was predominantly seen among children and AYA population in our study. Several studies have reported pediatric or adult populations separately. Pawar R et al[8] have reported three pediatric patients 14 patients. Sukumaran R et al have reported 5 (33%) pediatric cases.[12] In our study also, although the absolute number of cases were more in the pediatric and AYA group, the proportion of cases compared to other types of acute leukemia in that particular age group was similar across all ages ranging from 5 – 12%.

In our study male patients were more than female (16:5). Myint HH have reported a sex distribution of 3:1 ie 18 male patients and 6 female patients.[13]

The median age of EGIL only cohort was 14 and WHO – EGIL cohort was 22. Huang et al have reported a higher median age among both populations.[11]

5.2. Clinical and laboratory details

Most common clinical feature was splenomegaly, similar to study at Kasturba Medical College, Mangalore. (10) Hepatomegaly and lymphadenopathy were other clinical features. CNS involvement as assessed by CSF evaluation was found in one patient. Testicular involvement was seen in none of the patients.

The details of clinical characteristics and laboratory findings have been summarised in Table **. We have found a higher hemoglobin %, platelet count and WBC count in our EGIL only cohort compared to WHO-EGIL cohort. In contrast, Huang J et al who have reported a higher WBC count in the WHO – EGIL cohort.[11] They have however found a similar higher hemoglobin and platelet count among EGIL only population. However, these findings not statistically significant.

5.3. Bone marrow morphology

Four patients had blasts of two different morphology (bilineal).

5.4. Immunophenotype

There were 14 MPAL cases (3.08%) and 20 BAL cases (4.41%). 6 cases (1.32%) were fulfilling the EGIL criteria exclusively. Figure 7 compares the diagnosed criteria fulfilled by each subset. In our study period, T/myeloid (n=9+2) was the most frequent phenotype, followed by B/myeloid (n=5+1) and Acute undifferentiated Leukemia(n=3). We have diagnosed one B/T MPAL. One South Indian study has published 9 cases of T/ myeloid, 5 cases of B/ myeloid and one case of B/T MPAL. (12). Similar reports have been published by St Jude Children's Research Hospital and a study at Morocco. (13) Other studies have reported B/ myeloid MPAL as the most frequent immunophenotype.[8,13]

CD34 was positive in 13/21 cases (61.9%). Gupta N et al have documented CD34 expression in 85.7% of the MPAL cases.[14] The study from Manipal had reported that 8/9 (88.8%) showed positivity for CD34 and HLA DR indicating their origin from an early precursor.[10]

5.5. Cytogenetics

Cytogenetic evaluation was possible in nine patients. Normal karyotype was most common finding(n=5). One B/ Myeloid MPAL case had 94, XXYY, +2mar [20]/46, XY[30] 18/04/2020; 46,XY[50] and another patient had complex karyotype with t(4;11)(q21;q23); t(4;11) which was present in B-ALL diagnosed a few months earlier also. t (10; 13) (q24; q12) was seen in an Acute Undifferentiated Leukemia. A case of T / myeloid MPAL had t (9;22) t (2;6).

WHO classification of myeloid malignancies specifies that AML with complex karyotype should be classified as AML – MRC. Several studies have identified complex

karyotype as the most common cytogenetic abnormality in MPAL.[4] have opined that karyotype should not be the sole deciding factor to distinguish MPAL from AML – MRC. This raises the question about the place of MPAL arising in a known case of MDS, in the current classification systems and diagnostic criteria.

5.6. Diagnostic difficulties

Diagnostic challenges faced during this study were: (Secondary objective):

5.6.1. Discrepancy with reports here and outside:

Three T/Myeloid MPAL were diagnosed in outside hospital as ETP ALL because a limited panel of markers were used for immunophenotyping. Gupta Nishit et al have also highlighted that ETP – ALL immunophenotype resembles the earliest thymic precursors with both T and myeloid lineage potential. Corroborating with this truly biphenotypic potential, they have noted cMPO positivity in one out of the six ETP – ALL patients.[14] In our series, we have one encountered any case with cMPO positivity among twelve ETP – ALL cases presenting to our centre during the study period.

5.6.2. Cases with more than one blast population on morphology and its immunophenotype:

During the study period four cases had a dual blast population. UPN1 had myeloid population of 36.7% and T cell population of 15%. UPN6 had presented with features of B cell ALL at outside centre. At presentation this patient had in addition – myeloid blasts and undifferentiated blasts. UPN12 was a case of Ph pos MPAL with 64% T lymphoblasts and 10% myeloblasts. UPN16 had B lymphoblast (33.1%) and myeloblasts (27.3%).

The WHO criteria for bilineal MPAL require that the sum of the 2 blast populations is at least 20% of nucleated cells. (4) No minimum count is mandated for the minor population as long as the sum is 20% or greater. The biggest challenge here is that a sufficient number of events have to be analysed by FCM (≥1000 blasts and ≥20 000 total events per tube), so that a minor secondary blast population is not overlooked.[4] In our study, UPN17 qualified as MPAL because of this reason, however it did not fulfil a score more than 2 on EGIL scoring (the blast population being <10% strong positive).

5.6.3. Mismatch of morphology and expected IPT

A patient was excluded from further analysis at the stage of exclusion: A 55/F who had morphological features of plasma cell leukaemia. IPT features were of AUL. This case highlights the importance of morphological assessment.

5.6.4. Difficulty in selecting the panel of antibody in particular cases:

While selecting the baseline panel for analysis of Acute Leukaemia, morphology is assessed and orientation tube with cCD19 for B lineage, cCD3 for T lineage and MPO for myeloid lineage are included. Further panel is selected based on the results of first tube. Use of 8 colour flow cytometry is useful. However, in earlier days when 4 colour flow cytometry was done there was a difficulty in assessing different expression pattern of various antigens simultaneously by same population of blast cells. Also repeated aliquoting of the sample had a possibility of causing dilution of blasts if the bone marrow volume collected was minimal / inadequate.

5.6.5. Discrepancy between EGIL and WHO diagnosis:

In our study period about 28 patients had flow cytometry reports where more than two lineage were equal to or more than a score of two. Weinberg OK et al in their review in 2010 have recorded that a typographical error was made in the WHO classification 2001 in the second printed version of this work. EGIL score of ≥ 2 was mentioned instead of >2. This error led to the misconception of loosening of the criteria for biphenotypic leukemia and added to confusion.[16] In this review also they have enumerated studies where a higher number of cases were reported because of this confusion. [16]

Discrepency between EGIL and WHO diagnosis was noted in 7 cases. 6 cases could be defined as BAL by EGIL criteria, whereas one case was MPAL based on WHO criteria only.

The new consensus criteria for MPAL were published in the 4th Edition OF WHO and remain largely unchanged in 2016 update of the classification. Whereas, The EGIL approach of scoring included a detailed blast immunophenotype with numerous markers, WHO criteria emphasize on a few lineage – defining markers with particular emphasis on CD19 for B lineage, CD3 for T lineage and MPO for myeloid lineage. The WHO approach relies heavily on the sensitivity and specificity of a few markers. Also, the WHO classification does not specify thresholds for positivity of these key markers, leaving it up to individual laboratories to decide on the definition of significant expression. (4) In practice, the most frequent challenge in applying the WHO criteria for MPAL is interpretation of MPO expression in cases that are otherwise consistent with B – ALL or T – ALL.[4] MPO detection by RT PCR is most sensitive method followed by IHC and FC among other methods like EC and mRNA detection.

Charles NJ and Boyer DF have further opined that WHO has intentionally omitted thresholds for significant expression of MPO or other markers by FCM, stating that the threshold defined in either EGIL or FAB classification were a safe threshold to exclude nonspecific staining based on the techniques used at the time.[4]

In parallel, the EGIL criteria include a broad spectrum of immunophenotype under the category of ALAL, i.e., undifferentiated leukaemia where blasts that express CD34, HLA – DR, and / or CD38 and sometimes TdT but lack specific myeloid or lymphoid antigens; and Leukaemias with biphenotypic / bilineal antigen expression.[17]

5.6.6. Difficulty in assessing expression of a particular antigen

Marker due to lack of corresponding normal internal (de – novo) control population for a selected antigen marker. Absence of normal population of cells is seen in samples from patients with a packed marrow. For example, in case of packed marrow normal population of B lymphocytes with CD19 expression may be absent. Also, the expression of cCD79a and cCD22 may be dim. Similarly, the control population for MPO is granulocytes, which may be reduced in certain cases.

Blasts have greater autofluorescence than mature lymphocytes, and therefore a negative blast population will have a higher median fluorescence intensity than a negative lymphocyte population.. Because of this difference, it is important to know what negative control was used when interpreting partial positivity for MPO by FCM.[4]

In addition, one needs to be cautious when using polyclonal anti – CD3 in IHC as it may not be specific.[1]

5.6.7. Challenges in separating specific entities under the new WHO:

1. In this study population we encountered 28 cases where the EGIL score was equal to 2 for more than one lineage. They did not have expression of key lineage specific markers like cMPO, cCD3 or CD19 either.
2. A 30/ F who presented with Ph positive leukemia and high B lymphoid blast count. She also had splenomegaly. However, in the absence of recent blood counts or any kind of past medical records it was difficult to distinguish whether it was Ph positive MPAL or CML – BP. One case of CML BP had a minor population of undifferentiated blasts. These two cases were excluded from the study. However, some case series and studies have included CML BP with both lymphoid and myeloid blasts under MPAL.[7] They have further stated that there are only seven such cases reported so far, including their case. Hence, our cases would be eight and ninth. Nonetheless, several such cases would have gone unnoticed in the archives of laboratories.

We have excluded therapy related Acute Leukemia, AML – MRC, AML with specific translocations and CML – BP in the earliest stage of exclusion criteria although some cases fulfilled the EGIL criteria. The impact of MPAL immunophenotype on the outcomes in AML – MRC has not been specifically addressed.[4]

5.7. Treatment and outcomes

Overall survival for the total study population, irrespective of treatment modality, at Day 30, Day 60, Day 90, 6 months and 1 year was 90.5%, 85.7%, 85.7%, 71.4% and 46.8% respectively.

An earlier study of ALL(n=70) from our centre, showed a higher survival, even at 24 months, about 97% and 58% among MRD negative and MRD positive patients, respectively. However, the survival among AML(n=34) cohort between Nov 2017 to May 2018 at our centre, followed up for a year was 34.5% at 1 year.

In this study we have compared the outcomes among patients fulfilling EGIL exclusively (BAL) versus both WHO and EGIL(MPAL). Our study is the first such study from India looking at the outcome of EGIL only cases versus cases diagnosed by WHO – EGIL. We could not compare with cases who fulfilled diagnosis of MPAL only as there was only one such case in our study. Although the survival of EGIL only group was better upto Day60 (100%), at 6 months and one year the survival was comparable between the two groups. Median OS of EGIL only population was 12 months and EGIL-WHO population was 11.83 months. Huang et al have also called attention to previous EGIL criteria eliminating a subset of patients from ALAL. In their study they have questioned whether diagnosis based on WHO and treatment accordingly for this subset of patients are appropriate.[11] In contrast to our study population, they found the patients excluded by WHO criteria had an even worse prognosis than those patients included, characterized as shorter PFS (Log rank p=0.016) and OS (Log rank p=0.016).

An attempt was made to analyse the survival of patients based on the lineage subset, type of induction therapy, MRD status and whether received transplant or not.

Among these prognostic variables, we found that B/myeloid BAL patients, ALL type induction, MRD negative patients and those who were transplanted did better. However, the number in each subset was too small to achieve statistical significance. Also patients on AML induction were lesser in number. MRD status was not known in 29.4% overall and in 42.85% patients on AML type induction.

Causes of death included febrile neutropenia, disease relapse, marrow infiltrative disease, >Grade III graft versus host disease, acute intracerebral bleed, >Grade 3 mucositis, CMV infection, drug resistant bacteremia, septicemia, pneumonia, refractory septic shock and hemophagocytic lymphohistiocytosis.

6. Conclusion

Mixed Phenotypic Acute Leukaemia is a heterogenous group of disorders. Acute Leukemia of Ambiguous Lineage as defined by European Group of IL includes a more diverse group.

These are difficult diagnostic subsets of Acute Leukemia.

EGIL/WHO help identify this type of leukemia but both are not exclusive.

Identifying this subset, including the cases diagnosed by EGIL criteria exclusively has prognostic value. Correlation with molecular data will add further value.

7. Conflict of Interest

None.

8. Source of Funding

None.

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Cite this article: Gudibande T, Damodar S, Deepak MB, Chougule S, Prabhu S. Mixed phenotypic acute leukemia, diagnostic challenges and significance of various prognostic markers - A one year follow up a retrospective cohort study. *IP J Diagn Pathol Oncol*. 2025;11(3):126-137.