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Case Series

Oncology Section

# Navigating the Grey Zone: A Series of Three Cases on Mixed-phenotype Acute Leukaemia

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## ABSTRACT

Mixed-phenotype Acute leukaemia (MPAL) may consist of a single population of abnormal progenitors (morphological blasts) expressing antigens from two or more lineages. MPAL remains one of the most diagnostically challenging groups of acute leukaemias because of its intrinsic biological heterogeneity and overlap with both Acute Myeloid Leukaemia (AML) and Acute Lymphoblastic Leukaemia (ALL). Lineage assignment is best performed by Flow Cytometric Immunophenotyping (FCM IPT) in order to correlate the patterns of antigen expression on one or more cell populations. Authors present three cases of mixed phenotype acute leukaemia to highlight the breadth of MPAL presentations across age groups, ranging from neonates to elderly adults. Case 1 was of a 62-year-old male diagnosed with B/Myeloid MPAL whose molecular analysis revealed IDH2, SRSF2, and CUX1 mutations with del(7q), suggesting evolution from a myeloid clonal background. The high VAF of CUX1 (77.39%) in the context of del(7q) likely indicates Loss Of Heterozygosity (LOH). Case 2 (64-year-old male) presented with Central Nervous System (CNS) infiltration. In Case 3, 85-day-old male infant with B/T MPAL presented with extreme hyperleukocytosis and rapid clinical deterioration. This series underscores the marked biological and clinical heterogeneity of MPAL across age groups and disease subtypes, reinforces the critical role of multiparameter flow cytometry in objective lineage assignment, and highlights the importance of integrating immunophenotypic, cytogenetic, and molecular data to establish an accurate diagnosis. The varied clinical courses observed also reflect the prognostic unpredictability of MPAL and the practical challenges of managing this rare entity, particularly in resource-limited settings, emphasising the need for early recognition and comprehensive diagnostic evaluation to guide optimal therapy.

**Keywords:** Ambiguous lineage, Flow cytometry, Immunophenotyping, Multiparameter flow cytometry, Myeloperoxidase

## INTRODUCTION

The MPAL may consist of a single population of abnormal progenitors (morphological blasts) expressing antigens from two or more lineages, termed biphenotypic, or two populations of abnormal progenitors each of a different lineage, termed bilineal or bilineage cells [1]. In bilineal MPAL cases, each abnormal progenitor population must meet the immunophenotypic criteria for that lineage, but the numerical blast criterion of ≥ 20% need only be met in aggregate. MPAL remains one of the most diagnostically challenging groups of acute leukaemias because of its intrinsic biological heterogeneity and overlap with both Acute Myeloid Leukaemia (AML) and Acute Lymphocytic Leukaemia (ALL) [2]. Modern classification systems, including the World Health Organisation (WHO) 5$^{th}$ edition and the International Consensus Classification, emphasise the importance of objective, flow cytometry-driven lineage assignment and recurrent genetic alterations in establishing a definitive diagnosis [3].

Lineage assignment is best performed by FCM IPT in order to correlate the patterns of antigen expression on one or more cell populations. Expression of CD19 approaching the level seen on normal B progenitors (exceeding 50% of the level seen on normal B progenitors on at least a portion of the leukaemia) has considerable specificity for B lineage, but it still should occur with expression of one or more other B-lineage antigens (CD10, CD22, or CD79a) to define B lineage [4]. The most specific feature of myeloid lineage is the expression of Myeloperoxidase (MPO) in the cytoplasm of the leukaemic blasts, particularly when its level exceeds 50% of that seen on mature neutrophils and/or it shows a variable pattern reminiscent of that seen on normal CD34-positive myeloid progenitors [4].

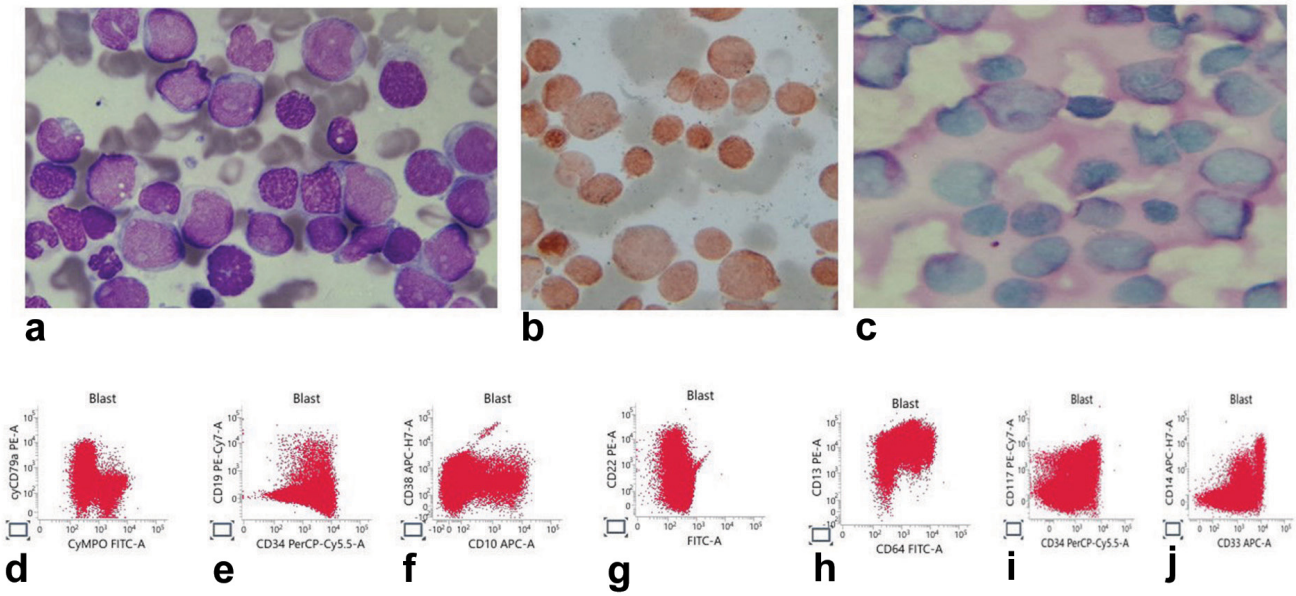
Updated diagnostic approaches emphasise genotype-phenotype correlations and use of high-parameter flow plus molecular data for accurate lineage assignment [5]. Recent genomic and single-cell studies have revealed stem-like and distinct transcriptomic programs in MPAL blasts, refining the understanding of its biology [6]. The current case series adds to the literature by highlighting: 1) An adult B/Myeloid MPAL with concurrent molecular and cytogenetic abnormalities; 2) Diagnostic and therapeutic challenges in resource-limited settings (e.g., limited access to allogeneic stem cell transplantation or advanced targeted therapies); and 3) A rare infantile B/T-type MPAL with rapid fatal progression.

## CASE SERIES

### Case 1

A 62-year-old male presented with breakthrough seizures, generalised tiredness, and vomiting for one month. Examination revealed moderate splenomegaly. Laboratory investigations showed haemoglobin (Hb) 6.5 g/dL, WBC 14,000/µL, and platelets 95,000/µL. Peripheral smear showed 49% blasts. Bone marrow aspiration revealed 71% blasts, negative for Sudan Black B (SBB) and Periodic Acid-Schiff (PAS) [Table/Fig-1].

The CNS imaging did not reveal any metastatic infiltration. Due to splenomegaly and leucocytosis clinically lymphoma/leukaemia was kept as provisional diagnosis. Peripheral smear showed 49% blasts. Bone marrow aspiration showed 71% blasts, which were negative for SBB and PAS stains [Table/Fig-1a-c]. Routine flow cytometry immunophenotyping was performed with 10 colour flow cytometer BD Facs Lyric using markers, such as CD 13, CD 33, cy MPO, CD 71, CD 41a, CD 64, CD 11c, CD 14, CD 19, CD 20. CD 22, CD 79a, cy CD 3, sm CD3, CD 5, CD7, CD 2, CD 4, CD 8, CD 1a, CD 34, Tdt, HLA DR, CD 10, CD 38, CD 45,and CD 117. FCM IPT of bone marrow aspirates showed blasts expressing MPO, CD34, CD38, CD13, CD64, CD117, CD14 and CD33 as myeloid lineage [Table/Fig-1d-j]. CD79a, CD19, CD10, and CD22 represents B-cell lineage. These expressions together confirm the case as MPAL (B/Myeloid). Mutational analysis revealed IDH2 (VAF




**[Table/Fig-1]:** a) Bone marrow aspirate smears showing blasts with moderate to scant eosinophilic cytoplasm, irregular nuclear membrane, open chromatin, multiple prominent nucleoli (May-Grunwald-Giemsa stain, 100x); b) Cytochemical staining for MPO reveals that the blasts are negative for MPO, supporting a non-myeloid lineage; c) Cytochemical staining for Periodic Acid Schiff (PAS) reveals that the blasts are negative for PAS, supporting a non-lymphoblastic lineage; and d to j) FCM IPT of bone marrow aspirate shows blasts expressing CD 79a, MPO, CD19, CD34, CD38, CD10, CD22, CD13, CD64, CD117, CD14 & CD33 consistent with MPAL (B/Myeloid).

45.93%), SRSF2 (VAF 50.6%), and CUX1 (VAF 77.39%). Given that CUX1 is located on 7q, the high VAF combined with the karyotypic finding of del(7q22) strongly suggests LOH or a founding germline variant. He was started on chemotherapy with hyper-fractionated cyclophosphamide, vincristine, Adriamycin and dexamethasone (HCVAD) regimen, six cycles over six months. He tolerated chemotherapy as well. On Measurable Residual Disease (MRD) analysis during post cycle 2 of HCVAD showed he had 8.5% MRD positive. HCVAD six cycles was restarted for six months followed by maintenance chemotherapy of 6-Mercaptopurine, methotrexate, vincristine and Wysolone. Post re-induction MRD is negative and patient is in clinical remission currently. He refused to transplant due to financial constraints.

### Case 2

A 64-year-old male with history of hypertension, dyslipidaemia, and hypothyroidism presented with complaints of body pains, epistaxis and haemoptysis for one month. On examination there was no hepatosplenomegaly. Lab investigation showed haemoglobin of 6.2g/dL, WBC count of 16,500/ µL, platelet count of 14,000/µL. Peripheral smear was sent on provisional diagnosis of leukaemia which showed 59% blasts, SBB positive, PAS negative [Table/Fig-2a-c]. Flow cytometry immunophenotyping was performed with 10 colour flow cytometer BD Facs Lyric using markers as described in the above case. Immunophenotyping by multiparameter flow cytometry showed expression of both myeloid markers (CD13, cMPO, CD117) and B markers (CD19, CD22, CD79a) suggesting diagnosis of MPAL (B/Myeloid) [Table/Fig-2]. FISH for BCR-ABL and PML RARA was negative. Molecular studies and karyotyping were normal. During hospital stay, the patient became drowsy and Computed Tomography (CT) brain showed leukaemic infiltrates. The 6-Mercaptopurine, Methothrexate, vincrisitine and Wysolone was started with palliative intent. He was discharged against medical advice and was lost to follow-up.

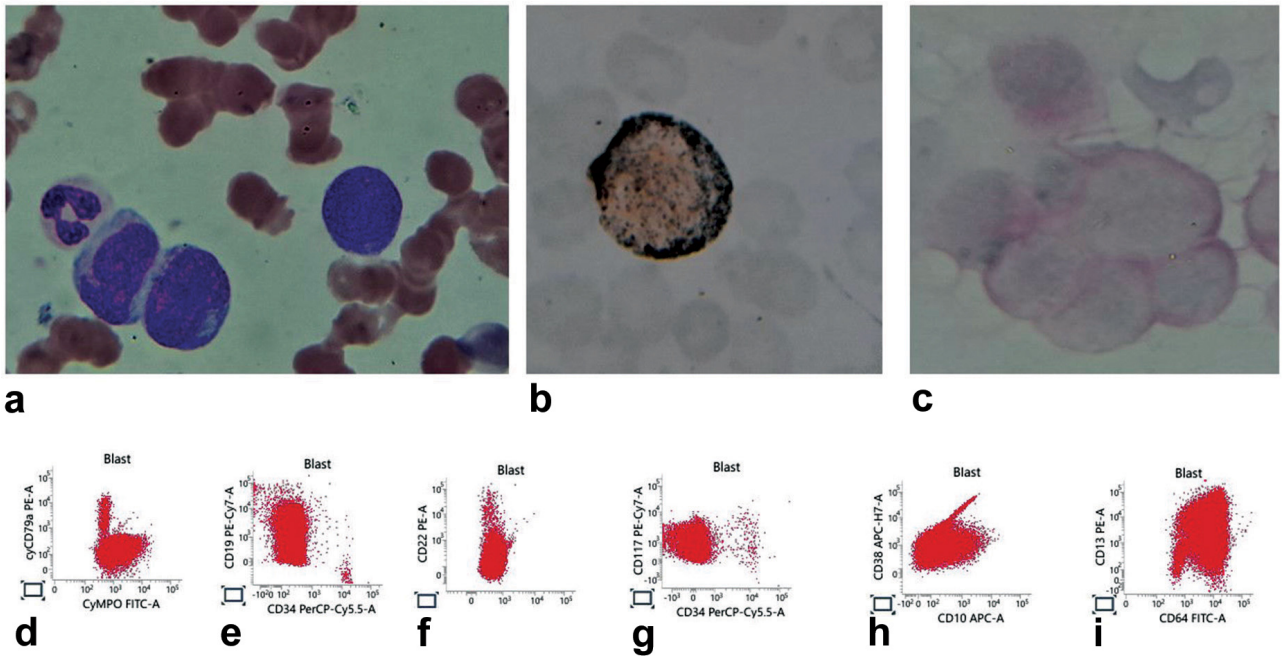
### Case 3

An 85-day-old male infant was brought to emergency medicine with fever, lethargy, and breathing difficulty. On examination there was no hepatosplenomegaly. Due to extreme hyperleukocytosis leukaemia was the working diagnosis. Hb, 2.8g/dL, WBC, 744.06 X $10^3$/µL, platelet count of 30000/µL. Peripheral smear had 99% blasts which were PAS positive and SBB negative [Table/Fig-3]. Flow cytometry immunophenotyping was performed with 10 colour flow cytometer BD Facs Lyric using markers as described in the above case. Immunophenotyping by multiparameter flow cytometryshowed MPAL with expression of both B markers (CD79a, CD22, and CD10) and T markers (CyCD3, CD7, CD2, and CD5). Blood gas analysis showed severe metabolic acidosis. His condition deteriorated quickly and the child was intubated, resuscitated with i.v. fluids of volume 20 mL/kg NS bolus. Inotropic support with Noradrenaline 0.6 mg/kg/min and Dobutamine 4 mg/kg/min. Packed red blood cell and platelet transfusions were done. Despite the management, the child succumbed to illness before further diagnostic evaluations or definitive therapy could be attempted.

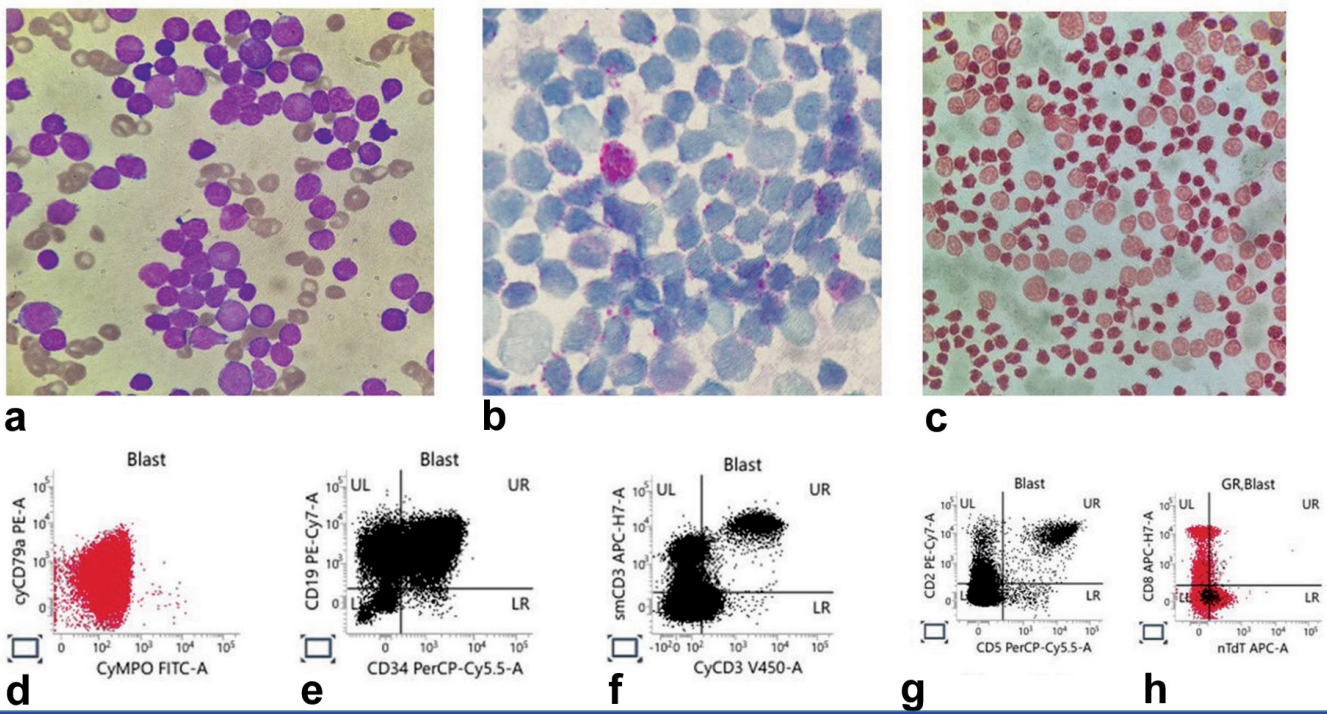
## DISCUSSION

Based on the antigenic characteristics of the blasts, most acute leukaemias can be categorised as myeloid, B, or lymphoid in origin. However, in a small percentage of situations, lineage assignment is not feasible because of the expression of both myeloid and lymphoid lineage-specific antigens in blast cells. These instances have been referred to in the literature as mixed-lineage acute leukaemias, and the WHO has recently suggested calling them MPAL [7]. Information on the characteristic features of MPAL is very limited because of the rarity of these leukaemias

The three cases presented in this series highlight the breadth of MPAL presentations across age groups, ranging from neonates to elderly adults, and the wide clinical variability associated with this disease [Table/Fig-4]. B-lineage and myeloid markers reduce diagnostic ambiguity and allow for reproducibility across laboratories [8]. The current case series both expands upon and closely resembles the range of MPAL reported in the recent literature. The presented cases, which cover infancy to the seventh decade of life, are consistent with the bimodal age distribution of MPAL reported by large cohort studies and expert reviews, with a predominance in children and older adults [2]. In a study conducted by Matutes E et al., the incidence of MPAL-B/ myeloid was 59%, T/myeloid 35%, B/T 4%, and trilineage combination 2% of cases [4]. Ichikawa et al. recently described the first reported instance of non-leukemic MPAL with trilineage differentiation presenting as a mediastinal tumor, underscoring the potential for unusual extramedullary manifestations in this entity [9]. B/myeloid MPAL remain the most frequently encountered subtype in adults, accounting for approximately 60-70% of cases, which is consistent with the Cases 1 and 2 in the present series. The rare B/T




**[Table/Fig-2]:** a) Peripheral blood smears showing blasts with scant to moderate basophilic cytoplasm, irregular nuclear membrane, open chromatin, prominent nucleoli (May-Grunwald-Giemsa stain, 100x); b) Cytochemical staining for MPO reveals that the blasts are positive for MPO, supporting a myeloid lineage; c). Cytochemical staining for Periodic Acid Schiff (PAS) reveals that the blasts are negative for PAS, supporting a non-lymphoblastic lineage; and d to i). FCM IPT of bone marrow aspirate shows blasts expressing CD 79a, cMPO, CD19, CD22, CD117, CD38, CD 10, CD13 & CD 64, consistent with MPAL (B/Myeloid).




**[Table/Fig-3]:** a) Peripheral blood smears showing blasts with scanty cytoplasm, open chromatin, prominent nucleoli (May-Grunwald-Giemsa stain, 100x, 40x); b) Cytochemical staining for Periodic Acid Schiff (PAS) reveals that the blasts are positive for PAS, supporting a lymphoblastic lineage; c) Cytochemical staining for MPO reveals that the blasts are negative for MPO, supporting a non-myeloid lineage; and d to g) Flow cytometric immunophenotyping (FCM IPT) of bone marrow aspirate shows blasts expressing CD34, cy CD79a, CD 19, cy CD3, CD 2 & CD 5 supporting diagnosis of MPAL-B/T.

MPAL phenotype observed in our infant case is distinctly uncommon, comprising <5% of MPAL cases, and is typically associated with aggressive disease biology and poor early outcomes, as documented in isolated reports and small paediatric series [10].

Consistent with the WHO 5th edition and International Consensus Classification (ICC) guidelines, lineage assignment in our cases relied on objective flow cytometric criteria rather than cytochemistry alone. Strong CD19 expression with additional B-lineage markers (CD79a, CD22, and CD10) and cytoplasmic MPO positivity were pivotal in classification, reinforcing published observations that cytochemical stains, such as PAS and SBB, are often insufficient or misleading in MPAL [11]. In the present study, case 1 had negative cytochemical staining with strong MPO positivity by flow cytometry, although in the other two cases, cytochemical stains were contributory to the diagnosis, supporting the findings of Fuda F et al., [11].

Adult MPAL has increasingly been shown to harbour molecular features overlapping with myeloid neoplasms, including mutations in IDH2, SRSF2, and CUX1, as well as chromosome seven abnormalities, all of which were present in Case 1 and are recognised adverse prognostic indicators in prior genomic studies.These findings support the evolving concept that many adult MPALs arise from a founding myeloid clone with retained lineage plasticity rather than representing true dual-lineage leukaemias [12]. As published by Munker R et al., extramedullary involvement, including CNS disease, has been reported more frequently in MPAL than in lineage-restricted AML, particularly in B/myeloid subtypes, aligning with the CNS infiltration observed in Case

| Parameters | Case 1 | Case 2 | Case 3 |
|---|---|---|---|
| Age and sex | 62, Male | 64, Male | 85 day, Male |
| Presenting symptoms | Tiredness, vomiting, Breakthrough seizures | Body pain, epistaxis, haemoptysis | Fever |
| Peripheral blood smear findings | 49% blasts, SBB Negative PAS Negative | 59% blasts, SBB positive, PAS negative | 99% blasts, PAS positive, SBB negative |
| Myeloid markers | MPO, CD13, CD64, CD117, CD14 & CD33 | cMPO, CD117,CD13 & CD 64 | cyCD79a, CD 19, |
| T -lineage Markers | Nil | Nil | cy CD3,CD 2 & CD 5 |
| B-lineage Markers | CD 79a, CD19, CD10,CD22 | CD 79a, CD19, CD22, CD 10 | Nil |
| Cytogenetics and molecular analysis | Karyotyping showed del7q22 [5] Molecular: (IDH2) (VAF: 45.93%), (SRSF2) (VAF: 50.6%) & CUX1 (VAF: 77.39%) | Normal | Not attempted |
| Diagnostic criteria met | WHO MPAL (Myeloid/B) | WHO MPAL (Myeloid/B) | WHO MPAL (B/T) |
| Initial treatment | HCVAD | 6MP Methotrexate, Vincristine, Wysolone | Succumbed within hours of presentation |

**[Table/Fig-4]:** Comparison of three cases [5].

2 [13]. This highlights the aggressive biology of these neoplasms. Infant leukaemias are known to harbour distinct epigenetic vulnerabilities and high proliferative indices, explaining the profound leukocytosis and rapid clinical decline, as evidenced in Case 3 [14].

Across all cases, the unifying theme was the complexity of MPAL treatment protocols. Historically, the choice of therapy fluctuated between AML-type-and ALL-type regimens; however, recent data suggest that ALL-directed induction followed by allogeneic hematopoietic stem cell transplantation in first remission improves survival in adults [15]. This was followed in Case 1; however, due to the aggressive nature of the disease, the patient had MRD positivity. Collectively, our cases corroborate established diagnostic and prognostic paradigms while emphasising the marked clinical, immunophenotypic, and molecular heterogeneity of MPAL across different age groups.

### Limitation(s)

The present case series had several limitations. Two of the three patients lacked long-term follow-up data, which limited the meaningful assessment of treatment durability and survival outcomes. Molecular testing was not uniformly performed across all cases, restricting genotype-phenotype correlations. Functional or single-cell analyses were not performed to elucidate clonal relationships or lineage evolution. Treatment strategies were heterogeneous and were influenced by real-world resource constraints, further limiting comparability.

## CONCLUSION(S)

The MPAL is characterised by marked biological diversity. Multi-parameter flow cytometry, interpreted using objective lineage-defining thresholds, is indispensable for diagnosing this disease. Strengthening access to comprehensive diagnostic testing and timely treatment is particularly important in resource-limited settings, where therapeutic constraints can further worsen outcomes. This series emphasises the importance of integrating immunophenotypic, cytogenetic, and molecular data to guide therapy and improve outcomes in this rare and aggressive disease entity.

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