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Acute leukemia of ambiguous lineage:

the known and the uncertain

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Abstract

Acute leukemia of ambiguous lineage (ALAL) is a rare, high-risk form of acute leukemia. It is characterized by the inability to assign a single lineage of differentiation to the leukemia and can manifest with more than one lineage-defining marker, called mixed phenotype acute leukemia (MPAL), or the complete absence of such markers, defined as acute undifferentiated leukemia (AUL). Recent genetic, epigenetic and metabolic insights refine diagnostic frameworks, inform classification and risk-stratification, and expose potential targetable vulnerabilities. However, the rarity and heterogeneous manifestations of ALAL result in ongoing diagnostic and therapeutic uncertainty. The most recent World Health Organization (WHO) and International Consensus Classification (ICC) manuscripts provide a pragmatic framework integrating immunophenotypic and genetic criteria for classification, with recognition of specific somatic genetic alterations that define disease biology. These include rearrangements involving *BCR::ABL1*, *KMT2A*, *ZNF384*, and *BCL11B* activation.

Current evidence supports the use of ALL-type induction regimens (with the addition of tyrosine kinase inhibitors (TKI) for Philadelphia-positive MPAL) over AML or hybrid approaches. For AUL the optimal therapeutic approach is uncertain. Incorporation of targeted therapies in combination with intensive, and lower-intensity chemotherapy backbones based on the specific biological and genetic characteristics of ALAL is an appealing approach and is increasingly reported. The use of lineage-specific targeted approaches may result in therapeutic pressure and lineage switch in patients with acute leukaemia with multi-phenotypic potential. The role and optimal platform for minimal residual disease surveillance in ALAL to guide therapy, and inform transplantation is unclear, given the paucity of prospective controlled data.

Keywords:

Acute leukaemia of ambiguous lineage (ALAL), Mixed phenotype acute leukaemia (MPAL), Acute undifferentiated leukaemia (AUL)

Introduction

For most patients with acute leukaemia a single lineage of differentiation can be assigned based on the expression of lineage-defining markers. Unfrequently, more than one lineage can be assigned to the acute leukemia, termed Mixed Phenotype Acute Leukaemia (MPAL), or no specific lineage can be assigned to the disease, called Acute Undifferentiated Leukaemia (AUL)[1]. According to the most recent World Health Organization (WHO) manuscript MPAL and AUL are collectively referred to as Acute Leukaemia of Ambiguous Lineage (ALAL). Over the years different terms were coined, and various classification criteria were applied to define these entities moving from immunophenotypic-only inclusive criteria to more genetic, and biological-driven stringent criteria that resulted in less patients diagnosed with *bona fide* ALAL. With more recent classifications, it is estimated that ALAL is diagnosed in 2-3% of patients with acute leukaemia[2,3], with a Surveillance, Epidemiology and End Results (SEER)-reported incidence of 0.35 per 1,000,000 person-years[2]. The rarity of ALAL along with complex diagnostic and clinical challenges result in much uncertainty about how to diagnose and treat these acute leukaemias. Recent insights on the processes driving hematopoiesis and lineage commitment, as well as on the role of somatic genetic alterations in driving ALAL further inform classification systems for ALAL and expose potential therapeutic vulnerabilities.

How to diagnose and classify ALAL

Historically, the classification systems used to identify bi-phenotypic leukemias (more than one lineage expressed on the same blast population) and bi-lineal leukemias (two discreet blast population of divergent lineage in the same patient) relied on the expression of lineage markers as assessed by immunophenotype and cytochemistry assays[3,4,5,6,7], as reflected in the European Group for the Immunological Characterization of Leukemias (EGIL) classification[8]. The major drawback of using a phenotype-based classification system is that many *bona fide* AML syndromes, as defined by founding genetic events, are associated with multi-phenotypic expression. For example, core-binding factor AML can express lymphoid markers including CD19[9,10].

The most recent WHO and International Consensus Classification (ICC) manuscripts aim to integrate genetic, immunophenotypic and clinical context to provide a practical classification of ALAL (Figure 1)[1,11,12]. In these classification systems ≥20% of blasts in the blood or marrow are required for ALAL diagnosis. A limited set of lineage-defining markers are utilized. Myeloperoxidase (MPO) or 2 of 5 monoblastic markers (CD11c, CD14, CD64, lysozyme or non-specific esterase [NSE]) define a myeloid lineage, CD3 (membranous or cytoplasmatic) defines T-lymphoid lineage, and CD19 with 1 or 2 associated markers (CD79a, cytoCD22 and CD10) define B-lymphoid commitment. In contrast, AUL lacks lineage-defining markers and may express no more than one lineage-associated marker for any given lineage. Most cases show a single myeloid marker alongside stem-cell markers such as CD34, HLA-DR, and TdT[13].

A key principle is that other WHO-defined AML entities take precedence over ALAL. Thus, ALAL can only be diagnosed after excluding well-defined AML subtypes that may exhibit mixed phenotypes. Once diagnosed, ALAL is further subclassified by genetic drivers including rearrangements in *BCR::ABL*1, *KMT2A, ZNF384*, or *BCL11B* activation, with a secondary mention of associate lineage mixture. Remaining cases are categorized by lineage combinations, with B/myeloid MPAL being most common (~two-thirds of patients), followed by T/myeloid. Rare forms include B/T, B/T/myeloid, and the more recently defined T/megakaryoblastic subtype, in which megakaryocytic markers (e.g. CD41, CD42, and/or CD61) appear with cCD3 (often as the only T-lineage marker), usually with lineage-associated myeloid, lymphoid and stem-cell markers[14,15,16].

While this classification provides a coherent and pragmatic framework for the pathologist and clinician, there are still several potential caveats and challenges in diagnosis and classification.

<u>How to define a positive lineage marker?</u>

The diagnosis of ALAL requires integration of clinical, immunophenotypic and genetic information that can only be achieved through close communication between the clinician, the pathologist, and supporting laboratories. In the most recent WHO manuscript, for CD3 to be regarded as positive its' intensity should exceed 50% of mature T-cell level by flow cytometry (FC) in the same sample, at least on a portion of the leukaemic cells. CD19 intensity should

exceed 50% of B-cell progenitor by FC , and MPO should exceed 50% of mature neutrophils or show a variable pattern reminiscent of that seen on normal CD34 positive myeloid progenitors[12,17].

MPO is considered a myeloid-defining marker, and no specific clone size or threshold is defined. The interpretation of MPO positivity should always be put in the clinical and pathological context, especially when addressing smaller clones (e.g. <10% by FC). For example, MPO positivity may be the sole differentiating feature between early T-cell acute lymphoblastic leukemia (ETP-ALL) and T/Myeloid MPAL[18]. The impact of isolated MPO expression in the context of an otherwise *bona fide* B-cell precursor (BCP) ALL may not change the biology and natural history of this disease. Weinberg et al. compared adult patients with MPAL B/Myeloid and MPO as their only myeloid-defining marker ('MPAL$^{\text{IsoMPO}}$') to patients with other types of MPAL and demonstrated improved overall survival (OS) for patients with MPAL$^{\text{IsoMPO}}$ (n=13) as compared to patients with other types of MPAL (n=10; median OS not-reached *vs.* 16 months, p<0.05), and a similar OS to that of patients with immunophenotypically unremarkable BCP-ALL. In this analysis, 'MPAL$^{\text{IsoMPO}}$' did not differ significantly from other MPAL subtypes in terms of cytogenetics, showing similar rates of abnormal karyotypes, comparable frequencies of *t(9;22)/BCR::ABL1* rearrangements, and absence of *KMT2A* rearrangements. Results from retrospective paediatric series focusing on 'MPAL$^{\text{IsoMPO}}$' were less consistent and showed mixed results[19,20,21].

Bi-phenotypic *vs.* bi-lineal leukemia

MPAL can present as a single blast population with multi-lineage expression (bi-phenotypic) or less frequently, as 2 discrete blast population of different lineage assignments (bi-lineal). For the latter, a total of ≥20% blast should be demonstrated in aggregate for the different blast populations. Although there is no clear biologic or diagnostic distinction between these two entities in the WHO or ICC manuscripts, retrospective observations suggest that bi-lineal MPAL is associated with inferior outcomes[5]. In one large international multi-center analysis (iBFM-AMBI2012), 20% of 221 paediatric and adolescent patients with MPAL had bi-lineal MPAL. These patients had a significantly shorter EFS and OS as compared to patients with bi-phenotypic

MPAL regardless of the induction approach applied, although this did not maintain significance in multivariate analysis[22].

Many times small lineage-aberrant clones can be identified alongside a dominant clone and may lead to diagnostic uncertainty[23]. Guidance from a recent ICC publication suggests that blast subpopulations of divergent lineage larger than 5% may be sufficient for diagnosis of bi-lineal MPAL while in cases of smaller clones, the diagnosis should be made according to the major clone with addition of a descriptive modifier annotating the small divergent aberrant clone[12,23]. The mention of a minute aberrant clone may prove to be of biological and clinical significance, since these small clones can be the basis for subsequent relapse and lineage switch (LS). Care must be taken to avoid misclassifying residual normal myeloid blasts or hematogones as pathologic divergent lineage populations[16,23].

Does my patient have MPAL or secondary AML with a 'mixed phenotype'?

The current WHO and ICC manuscripts moved towards classification based on somatic genetic aberrations including AML with myelodysplasia-related cytogenetics and AML with myelodysplasia-related mutations[24]. These well-defined AML syndromes can be associated with expression of aberrant lineage markers but should be classified according to their primary WHO diagnostic ascription rather than ALAL. This presents a specific challenge in the context of ALAL since some of the 'myelodysplasia-related' AML defining cytogenetic aberrations were reported in patients with *bona fide* ALL. For example, in the UKALLXII/ECOG2993 trial 2-5% of patients harbored 'MDS-type' cytogenetic aberrations (e.g. complex karyotype, del7, del17p, trisomy 8, and del13q)[25].

Historically, many ALAL cohorts included patients with 'secondary-type' defining genetics that probably affected the epidemiology, prognosis and therapeutic outcomes of these patients[26]. For example, in one pivotal report of 100 patients with MPAL according to the WHO 2008 criteria, the most frequent cytogenetic abnormality was complex karyotype (in 32% of patients) [27]. More recent reports utilizing up-to-date WHO criteria for ALAL validate the high frequency of myelodysplasia-related cytogenetics in patient series of ALAL. In an analysis of 28 adult patients with MPAL (per WHO 2016), complex karyotype was reported in 28% of patients while other

myelodysplasia defining cytogenetics such as del5q/-5, del7q/-7, del17p/-17, trisomy 8, del20q, del9q and del13q were reported in aggregate in 32% of patients[28].

Galera et al. compared the clinical characteristics and outcome of patients with secondary AML with a 'mixed-phenotype' (sAML-MP; n=55; therapy-related AML and AML with myelodysplasia-related changes according to the WHO 2016 criteria) with patients with *bona fide* MPAL (n=45) and AML (n=100). Patients with sAML-MP had worse outcomes than patients with MPAL. Secondary type mutations (STM) as well as myelodysplasia-related cytogenetics were a common finding in the secondary AML group and very uncommon in the MPAL group except for a complex karyotype and mutations in *RUNX1* (identified in 20% and 23% of patients with MPAL, respectively). No *TP53* mutations were found in the MPAL group (Figure 2). As compared to patients with MPAL, patients with sAML-MP had lower remission rates with intensive induction (37.5% vs 90%, p<.001) and inferior survival (median OS, 10.3 vs 42.8 months, p<.0001; HR, 2.3, 95% CI, 1.4-4.0, p=.002), comparable to the outcome of the 'control' AML cohort. ALL-type induction was very effective in patients with MPAL and was largely ineffective in sAML-MP (complete remission [CR] 96.6% *vs.* 14.3%, p=0.001). AML-type induction performed better in sAML-MP. Multivariate analyses demonstrated that ALL-directed therapy for sAML-MP was associated with inferior OS (HR 5.68; 95% CI 1.39-23.4; p = 0.016), whereas MPAL had significantly improved OS with ALL-directed regimens (p = 0.0001). Across all ALL-treated patients, age > 65 y (HR 9.0; 95% CI 2.28-35.6; p = 0.001) and sAML-MP subtype (HR 12.5; 95% CI 2.72-57.8; p = 0.001) predicted worse OS. With AML-directed intensive therapy, remission was achieved in 12/20 AML-MP cases (60%) versus 0/4 MPAL cases (p = 0.09); here, only age > 65 y independently predicted inferior OS (HR 3.0; 95% CI 1.1-8.0; p = 0.02). Interestingly, lymphoid LS at progression was much more common in MPAL than in sAML-MP (35.7% *vs.* 2.5%, p=.0003). On the transcriptional level, sAML-MP was enriched for stemness signatures, and demonstrated a relative deficit of transcription factors central for myeloid and lymphoid differentiation (similar to the AML cohort), as compared to MPAL[29]. Kirtek et al. investigated the impact of STM *(SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*, *ASXL1*, *EZH2*, *BCOR*, *STAG2*, and/or *RUNX1*) on the characteristics and outcome of patients with ALAL (n=23) or AML (n=167). ALAL and AML with STM were overall comparable in terms of baseline characteristics, other than higher blast counts and rates of abnormal

karyotype in patients with ALAL. The clinical course and outcomes were very similar between the two groups suggesting that STM, not immunophenotype, drive the biology and clinical outcome for these patients[30]. A similar study comparing the impact of complex karyotype (defined as ≥3 structural abnormalities) on outcome of patients with MPAL, AML or ALL confirmed that complex karyotype, not immunophenotype, drives the poor outcome for these patients[31], although this observation was not confirmed in the study by Galera et al[29].

Taken together, we recommend that patients with myelodysplasia-related defining cytogenetics and/or myelodysplasia-related defining mutations (STMs) be defined as such, regardless of their lineage-mix, and treated with myelodysplasia-related AML directed therapies. One exception are *RUNX1*-mutated cases (regarded as STMs by the ICC, but not by the WHO) that are enriched in MPAL cases and seem to share biological and prognostic similarities with other MPALs.

Genetic and epigenetic alterations in ALAL

Somatic genetic alterations initiate and drive the biology of acute leukaemia. In ALAL, unique patterns of genetic alterations are described with specific associations between genotype and immunophenotype. *BCR::ABL1* and *KMT2A* rearrangements (*BCR::ABL1*r, *KMT2Ar*) were the first specific MPAL subgroup-defining alterations. *BCR::ABL1r* account for 15-20% of MPAL cases as are associated with older age and a B/Myeloid immunophenotype. ALAL with *KMT2Ar* can be found in approximately 10% of cases, mostly in infants and children. The most common expected 5 *KMT2A* partners are *AFF1* (*AF4*), *MLLT3* (*AF9*), and *MLLT1* (*ENL*), and most cases will present as B/Myeloid immunophenotype although rare cases of *KMT2Ar* AUL and MPAL B/T were reported[1]. More recently, two additional ALAL subgroup-defining rearrangements were recognized and incorporated into the formal WHO and ICC classifications.

ZNF384-rearranged (*ZNF384r*) MPAL was shown to occur in up to 50% of paediatric patients with B/Myeloid MPAL[32], but rarely in adults[33]. Different fusion partners including transcription factors (*TAF15* and *TCF3*) and chromatin modifiers (*CREBBP, EP300, SMARCA2, and ARID1B*) were reported, with *EP300* being the most common. *ZNF384r* MPAL shares genetic and

transcriptional similarities with *ZNF384r* BCP-ALL (that is frequently characterized by aberrant expression of myeloid markers)[34]. These leukemias were shown to overexpress the *FLT3* gene [32,35], and the specific type of fusion partner was suggested to impact patient outcome (e.g. *EP300-ZNF384* and *TCF3-ZNF384* fusions associated with favorable and poor outcomes, respectively)[32,36].

BCL11B is a master regulator of T-lineage commitment. *BCL11B*-activated ALAL defines a genetic subgroup of ALAL accounting for 10–15% of patients with MPAL and about one third of patients with T/myeloid MPAL[1]. It can also be found in cases of AUL, ETP-ALL and AML[37]. *BCL11B* activation and overexpression are the result of enhancer hijacking created by repositioning of regulatory sequences (*e.g. ARID1B*, *MYC-BENC*, or *CDK6*) upstream or downstream of the *BCL11B* gene, or from an amplification of a 2.5 kb region downstream of *BCL11B* (*BCL11B* enhancer tandem amplification). These alterations are frequently associated with JAK-STAT pathway activation and *FLT3-ITD* mutations[37,38,39], and should not be confused with loss-of-function mutations, deletions, and oncogenic rearrangements of *BCL11B* typically seen in T-ALL.

Ph-like alterations in patients with MPAL are infrequently reported in the literature[40]. For example, in one pivotal analysis of gene expression profiles, only 3 of 95 patients assessed harbored Ph-like driving alterations (including *EBF1–PDGFRB, IGH–CRLF2* and one case lacking an identified kinase lesion)[32].

The advent of comprehensive platforms for detection of translocations and copy number changes such as optical genome mapping (OGM) and RNA sequencing are instrumental for systematically detecting disease-defining genetic alterations, including additional rare recurrent translocations associated with ALAL such as *PICALM::MLLT10, NUP98::NSD1 and SET::NUP214* [41].

The landscape of small sequence variants in ALAL encompasses a combination of somatic alterations seen in AML and ALL. Different data sets consisting of different, heterogenous populations report varying patterns of somatic mutations[28,29,33,42,43,44,45,46]. *Bona fide* MPAL (as compared to sAML-MP) rarely exhibit *TP53* mutations or STM[29] with the exception of *RUNX1*

mutations, shown to be enriched in patients with MPAL[28,29,32,33,42,43, 47]. B/Myeloid and T/Myeloid MPAL display different mutational patterns (Figure 2). In one analysis of 115 paediatric and adolescent patients with MPAL, T/Myeloid MPAL was characterized by high mutational burden involving transcriptional regulators such as *WT1*, *ETV6*, *RUNX1* and *CEBPA* that were mutually exclusive, as well as high rates of JAK-STAT alterations, and mutations in epigenetic regulators (e.g. *EZH2* and *PHF6*) and *FLT3* mutations. B/Myeloid MPAL was associated with lower mutational burden and involved the transcription factors *IKZF1* and *PAX5*, and RAS pathway alterations (most commonly *NRAS* and *PTPN11*). T/Myeloid MPAL and ETP-ALL demonstrated significant overlap in mutational patterns[32]. Other retrospective cohorts of adult or mixed paediatric and adult populations associated T/Myeloid MPAL with higher frequencies of mutations in *CEBPA*[42], *DNMT3A*[42,43], *PHF6*[43], and *NOTCH1*[33,42] while B/Myeloid MPAL was associated with mutations in *RUNX1*[33] and in the RAS pathway[42]. Data on the genetic makeup of AUL is scarce. In one analysis of 24 adults with AUL, the most common genetic alterations were trisomy 13 and mutations in *PHF6, SRSF2, RUNX1, ASXL1* and *BCOR*. Mutations in *PHF6* and *SRSF2* were enriched in AUL as compared to patients with AML and minimal differentiation[13].

Recent studies demonstrate that epigenetic profiling, including DNA methylation signatures and chromatin accessibility landscapes are powerful tools to identify lineage identity[48,49,50]. For example, in *KMT2Ar* MPAL, epigenetic patterns often align with a dominant lineage identity, typically myeloid or lymphoid, despite immunophenotypic ambiguity[48].

Takahashi et al. performed integrated methylome and transcriptome analysis in 31 MPAL patients, revealing distinct epigenetic programs that stratify MPAL by lineage. T/myeloid MPAL showed hypomethylation patterns supporting T-cell receptor (TCR) signaling pathways while B/myeloid MPAL demonstrated patterns driving the B-lineage transcriptional axis. Gene set enrichment confirmed NF-κB and B cell receptor (BCR) signaling in B/myeloid MPAL. Methylation-based clustering identified two MPAL subtypes: an 'ALL-like' subtype that clustered with B-ALL or T-ALL, and an 'AML-like' MPAL that aligned with *bona-fida* AML. Patients that received an induction approach aligned with their methylone-based lineage assignment (e.g. an ALL-type induction for an 'ALL-like methylome') had significantly higher complete remission (CR)

rates compared to those receiving mismatched treatments (72% vs. 22%, *P* = 0.037), although this did not translate into better survival [33].

The cellular origin of MPAL

The mechanisms underlying multi-phenotypic expression in leukaemia have been the focus of investigation. A central unresolved question has been whether phenotypic heterogeneity arises primarily from the stepwise acquisition of somatic mutations ('clonal evolution'), or from intrinsic plasticity of leukaemic stem-cells (LSC) with a largely stable genetic make-up.[51,52,53,54,55,56,57,58,59,60,61]. Alexander et al. presented multiple compelling observations to support the existence of a multipotent LSC rather than clonal evolution as the cellular basis for the development of MPAL[32]. The authors demonstrate that leukaemia initiating mutations occur at the stem-cell level. These somatic genomic alterations were shown to have the potential to produce different lineage phenotypes with similar biologic implications (such as in the case of *ZNF384r* BCP-ALL or B/Myeloid MPAL) and remain stable across phenotypic subclones. In a key experiment, flow-sorted subpopulations from primary ALAL samples were transplanted into NSG-SGM3 mice and successfully reconstituted the full phenotypic spectrum of the original leukaemia, even in the absence of therapeutic pressure[32]. In line with these observations, Xiao et al. and Kotrova et al. demonstrated that phenotypically diverse blast sub-populations from patients with MPAL harbor similar genetic alterations[43,62] and Galera et al. demonstrated similar gene expression patterns in sorted blasts of myeloid and T-cell lineage origin from the same patient[29]. A study of patients with acute leukaemia and a CpG island methylator phenotype, an immature form of leukaemia associated with a mixed phenotype, demonstrated a similar hybrid epigenetic landscape despite varying somatic initiating mutations suggesting that epigenetic dysregulation rather than genetic lesions drive the mixed phenotype in these ambiguous leukaemias[63]. Taken together these observations support the concept that phenotypic diversity results from mutation acquisition in a multipotent stem-cell/progenitor cell rather than phenotypic diversity that results from ongoing genomic evolution.

Therapeutic approach

ALAL is considered a high-risk form of leukaemia with increased relapse rates and poor long-term survival. A SEER registry analysis demonstrated a 59% and 26% increase in the risk of death for patients with MPAL as compared to patients with ALL and AML, respectively[64]. The high-risk nature of ALAL was further highlighted in a recent retrospective analysis of real-world outcomes of patients with MPAL in the United States (n=4,756 adults; median age 68 years). Median OS was poor at 4.2 months (95% CI 3.6–4.9) with 30-day mortality of 33%. Subtype analysis revealed markedly improved outcomes for patients with Philadelphia-positive (Ph+) MPAL (median OS 53.6 months, 5-year OS 49%), and poor outcome for AUL (median OS 1.4 months, 5-year OS 14%)[65].

The optimal treatment strategy for ALAL remains undefined. Current approaches are mostly informed by retrospective heterogeneous cohorts, limiting firm conclusions. Consequently, therapy is often individualized, with no clear consensus on induction or long-term management [66,67,68]. Despite these limitations, a structured approach can guide therapy selection. This includes evaluating patient-specific factors (e.g. age, comorbidities, and fitness) alongside comprehensive analysis of blast morphology, immunophenotype, cytogenetics, and molecular genetics (Figure 3)[68].

What is the optimal induction regimen for ALAL?

Over the years, multiple retrospective analyses have compared induction strategies for ALAL (Table 1). A systematic review and meta-analysis examined outcomes in 1,499 patients with MPAL, classified according to WHO 2008 or EGIL criteria based on the type of induction regimen administered: ALL-type, AML-type, or hybrid. The analysis showed that ALL-based induction was significantly more effective in achieving CR than AML-based therapy (WHO-based, OR = 0.33; 95% CI, 0.18–0.58). Multivariable analysis of pooled patient-level data confirmed inferior remission rates with AML induction (OR = 0.45; 95% CI, 0.27–0.77). OS also favored ALL-type regimens (WHO-based, OR = 0.45; 95% CI, 0.26–0.77). While AML induction was not

independently associated with worse OS, hybrid regimens were linked to significantly inferior survival (HR = 2.11)[69].

The International Berlin-Frankfurt-Münster (iBFM-AMBI2012) Study of ALAL reported on a large retrospective multinational cohort of 233 children and adolescents with ALAL. ALL–type primary therapy resulted in superior outcome (5y EFS 80% ± 4%) as compared to AML–type or combined-type approach (36% ± 7.2% and 50% ± 12%, respectively, p<0.0001)[22]. Rasekh et al. analyzed 102 patients with MPAL (54 paediatric patients and 48 adults) and showed that ALL-like regimens were associated with significantly better response rates as compared to AML-type therapies regardless of age (p = 0.001)[70]. Conversely, a report from the Children's Oncology Group (COG) retrospectively analyzed 54 centrally-reviewed patients with MPAL and demonstrated that both ALL- and AML-based induction regimens resulted in comparable outcomes[71].

The utility of hyperCVAD protocol (fractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone) for patients with ALAL was assessed in a retrospective study of 25 patients from 5 centers. HyperCVAD-based therapy resulted in CR/CRi in 84% of patients. Median OS was not reached (median follow-up of 31.6 months) and two-thirds of responding patients proceeded to allogeneic transplantation (HSCT)[72].

Patients with *BCR::ABL1*r MPAL should receive tyrosine kinase inhibitor (TKI)-based therapy. The addition of TKIs to the therapeutic protocol of patients with Ph+ MPAL is associated with better OS in comparison to other MPAL patients, and comparable to the outcome of patients with *bona fida* Ph+-ALL[73,74]. For example, a SEER analysis of 241 patients with 2008 WHO defined MPAL (after the standard incorporation of TKIs) demonstrated that Ph+ MPAL patients had reduced risk of death in comparison to other patients with MPAL (hazard ratio [HR] = 0.28, P = 0.002). KMT2Ar patients with MPAL had the worst outcome with a 10-fold increased risk of death in comparison to Ph+ MPAL (HR = 10.2, P < .001). Outcome of Ph+ MPAL was comparable to Ph+ ALL in a 1:1 matched case-control analysis[74].

The historically poor outcomes in MPAL have prompted the development of hybrid protocols incorporating elements of both AML-like and ALL-like regimens. While several studies reported

inferior outcomes with this approach[27,69], possibly reflecting the increased toxicity associated with such intensive protocols, other reports suggest potential benefit in selected patients[75,76]. A Polish Adult Leukemia Group (PALG) study, reported on 16 adult patients with MPAL treated with CLAG-M hybrid protocol (cladribine, cytarabine, mitoxantrone and GCSF). Eight patients received CLAG-M as first-line therapy with an ORR of 100% (CR, n=6 and PR, n=2). Toxicity was acceptable and median time for recovery of neutropenia and thrombocytopenia were 22 days (range, 16–24) and 17 days (range, 12–24), respectively; grade 3-4 infections were observed in 12 cases[77].

The optimal approach to AUL is unknown as systematic data to guide therapy for this extremely rare entity are lacking. Of the 223 patients reported in the IBFM retrospective study, 5 children fulfilled criteria for AUL. In these few patients ALL-type treatment appeared particularly ineffictive[22].

Regardless of the therapeutic approach chosen, central nervous system assessment, prophylaxis and treatment as indicated should be pursued in a proactive, protocol-driven manner [68,71,78].

Accordingly, our current approach and recommendation is to treat most patients with MPAL with an ALL-type induction, with the addition of a TKI for patients with a *BCR::ABL1*r. The optimal induction approach for AUL is not defined and should be individualized (Figure 3).

Novel agents for ALAL – promise and pitfalls

The incorporation of targeted novel agents is increasingly utilized in ALAL (Table 2). Preclinical observations demonstrate BCL2-dependancy in ETP-ALL that is lost in mature forms of T-ALL[79], prompting interest in the clinical utility of Venetoclax (Ven) in ALAL[79,80,81,82]. In one case series, three patients with MPAL were treated with Ven in combination with fludarabine, cytarabine, idarubicin, and filgrastim (FLAG), with or without idarubicin or gemtuzumab ozogamicin (GO). Following induction, all patients achieved a sustained CR with undetectable minimal residual disease (MRD) by FC[83]. An additional report of 4 patients with ALAL that received FLAG-IDA-Ven demonstrated encouraging responses in the 2 patients sequenced early with this approach (first-line and first salvage) as compared to poor outcomes in patients treated for advanced

disease[81]. The potential efficacy of FLAG-Ida-Ven should be balanced against the potential increase in infectious complications and treatment-related mortality that were previously reported for this protocol, especially in the relapsed and refractory (R/R) setting[81,84,85,86,87].

In older or medically unfit patients, standard high-intensity AML or ALL induction regimens may be inappropriate. Such patients may be candidates for lower-intensity therapeutic approaches such as the combination of Ven with azacitidine or low dose cytarabine (LDAC) as described in few case reports[88,89,90].

The incorporation of Menin-inhibitors for the *KMT2Ar* subset of ALAL is appealing given the activity of these agents in *KMT2Ar* acute leukaemia. In the phase 2 AUGMENT-101 trial that led to the approval of revumenib for *KMT2Ar* R/R acute leukaemia, only one patient with MPAL was included (and achieved a morphologic remission)[91].

In Ph-negative ALL, alterations in the JAK-STAT pathway can be found in approximately 10-20% of patients, most frequently in the context of *JAK1/2, IL7R and CRLF2* Ph-like mutations and rearrangements[92]. Targeting these high-risk genetic alterations with JAK inhibitors was shown to be effective in pre-clinical observations[93,94], and ongoing clinical studies are assessing the safety and efficacy of incorporating JAK-inhibitors in combination with chemotherapy in JAK-STAT-altered ALL[95] (Table 2). Whether the incorporation of JAK-inhibitors in ALAL subtypes that overexpress the JAK-STAT pathway such as T/Myeloid MPAL and *BCL11B*-activated ALAL is effective is unclear to date.

Antibodies directed towards common 'stem-cell' markers that are highly expressed in these leukaemias (e.g. CD38 and CD123) may be an attractive therapeutic avenue[96,97]. There are several case-reports on the use of blinatumomab, a bispecific T-cell engager, in ALAL[98], including in combination with Ven[99,100]. Lower-Intensity chemo-Immunotherapy with cladribine, low-dose cytarabine, Ven and sequential blinatumomab was reported in four patients with *BCR::ABL1*-Negative B/myeloid MPAL (median age 71 years [range, 55–77]). Three patients received this regimen as frontline therapy and one as salvage after decitabine-Ven. All patients achieved CRc with undetectable MRD by FC after one cycle. Toxicity was manageable with only low-grade

neurotoxicity reported. At last follow-up, three patients remained alive while one patient relapsed with AML[101].

CD19-directed chimeric antigen receptor T-cell (CAR-T) therapy in CD19-positive ALAL may be a potentially effective treatment strategy as previously reported for patients with CD19-positive core-binding factor AML[102,103].

Lineage-directed targeted therapies and lineage-switch

LS is defined as a change in lineage during therapy, or at relapse. Although LS is a well-described phenomena with historically poor outcomes, it has recently emerged as a unique clinical phenomena and mechanism of disease resistance following lineage-directed targeted immunotherapy[104,105], with an incidence approaching 8% of patients with B-ALL, following CAR-T cell therapy [106].

The potential for LS may already exist at the time of diagnosis. In one study of 223 cases of *de novo* BCP-ALL, 31 (13.9%) harbored *CRLF2* rearrangements (*CRLF2r*), and among these, 7 (22.6%) exhibited partial monocytic differentiation. These findings suggest that monocytic features in *CRLF2r* B-ALL are relatively common and may predispose to subsequent LS[107].

Identification and awareness of B-ALL with genotype-linked myeloid/monocytic aberrancies is critical to avoid inappropriate deviation from effective ALL-directed therapy. Certain genetically defined B-ALL subtypes, notably *DUX4r, PAX5-P80R*-mutated, and *ZNF384r* cases, may develop a pronounced monocytic switch early during therapy, with acquisition of CD14, CD64, or other myeloid/monocytic markers. Novakova et al. showed that these populations remained clonally identical to the original B-lymphoblasts by Immunoglobulin/T-cell receptor rearrangement analysis, retained their defining genetic lesions, and that their emergence was not associated with inferior outcome with ALL-directed therapy[108].

In one study, 33 patients with LS (defined by cytogenetic/molecular evidence for clonal relatedness between the original leukaemia and LS) were analyzed. Risk factors for LS included paediatric patients, the use of CD19- T-cell engaging therapies, recurrent alterations

in *EZH2* and *KMT2A* fusions[109]. In a recent study, single-cell transcriptome profiling revealed LS–prone B-ALL subtypes (*BCR::ABL1, KMT2A-R, DUX4-R*) that are enriched for multipotent progenitor-like states with *CEBPA* activation and myeloid potential. This phenotype underlies leukemic drift and is captured by a 'multi-potency score' associated with inferior survival[110].

A recent report (EVOLVE project) analyzed 70 cases of LS after exposure to immunotherapies. Most patients (n=53, 75.7%) transitioned from BCP-ALL to AML, while 17 patients (24.3%) had a BCP-ALL to B/Myeloid MPAL or AUL switch. The immunotherapy most proximal to LS included CAR-T cells (n=34, 48.6%) and blinatumomab (n=31, 44.3%), and LS emerged within 6 months of the most recent immunotherapy in 57 patients (81.4%), including during immunotherapy. Only 20 of 65 patients (30.8%) achieved CR to first-line LS-directed therapy. Overall, remission rates were <40%, and median OS following LS diagnosis was 4.8 months. The predominant cause of death was resistant disease (n=52 of 61, 85.2%). Notably, 64% of BCP-ALL to AML LS cases harbored *KMT2Ar.* As compared to other patients, *KMT2Ar* patients were younger and had an earlier LS (median 1.1 vs. 3 months; p=0.16). CR rates post-LS were higher in the *KMT2Ar* group (17/40, 42.5%) than in the non-*KMT2Ar* group (6/25, 24%; p=0.18), though survival remained poor in both groups (15.6% vs. 4% survival, respectively)[104].

These data highlight the need for awareness and proactive surveillance for LS, especially in predisposed genetic subgroups, and after CD19-directed interventions. Combination of lineage-selective targeted therapies with chemotherapy backbone might offer a potential strategy to reduce LS and remains to be studied.

Can MRD guide therapy in ALAL?

MRD assessment is a powerful predictive tool that is used to make strategic therapeutic decisions for patients with ALL and AML [11,111]. Since patients with ALAL are poorly represented in large cooperative trials, the use of MRD assessments for risk stratifications and decision analysis are less established. Furthermore, the stability and biological and clinical utility of T-cell receptor or immunoglobulin heavy-chain clonal rearrangements for MRD surveillance are

uncertain. Several retrospective analyses demonstrated that MRD-negative responses during therapy[22,112,113], and before HSCT[114] are significantly associated with improved survival as compared to patients with sub-optimal MRD responses. The iBFM-AMBI2012 study demonstrated that patients with >5% leukemic cells at the end of induction (EOI) had poor 5-year EFS[71]. The predictive utility of flow-based MRD was reported in 112 pediatric patients diagnosed with MPAL. In this analysis, centrally reviewed flow-based MRD was assessed in patients receiving ALL-type regimens. With this approach, end of induction (EOI) MRD negative (<0.01%) remission was attained in most patients (70%). EOI MRD positivity was predictive of 5-year EFS (HR = 6.00, $p < 0.001$) and OS (HR = 9.57, $p = 0.003$). Earlier MRD clearance was associated with better survival[112]. Based on these data, the Children's Oncology Group Acute Leukemia of Ambiguous Lineage Task Force recommended that only patients with EOI blast clearance <5% and end-of-consolidation MRD <0.01% continue ALL therapy. The task force also advised that patients that fail to meet these thresholds be considered for early intensification with AML and/or SCT consolidation[71]. While we generally support re-evaluating treatment approach in patients with ALAL and MRD-failure, these recommendations should be validated within prospective clinical trials, and in the adult population.

Given limited data on the clinical utility of MRD in MPAL, we recommend it not be used as the sole basis for major clinical decisions such as HSCT or therapy de-escalation outside clinical trials. MRD should be utilized according to the therapeutic protocol applied and MRD-failure should prompt the clinician to re-assess and re-consider the therapeutic plan including consideration of HSCT. Combining different MRD platforms (e.g., FC with molecular or NGS assays) may improve predictive accuracy[111,112].

Allogeneic transplantation in first remission

Since ALAL is regarded as a high-risk leukaemia with increased relapse rates, HSCT in first remission (CR1) should be considered in eligible patients. Total body irradiation (TBI)-based conditioning may be preferred, when feasible, based on ALL guidelines[115]. A retrospective registry study evaluated 519 adult patients with MPAL who underwent allo-HSCT in first remission. Since the study spanned over several years (2000-2014), patients were classified by

different classification systems. At three years post-transplantation, the cumulative incidence of relapse was 31.4% (95% CI, 26.9–35.9), and non-relapse mortality was 22.1% (95% CI, 18.4–26.1). Leukemia-free survival (LFS) and OS rates were reported at 46.5% (95% CI, 41.7–51.4) and 56.3% (95% CI, 51.5–61.2), respectively. In a multivariate analysis, age and year of transplant had a strong impact on outcome. Myeloablative conditioning using TBI correlated with a better LFS[116]. Another study reported outcomes in 77 adult patients diagnosed with MPAL over 10 years (median age 49 years; ALL-type induction in 61%) and reported median OS for the whole cohort of 41.9 months. Fifty patients (65%) were transplanted and the 5-year OS for this group was 54% with better outcomes reported for patients who were MRD-negative prior to transplant (75.8% vs 45.2%, P = 0.06)[114].

In paediatric MPAL, allo-HSCT offers no clear survival benefit. Most patients achieve durable remissions with ALL-based therapy alone, and HSCT is typically reserved for select high-risk genetic or clinical features[71,116,117,118]. The decision on transplant adult patients in first remission is complex and should be guided by disease genetic-risk, MRD response (as assessed by multiple methods), patient co-morbidity burden, and fitness. A specific group of interest are patients with *BCR::ABL1* MPAL in whom transplant decision can be informed by the criteria applied for *BCR::ABL1* B-ALL.

Uncertainties and future challenges for ALAL

ALAL is a rare disease with several diagnostic and therapeutic uncertainties. Genetic, epigenetic and metabolic translational insights improve the ability to better classify and risk-stratify ALAL as reflected in the most recent WHO and ICC manuscripts. From the diagnostic perspective, close cooperation between the clinician, the pathologist and the laboratory services are needed to yield optimal diagnostic and therapeutic results.

Still, several diagnostic dilemmas exist. The reproducibility of diagnostic criteria for lineage commitment in the clinical setting across different laboratory services, is uncertain, and prospective validation of the most recent diagnostic schemes is needed. The clinical relevance of morphological correlations (e.g. Auer-Rod bodies in the context of ALAL) and the diagnostic

impact of small, minute divergent clones is uncertain. Current classification systems for AML are rapidly moving towards genetically based classification of disease ontogeny. The diagnostic, prognostic and therapeutic impact of myelodysplasia-related genetics including STM that may overlap between ALAL and secondary AML, need further clarification. The clinical utility of transcriptomic and epigenomic profiles to aid classification, and guide therapy require further investigation.

Although cumulative evidence on the optimal treatment approach for patients with ALAL largely supports an ALL-type induction for most patients, prospective controlled data are lacking. Novel, innovative approaches for specific and rare subtypes of ALAL with extremely poor outcomes such as AUL and LS are needed. Incorporation of novel, targeted therapies in combination with intensive, and lower-intensity chemotherapy backbones based on the specific biological and genetic characteristic of ALAL is an appealing approach and is increasingly reported. While promising, one must consider the potential for driving LS with the use of lineage-specific targeted approaches in acute leukaemia with multi-phenotypic potential. Lastly, the role and optimal platform for MRD surveillance in ALAL to guide therapy and transplantation is unclear given the paucity of prospective controlled data.

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Table 1. Comparing induction outcomes in ALAL

First Author	Classification system	Context	Outcome with induction approach	Message
Galera et al. 2025[29]	WHO 2022	33 adult patients with MPAL	CR • ALL-type 96.6% • AML-type 0%	ALL-type therapy is superior to AML-type in MPAL patients AML-type better in sAML-MP OS advantage for ALL-type confirmed in MVA*
Rasekh et al. 2021[70]	WHO 2008	102 adult and paediatric patients with MPAL	CR* • ALL-type 84.2% • AML-type 50% 3-year OS* • ALL-type 40.9% • AML-type 18.5%	ALL-type therapy is superior to AML-type; age impacts response.
Orgel et al. 2020[71]	WHO 2016	54 paediatric/AYA MPAL	CR • AML-type 69.2% • ALL-type 71.8% • Hybrid 100% 5-year EFS • AML-type 62±14% • ALL-type 73±10% • Hybrid NA 5-year OS • AML-type 69±14% • ALL-type 78±9% • Hybrid NA	No significant difference was found between ALL and AML induction regimens.
Hrusak et	WHO 2008	233 paediatric ALAL	5-year EFS*:	ALL-type therapy is superior to

al. 2018[22]			• AML-type 36%±7.2% • ALL-type 80%±4.0% • Hybrid 50%±12% •	AML-type, especially in CD19+ ALAL.
Heesch et al. 2013[46]	WHO 2008	26 adult patients with ALAL	CR • ALL-type 40% • AML-type 22.2% Median OS • ALL-type 21.5 months • AML-type 11 months	Outcomes in adult ALAL are poor with no statistically significant OS benefit with treatment protocol type.
Matutes et al. 2011[27]	WHO 2008	100 patients with MPAL	CR • ALL-type 85% • AML-type 41% OS mos.* • ALL-type 139 mos. (range, 8-270) • AM-type 11 mos. (range, 8-14)	ALL-type therapy is superior to AML-type.

*Statistically significant difference reported ($p<0.05$).

ALAL-acute leukemia of ambiguous lineage, MPAL-mixed phenotype acute leukaemia, sAML-MP-secondary AML with a mixed-phenotype, AML-type – acute myeloid leukaemia type induction therapy, ALL-type – acute lymphoblastic leukaemia type induction therapy WHO- world health organization, CR-complete remission, OS- overall survival, EFS event free survival, MVA – multivariate analysis.

Table 2. Potential novel agents for ALAL

Drug/agent	Rational	Target ALAL population	Clinical evidence	Ongoing trials
Venetoclax	ALAL may be *BCL2*-sensitive[80,79,81,82]	Nonspecific	Case reports and case series: Ven-low-intensity regimens[88,89,90,101] Ven-high intensity regimens[81,82,83]	NCT05901974 NCT03194932 NCT04872790
FLT3 inhibitors	Targeting *FLT3*-mutated ALAL Targeting *FLT3*-overexpressed ALAL	*ZNF384*-rearranged ALAL (overexpression) *BCL11B*-activated (*FLT3-ITD* mutation)	NA	NA
Menin inhibitors	Menin inhibition active in *KMT2A*-rearranged leukaemia	*KMT2A*-rearrannged ALAL	AUGMENT 101 (revumenib) N=68; ORR 53% (1 pt. with MPAL)[91]	NCT05326516 NCT05761171
JAK inhibitors	Targeting overexpressed *JAK-STAT* pathway	T/Myeloid MPAL *BCL11B*-activated ALAL	Pre-clinical and clinical reports in ALL [93,94,95]	NCT02723994 NCT02115295
CD123-directed therapies	Highly expressed 'stem-cell ' marker	CD123-positive ALAL	Pre-clinical data[96]	NCT06034470
CD38-directed therapies	Highly expressed 'stem-cell ' marker	CD38-positive ALAL	Clinical trial in R/R paediatric ALL/LBL (daratumomab)[97]	NA
Blinatumomab	Targets B-lineage defining marker	CD19-positive ALAL	Case series and case reports, also in combination[98,99,100,101]	NCT04827745 NCT06991920 NCT04872790
CAR-T cell therapy	Targets B-lineage defining marker	CD19-positive ALAL	Not data for ALAL Clinical reports in CD19 positive AML[102,103]	NCT06325748

ALAL-acute leukemia of ambiguous lineage, CAR-T cell- chimeric antigen receptor T-cell

Legend to Figure 1: Diagnosis and classification of ALAL. Pragmatic approach for the diagnosis and classification of ALAL.

*Myeloid lineage is defined by cytoplasmic MPO expression (>50% of mature neutrophils or variable pattern resembling that of normal CD34+ myeloid progenitors). Dim MPO is less specific but gains specificity with high light-scatter or co-expression of CD117/bright CD13/CD33. If MPO is dim/absent, myeloid lineage requires ≥2 monocytic markers (CD11c, CD14, CD36, CD64, lysozyme, or diffuse nonspecific esterase positivity). B-lineage requires CD19 (>50% of normal B progenitors at least in part of the leukaemia cells) plus ≥1 additional B-cell marker (CD10, CD22, CD79a); lower CD19 expression requires ≥2 additional markers. CD79a lacks specificity when T-lineage is in the differential. PAX5 is specific but tentative. Rarely, B-lineage can be established without CD19 if PAX5, CD79a, and CD22 are co-expressed. T-lineage requires cytoplasmic/membranous CD3 (expression >50% of mature T-cells at least on a portion of the leukaemic cells) or by immunohistochemistry, although may not be entirely T-lineage–specific since polyclonal antibodies may react with the CD3 ζ chain (also present in normal NK cells).

Cases of ALAL that do not satisfy the criteria for a specific ALAL category should be classified as ALAL, not otherwise specified (NOS).

ALAL-acute leukemia of ambiguous lineage, MPAL-mixed phenotype acute leukaemia, AUL-acute undifferentiated leukaemia, r- rearrangement

Legend to Figure 2: Frequency of somatic genetic alteration in B/Myeloid MPAL, T/Myeloid MPAL, and sAML-MP.

These are approximate frequency estimations based on data compiled from multiple genomic studies, including small cohorts with notable heterogeneity in age and reported mutation prevalence that may affect frequency (e.g. *ZNF384r* common in paediatric patients; *BCR::ABL1r* more common in adults).

sAML-MP –secondary AML with a mixed phenotype. STM: secondary type mutations (*ASXL1, BCOR, EZH2, SF3B1, SRSF2, STAG2, U2AF1, ZRZS2*; per WHO 5th edition). *EZH2* is also depicted by itself, due to reports of high frequency in T/Myeloid MPAL in some studies.

Legend to Figure 3: Management of patients with ALAL. A pragmatic approach for the workup and management of newly diagnosed adult patients with ALAL.

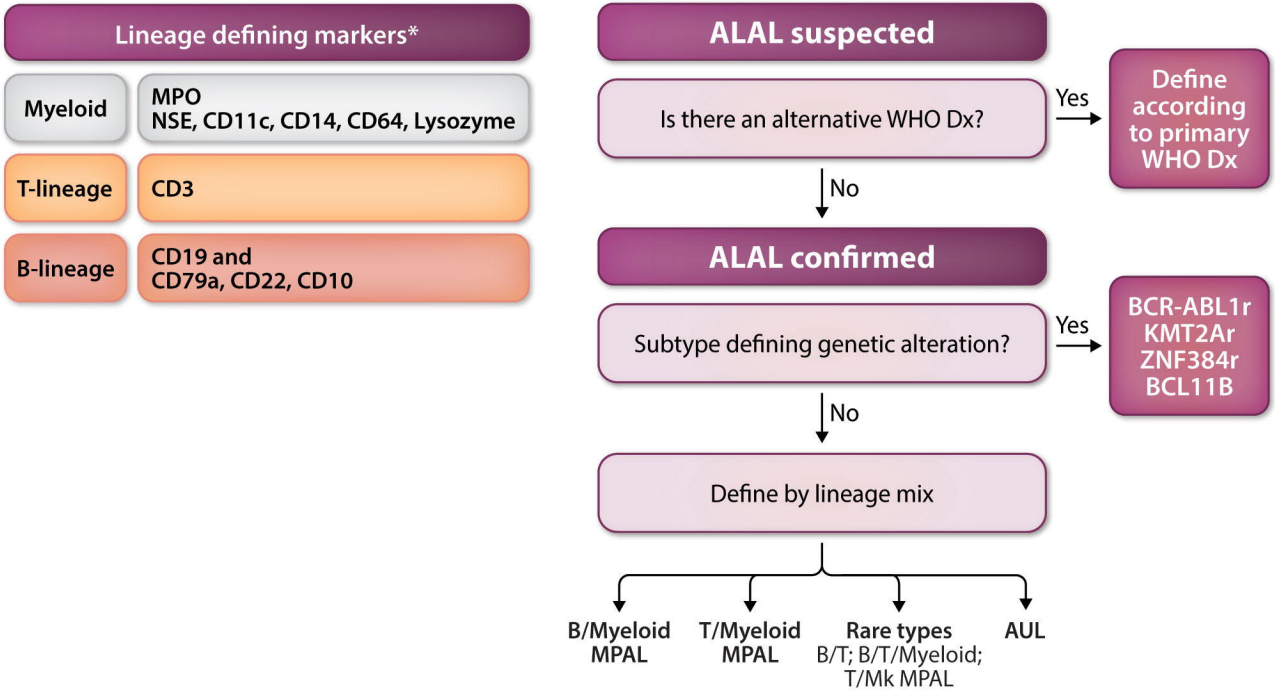
[*]Validated tools for fitness assessment, e.g. comprehensive geriatric assessment

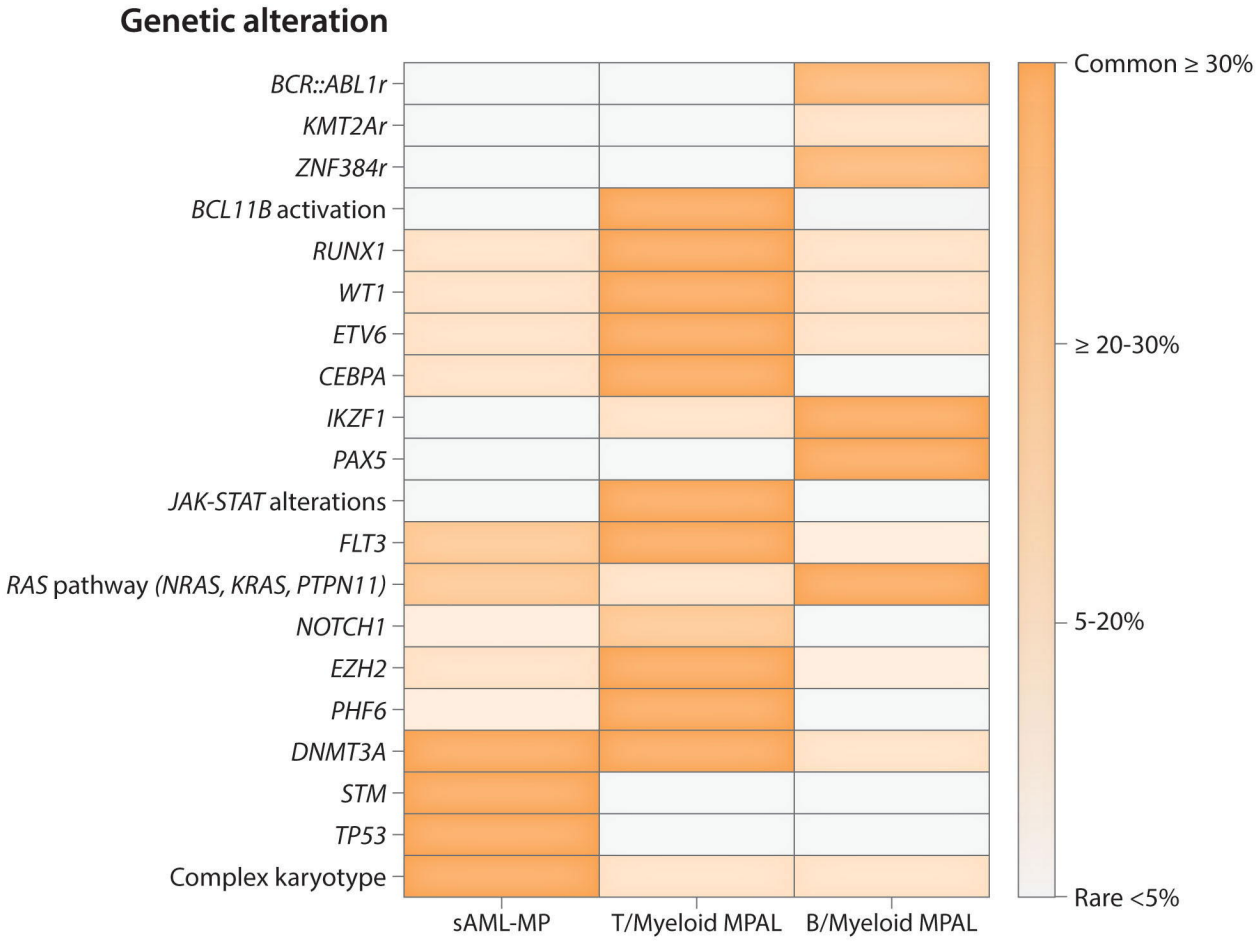
[**]Comorbidity assessment, e.g. Charlson comorbidity index.

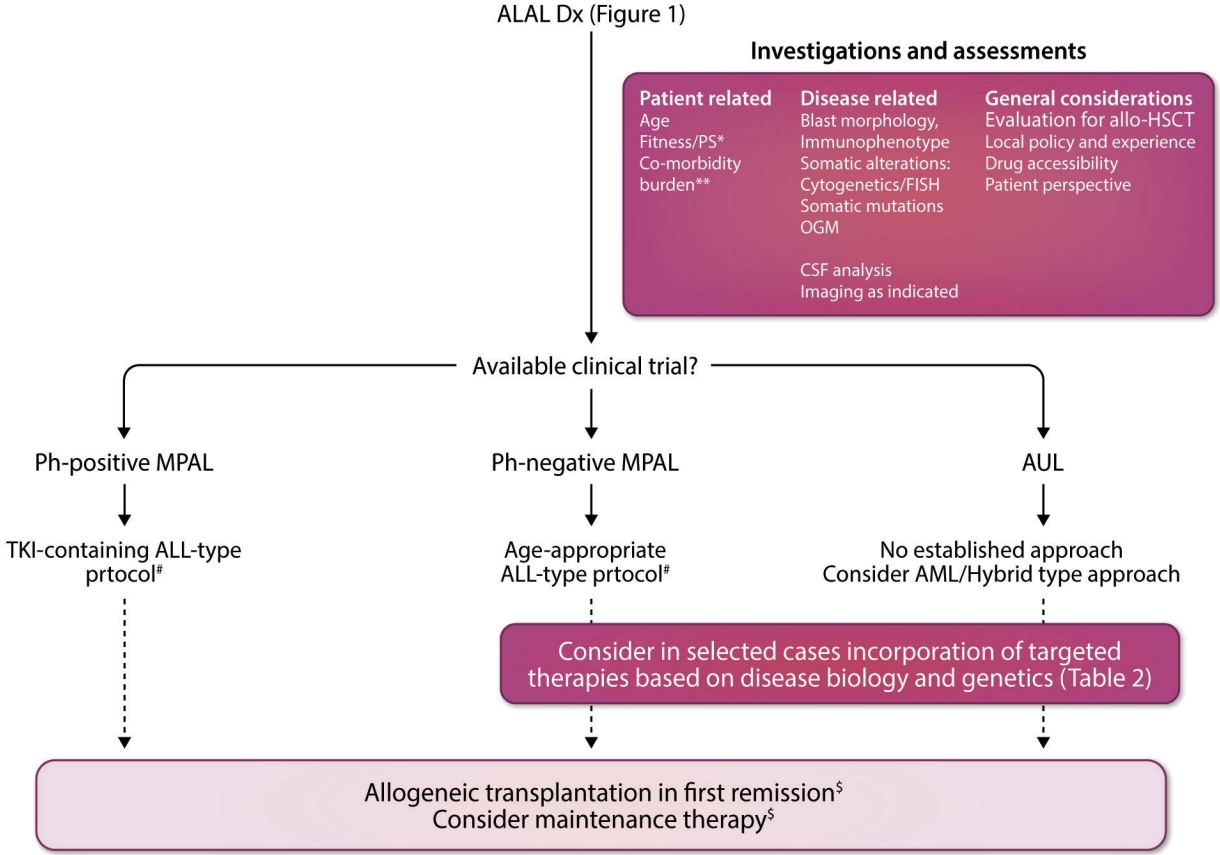
[#]It is extremely important to utilize established age-appropriate ALL protocols that the medical team are familiar and is experienced with.

[$]tools to aid decision include: MRD assessment with multiple methods (Flow-based, RT-PCE, NGS), disease genetic-risk, patient co-morbidity burden and fitness.

ALAL-acute leukaemia of ambiguous lineage, MPAL-mixed phenotype acute leukaemia, AUL – acute undifferentiated leukaemia, r- rearrangement


Lineage defining markers*
Myeloid
MPO
NSE, CD11c, CD14, CD64, Lysozyme
T-lineage
CD3
B-lineage
CD19 and
CD79a, CD22, CD10
ALAL suspected
Is there an alternative WHO Dx?
Yes
Define according to primary WHO Dx
No
ALAL confirmed
Subtype defining genetic alteration?
Yes
BCR-ABL1r
KMT2Ar
ZNF384r
BCL11B
No
Define by lineage mix
B/Myeloid MPAL
T/Myeloid MPAL
Rare types
B/T; B/T/Myeloid; T/Mk MPAL
AUL


Genetic alteration
BCR::ABL1r
KMT2Ar
ZNF384r
BCL11B activation
RUNX1
WT1
ETV6
CEBPA
IKZF1
PAX5
JAK-STAT alterations
FLT3
RAS pathway (NRAS, KRAS, PTPN11)
NOTCH1
EZH2
PHF6
DNMT3A
STM
TP53
Complex karyotype
sAML-MP
T/Myeloid MPAL
B/Myeloid MPAL
Common ≥ 30%
≥ 20-30%
5-20%
Rare <5%


ALAL Dx (Figure 1)
Investigations and assessments
Patient related
Age
Fitness/PS*
Co-morbidity burden**
Disease related
Blast morphology, Immunophenotype
Somatic alterations: Cytogenetics/FISH
Somatic mutations
OGM
CSF analysis
Imaging as indicated
General considerations
Evaluation for allo-HSCT
Local policy and experience
Drug accessibility
Patient perspective
Available clinical trial?
Ph-positive MPAL
Ph-negative MPAL
AUL
TKI-containing ALL-type prtocol#
Age-appropriate ALL-type prtocol#
No established approach
Consider AML/Hybrid type approach
Consider in selected cases incorporation of targeted therapies based on disease biology and genetics (Table 2)
Allogeneic transplantation in first remission$
Consider maintenance therapy$