

# T-lymphoid/myeloid mixed phenotype acute leukemia and early T-cell precursor lymphoblastic leukemia similarities with *NOTCH1* mutation as a good prognostic factor

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Elda Pereira Noronha  
Luísa Vieira Codeço Marques  
Francianne Gomes Andrade  
Ingrid Sardou-Cezar  
Filipe Vicente dos Santos-Bueno  
Carolina Da Paz Zampier  
Eugênia Terra-Granado  
Maria S Pombo-de-Oliveira

Paediatric Haematology-Oncology  
Program, Research Centre, Instituto  
Nacional de Câncer, Rio de Janeiro, RJ,  
Brazil

**Purpose:** T-lymphoid/Myeloid Mixed phenotype acute leukemia (T/M-MPAL) is ambiguous leukemia which overlaps with early T-cell precursor lymphoblastic leukemia (ETP-ALL). We have revisited the immunophenotyping profile of T/M-MPAL and ETP-ALL to identify differences and/or similarities, as these entities represent a therapeutic challenge in clinical practice.

**Patients and methods:** A total of 26 ETP-ALL and 10 T/M-MPAL cases were identified among 857 cases of childhood leukemia (T-ALL, n=266 and AML, n=591) before any treatment decisions. The variables analyzed were age strata, sex, clinical features, immunophenotyping, and molecular aberrations. Immunophenotyping was performed in all samples using a panel of cytoplasm and membrane antibodies to identify the lineage and blast differentiation. The mutational status of *STIL-TAL1*, *TLX3*, *RUNX1*, *NOTCH1*, *FBXW7*, *FLT3*, *IL7R*, *RAS*, *KMT2A*, and *CDKN2A/B* was tested using RT-PCR, FISH, and PCR sequencing methods. The outcomes were assessed in terms of overall survival (OS).

**Results:** The immunophenotypes were similar in ETP-ALL and T/M-MPAL, regarding the cellular expression of CD34, CD117, CD13/CD33, and CD11b, although CD2 and HLA-DR were more frequent in T/M-MPAL ( $p < 0.01$ ). aMPO positivity and myelomonocyte differentiation were definitive in separating both entities. *NOTCH1*, *FLT3-ITD*, and *NKRAS* mutations as well as *TLX3* and *KMT2A* rearrangements were found in both ETP-ALL and T/M-MPAL. Thirty-one patients received ALL protocol whereas five had AML therapy. The overall 5-year survival rate (pOS) was 56.4% for patients treated using ALL protocols. No differences were observed between T/M-MPAL (pOS of 57%) and ETP-ALL (pOS of 56%) patients. The prognostic value of *NOTCH1*<sup>mut</sup> was associated with significantly better OS (pOS 90%) than *NOTCH1*<sup>wt</sup> (pOS 37%) ( $p = 0.017$ ).

**Conclusion:** This research can potentially contribute to *NOTCH1* as targeted therapy and prognostic assessment of T-cell mixed phenotype leukemia.

**Keywords:** T-lymphoid/myeloid mixed phenotype acute leukemia, early T-cell precursor lymphoblastic leukemia, *NOTCH1* mutations

Correspondence: Maria S Pombo-de-Oliveira  
Paediatric Haematology-Oncology  
Research Program, Research Centre,  
Instituto Nacional de Câncer - INCA, Rua  
André Cavalcanti, 37, Rio de Janeiro/RJ  
20231-050, Brazil  
Tel +55 213 207 6532  
Email mpombo@inca.gov.br

## Introduction

Acute leukemia (AL) are heterogeneous diseases in which the precise diagnosis characterization of either B-, T-lymphoid or and myeloid lineage is essential to treatment strategy and outcome success. Since the advent of integrative diagnosis of

leukemia applying broad cell immunophenotype, karyotype and molecular profiles, unusual AL cases with mixed phenotype have been described.<sup>1</sup>

In the recent World Health Organization (WHO) classification, T/myeloid mixed phenotype acute leukemia (T/M-MPAL) and B/myeloid mixed phenotype acute leukemia (B/M-MPAL) are distinct entities within the same category of leukemia of ambiguous cell lineage. Even with myeloid markers, the early-T-cell precursor acute lymphoblastic leukemia (ETP-ALL) is not classified by the WHO as leukemia of ambiguous lineage.<sup>2</sup> ETP-ALL is classified under T-ALL groups with the strong argument that the behavior of ETP-ALL is like other T-ALL in the most recent children's oncology group trials and in some adult studies.<sup>3,4</sup> Recently, new leukemia of ambiguous cell lineage has been identified. It has been proposed that acute myeloid/T-lymphoblastic leukemia, a specific subset of minimally differentiated myeloid leukemia with T-cell receptor gene rearrangements (*TCR-r*), should be included in the WHO classification as a diagnostic entity with a distinct molecular profile compared to ETP-ALL and T/M-MPAL.<sup>5</sup>

In clinical practice, T/M-MPAL and ETP-ALL cases remain a therapeutic challenge. The genomic profiles of ETP-ALL and T/M-MPAL have demonstrated that both diseases might have gene aberrations with potential to target therapy.<sup>6,7</sup> It is important to highlight that B/M-MPAL, when associated with *BCR-ABL1* and *KMT2A/AFF1* genetic abnormalities, determines the treatment strategy.<sup>2</sup> The aim of this study was to compare the similarities in immunophenotyping, molecular aberrations, and outcomes in a series of ETP-ALL and T/M-MPAL cases and validate an algorithm of molecular tests at diagnosis to drive prognostic assessment and therapeutic decisions.

## Patients and methods

### Patients

Diagnostic tests obtained from bone marrow (BM) aspirates and/or peripheral blood samples sent to the Pediatric Haematology–Oncology Program, Research Centre, Instituto Nacional de Cancer, Rio de Janeiro, Brazil, before any treatment decisions, were revisited (2005–2017). Forty-four cases involving AL with an ambiguous phenotype were selected among 857 cases of childhood leukemia (only T-ALL, n=266 and AML, n=591 were included), in which immunophenotyping and molecular tests were performed. The exclusion criteria were

B-cell precursor ALL and cases lacking the complete panel of monoclonal antibodies to compare ETP-ALL and T/M-MPAL. Cases of AL with *BCR-ABL1* were also excluded.

The European Group for the Immunological Classification of Leukemias criterion<sup>8</sup> was used from 2005 to 2010, and all cases with an ambiguous phenotype were reviewed using the WHO classification.<sup>2</sup> The definition of ETP-ALL was based on criteria described by Coustan-Smith et al, (2009)<sup>9</sup> in all incident T-ALL cases with ambiguous phenotype since the year 2010. The scoring system proposed by Inukai et al, (2012)<sup>10</sup> was applied in all cases reviewed in this study. Minimally differentiated AML (AML-M0) with CD7 positivity without TCR rearrangements was included only for comparisons with immunophenotyping. The referring physicians provided an update of the patients' follow-up.

### Multiparametric flow cytometry

Multiparametric flow cytometry (M-FCM) tests were performed in all samples. The first screening panel was cytoplasmic (cy) CD3, cyCD79/CD22, aMPO, CD7, and CD19 antibodies (MoAb) to identify the leukemia lineage. Then, in samples positive for cyCD3 (T-cell lineage), CD7 and aMPO (Myeloid lineage), a panel of MoAb with T-cell, myeloid and stem cell-associated membrane antigens was applied. The six-color conjugated-MoAbs were performed as described in the supplemental material (Table S1). Samples were acquired in a FACS Canto Flow cytometry (Becton, Dickinson and Company, CA, USA). Data analyses were performed by Infinicyt version 1.8 (Cytognos, Salamanca, Spain) Software.

The analyses were first evaluated in a blast gate identified by CD45<sup>low/intermediate</sup> expression, then, followed by intracytoplasmic and membrane analysis of blast gate according to MoAb panel (Figure S1).

### Molecular tests

Samples were first subjected to DNA and RNA extraction using commercial kits. Genomic DNA and total RNA were isolated from mononuclear cells from BM samples with the QIAamp® DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) or the TRIzol™ reagent kit (Invitrogen, Carlsbad, USA), respectively; 2–2.5 µg of RNA were reverse transcribed using First-Strand cDNA Synthesis Kit™ (Amersham Pharmacia Biotech Inc., NJ, USA). The integrity of RNA and cDNA was examined by amplifying a fragment of the *GAPDH* gene.

The *STIL-TAL1*, *RUNX1-RUNX1T1*, *CBF $\beta$ -MYH11*, *BCR-ABL1*, and the presence of the *TLX3* transcript were assessed by the reverse transcriptase-polymerase chain reaction technique using primers and conditions as previously described.<sup>11,12</sup> *KMT2A* rearrangements (*KMT2A-r*) including fusion partners *AFF1* (*AF4*), *MLLT3* (*AF9*), *MLLT10* (*AF10*), *MLLT1* (*ENL*), and *MLLT4* (*AF6*), were identified by RT-PCR according to previous publications.<sup>13</sup> Additional fluorescence in situ hybridization with commercial DNA probes (*MLL* Dual Color Break Apart Rearrangement Probe; Cytocell, UK) was performed to identify *KMT2A-r* before RT-PCR to identify selected partner genes.

Mutational status of *NOTCH1*, *FBXW7*, *FLT3*, *IL7R*, *RAS*, and *CDKN2A/B* deletions was tested throughout direct sequencing according to methods previously described.<sup>14–16</sup>

## Treatments

Patients were not enrolled in any clinical trials, but received treatments either according to the Brazilian Group for Treatment of Childhood Lymphoblastic Leukemia (GBTLI-ALL 99/09) or to ALL Berlin-Frankfurt-Münster (BFM-ALL 95 and 2002) protocols.<sup>17,18</sup> Patients were assigned into the group of a high risk of relapse, according to age strata, leukocyte count ( $>50 \times 10^9/L$ ), and T-cell phenotype of both protocols. In summary, all patients received a 4-week induction phase, which included prednisone, vincristine, doxorubicin, L-asparaginase, intrathecal Methotrexate (MTX), Cytarabine (Ara-C), and dexamethasone (MADIT). In the GBTLI-ALL, the consolidation phase, Cyclophosphamide, Ara-C, and 6-mercaptopurine (6-MP) were included. Intensification treatment with MTX  $2 \text{ g/m}^2 \times 4$ , 6-MP and MADIT and maintenance of 6-MP, MTX, vincristine, dexamethasone, and MADIT. Patients with more than 5% blast cells in their cerebrospinal fluid after day 14 of induction therapy have received cranial radiotherapy (1200 Gy during the late consolidation phase).<sup>18</sup>

## Statistical analysis

To compare the distribution of categorical variables in leukemia subtypes with the Pearson Chi-square test. When the expected count in at least one cell of the table was less than five, the Fisher's exact test (two-tailed) was used. Cases were grouped in T/M-MPAL and ETP-ALL,

and variables analyzed were age range, sex, clinical features, immunophenotyping, and molecular markers.

Overall survival (OS) was measured from the date of diagnosis to the date of last follow-up or death from any cause. Patients who did not experience an event and/or lost to follow-up were censored. The Kaplan–Meier survival analysis method was used to calculate the 5-year OS. The estimated survival values in ETP-ALL and T/M-MPAL were compared using the log-rank test. *p*-values  $<0.05$  were considered statistically significant.

All analyses were performed using SPSS 21.0 (SPSS, Chicago, IL, USA, 2004).

## Results

The demographic and clinical-biological features of ETP-ALL and T/M-MPAL can be seen in Table 1. Forty-four selected cases were characterized as ETP-ALL ( $n=26$ ), T/M-MPAL ( $n=10$ ), and AML-M0 with CD7 positivity ( $n=8$ ), with equal frequency distribution of variables in each group. Five cases were treated in accordance with the AML BFM-2004 protocol (ETP-ALL,  $n=2$ ; T/M-MPAL,  $n=3$ ) and 31 cases were treated using ALL high-risk protocols. The AML-M0 cases were CD34/CD7<sup>pos</sup> and negative for CD41/CD61, cyCD3, and mCD3, in contrast to the ETP-ALL and T/M-MPAL cases (Table S2). AML-M0 cases positive for CD7 were included in comparisons between ETP-ALL and T/M-MPAL immunophenotype profiles only. AML-M0 positive for CD7 were cCD3 negative and *TCR*, *NOTCH1*, *FLT3*, and *RAS* wild-type.

The summary of the immunophenotyping and molecular analysis results for ETP-ALL and T/M-MPAL cases ( $n=36$ ) is shown in Figure 1. Detailed M-FCM results for aberrant phenotypes are shown in the supplemental material (Table S2). CD34, CD117, CD13/CD33, and CD11b had similar frequency distributions in both groups, whereas CD2 and HLA-DR were more frequent in the T/M-MPAL group ( $p<0.01$ ); cyCD3 was present in more than 30% of blast cells, and aMPO was found in more than 20% in T/M-MPAL. Seventeen ETP-ALL cases were previously diagnosed using the criteria initially suggested by Coustan-Smith et al,<sup>9</sup> and nine additional cases were identified by applying the scoring system proposed by Inukai et al.<sup>10</sup> Four cases (#29, #33, #34, #36), classified as T/M-MPAL, had scores that would classify them as ETP-ALL. In these cases, aMPO expression and/or blast cells with myelomonocyte differentiation markers were used to distinguish between T/M-MPAL and ETP-ALL, an

**Table 1** Demographic and main clinical-laboratorial features of early T-cell precursor-ALL, T-lymphoid/myeloid mixed-phenotype acute leukemia, 2005–2017, Brazil

	Total n (%)	ETP-ALL n (%)	MPAL T/Myeloid n (%)	p-value*
Age (yrs)				
<10	17 (47.2)	10 (38.5)	7 (70)	0.139
10–18	19 (52.8)	16 (61.5)	3 (30)	
Sex				
Males	23 (63.9)	16 (61.5)	7 (70)	0.716
Females	13 (36.1)	10 (38.5)	3 (30)	
Ethnicity/Skin color				
White	10 (27.8)	8 (30.8)	2 (20)	0.689
Non-White	26 (72.2)	18 (69.2)	8 (80)	
WBC ( $\times 10^9/L$ )				
<50	17 (47.2)	12 (46.2)	5 (50)	1.000
$\geq 50$	19 (52.8)	14 (53.8)	5 (50)	
Mediastinal mass				
Yes	11 (30.6)	7 (26.9)	4 (40)	0.454
No	25 (69.4)	19 (73.1)	6 (60)	
CNS infiltration				
Yes	3 (8.6)	2 (8.0)	1 (10)	1.000
No	32 (91.4)	23 (92)	9 (90)	
Hepatomegaly				
Yes	19 (52.8)	15 (57.7)	4 (40)	0.463
No	17 (47.2)	11 (42.3)	6 (60)	
Splenomegaly				
Yes	22 (61.1)	16 (61.5)	6 (60)	1.000
No	14 (38.9)	10 (38.5)	4 (40)	
Lymph nodes				
Yes	27 (75)	21 (80.8)	6 (60)	0.226
No	9 (25)	5 (19.2)	4 (40)	
Treatment				
ALL	31 (86.1)	24 (92.3)	7 (70)	-
AML	5 (13.9)	2 (7.7)	3 (30)	
Total	36 (100)	26 (100)	10 (100)	

**Notes:** \*p-value calculated by Pearson Chi-Square or Exact Fisher test comparing all subtypes.

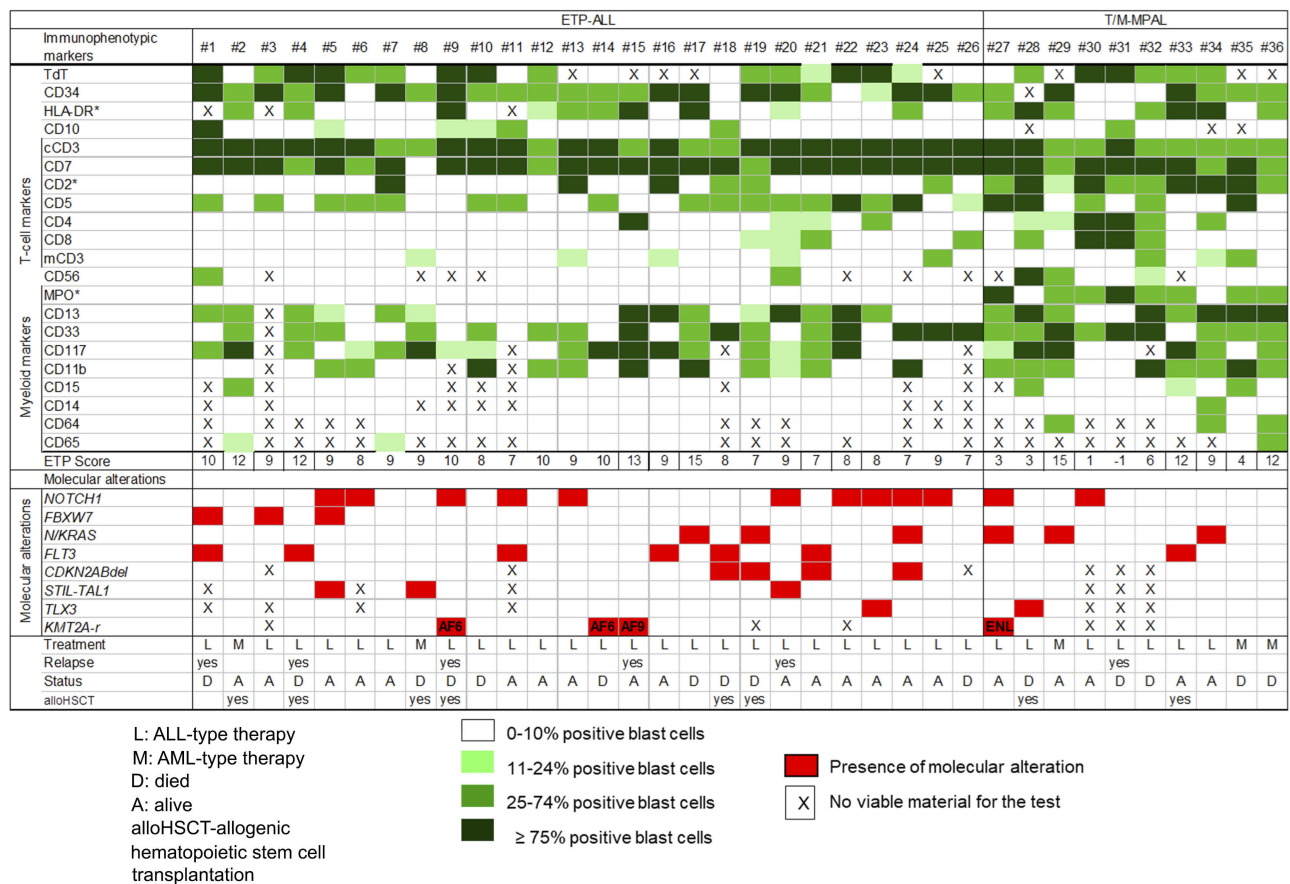
**Abbreviations:** ETP-ALL, early T-cell precursor acute lymphoblastic leukemia; Yrs, years; WBC, white blood cell count; CNS, central nervous system; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia.

approach that enhances the guidelines provided in the WHO criteria for MPAL.

In the current study, *NOTCH1*, *FLT3-ITD*, and *N/KRAS* mutations as well as *TLX3* and *KMT2A* rearrangements were found in both ETP-ALL and T/M-MPAL cases, whereas *FBXW7*<sup>mut</sup>, *CDKN2A*<sup>del</sup>, and *STIL-TAL1* were only found in ETP-ALL cases (Figure 1). *KMT2A-r* was present in 4/29 cases (13.8%), with two *MLLT4* (*AF6*) and one *MLLT3* (*AF9*) partner genes among the ETP-ALL

cases and one *MLLT1* (*ENL*) partner gene in the T/M-MPAL case. In our cohort, *NOTCH1*<sup>mut</sup> was the most frequent molecular aberration found in cases with an ambiguous phenotype (ETP-ALL, 38.5%; T/M-MPAL, 20%;  $p=0.438$ ), following *FLT3*<sup>mut</sup> (ETP-ALL, 23.1%; T/M-MPAL, 10%;  $p=0.645$ ) and *RAS*<sup>mut</sup> (ETP-ALL, 11.5%; T/M-MPAL, 30%;  $p=0.317$ ).

There were no differences in patient characteristics such as age strata, sex, white blood cell count (WBC) at



**Figure 1** Immunophenotypic and molecular profile of ETP-ALL and T/M-MPAL, Brazil, 2005–2017. The columns represent each patient. \*Most frequent markers in T/M-MPAL,  $p < 0.01$ .

**Abbreviations:** ETP-ALL, early T-cell precursor acute lymphoblastic leukemia; T/M-MPAL, T-lymphoid/myeloid mixed phenotype acute leukemia; mut, mutated; del, deleted; r, rearranged.

diagnosis and molecular alteration, with the exception of the NOTCH1 mutation ( $p=0.017$ ) as shown in Table 2. Regarding treatment regimen given, 5 out of 36 patients were treated using AML protocols (two ETP-ALL, three T/M-MPAL patients) and they were excluded from the survival analysis by protocol treatment due to small sample size. In four cases (one ETP-ALL and three T/M-MPAL), complete remission was not achieved and the disease progressed until the patients' death; one ETP-ALL (#2) was alive in the follow-up after undergoing allogeneic hematopoietic stem cell transplantation (alloHSCt). Thirty-one patients were treated initially with ALL protocols (ETP-ALL,  $n=24$ ; T/M-MPAL,  $n=7$ ). The overall 5-year survival rate of the 36 patients was 52.3%, mean/months was  $37.7 \pm 4.54$  and confidence intervals of 28.8–46.6 (Table 2). Eight patients underwent alloHSCt, being ETP-ALL ( $n=6$ ) and T/M-MPAL ( $n=2$ ) in the first relapse. Death occurred for six patients (ETP-ALL ( $n=5$ ) and T/M-MPAL ( $n=1$ ) after alloHSCt

due to relapse ( $n=2$ ) and toxicity ( $n=4$ ). Data are summarized in Figure 1.

The overall 5-year survival rate was 56.4% for 31 patients treated using ALL protocols. No differences were observed between T/M-MPAL (pOS of 57%) and ETP-ALL (pOS of 56%) patients (Figure 2A). As *NOTCH1*<sup>mut</sup> was the most frequent molecular aberration in the cohort and because the treatment administered was approximately the same in most cases, the prognostic value of *NOTCH1* status was tested. *NOTCH1*<sup>mut</sup> was associated with significantly better OS (pOS of 90%) compared to *NOTCH1*<sup>wt</sup> (pOS of 37%) ( $p=0.017$ ) (Figure 2B).

## Discussion

ETP-ALL and T/M-MPAL are closely related entities when it comes to immunophenotyping, genomic alterations, and outcomes.<sup>19</sup> Therefore, these entities are a diagnostic and therapeutic challenge owing to their

**Table 2** Overall survival of the T-lymphoid/myeloid mixed phenotype acute leukemia and early T-cell precursor lymphoblastic leukemia, 2005–2017, Brazil

Variables	pOS % (SE)	Mean* (SE)	95%CI	p-value
Age (years)				
<10	34.5 (0.15)	32.9 (6.53)	(20.12–45.7)	0.106
≥10	72.3 (0.12)	46.3 (6.04)	(34.4–58.1)	
Sex				
Males	55.4 (0.13)	41.1 (5.56)	(30.2–52.4)	0.903
Females	56.8 (0.17)	39.4 (8.23)	(23.3–55.6)	
WBC x10 <sup>9</sup> /L				
<50	63.3 (0.15)	44.8 (6.30)	(32.5–57.1)	0.510
≥50	51.3 (0.14)	37.6 (6.41)	(25.1–50.2)	
Subtypes				
ETP-ALL	56 (0.12)	40.7 (5.36)	(30.2–51.3)	0.996
T/M-MPAL	57 (0.19)	40.6 (8.91)	(23.1–58)	
Molecular Alterations				
<i>NOTCH1</i> <sup>mut</sup>	90.0 (0.95)	55.7 (4.10)	(47.7–63.7)	0.017
<i>NOTCH1</i> <sup>WT</sup>	36.9 (0.13)	32.0 (5.88)	(20.5–43.5)	
<i>N/KRAS</i> <sup>mut</sup>	53.3 (0.25)	38.9 (11.42)	(16.5–61.3)	0.671
<i>N/KRAS</i> <sup>wt</sup>	57.3 (0.11)	41.3 (4.95)	(31.5–51.0)	
<i>FLT3</i> <sup>mut</sup>	60.0 (0.22)	48.4 (6.36)	(35.9–60.9)	0.417
<i>FLT3</i> <sup>wt</sup>	55.0 (11.4)	38.1 (5.62)	(27.1–49.1)	
ALL treatment (n=31)	56.4 (0.10)	40.7 (5.60)	(31.7–49.7)	
Overall cases (n=36)**	52.3 (0.09)	37.7 (4.54)	(28.8–46.6)	

**Notes:** \*Mean survival in months; \*\*Including five patients treated with AML protocol.

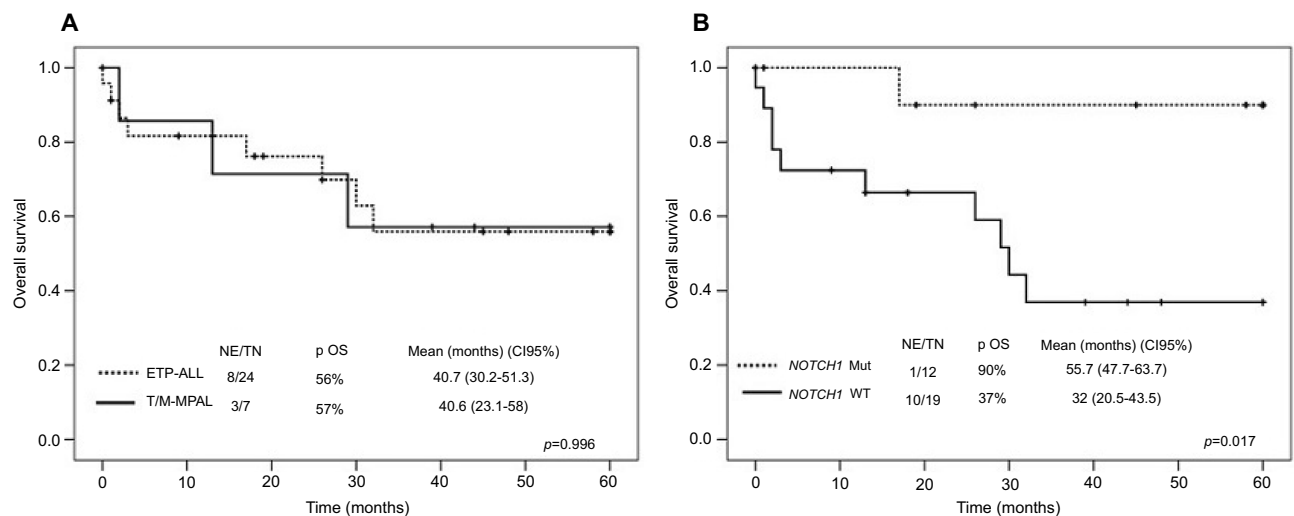
**Abbreviations:** pOS, probability of overall survival; SE, standard error; CI, confidence interval, mut, mutated; WT, wild type.

heterogeneity with ambiguous phenotypes and overlapping genomic alterations.

Since childhood ETP-ALL cases can be classified as T/M-MPAL, we revisited the immunophenotype and molecular on a cohort of childhood AL ambiguous phenotypes to test the value of identifying differences or similarities between

these diseases in clinical outcomes. Hence, the first step of this study was to determine the methodological criteria to improve the identification of each type. In our cohort, by revisiting the M-FCM, we identified three possible diagnostic categories: ETP-ALL, T/M-MPAL, and AML-M0 with CD7 expression. We were able to demonstrate 9.7% of ETP-ALL cases amongst T-ALL, and prevalence in patients older than 10 years of age at diagnosis. The frequency may be lower than previously reported (11% rather than 17%).<sup>9,20,21</sup> However, this aspect should take into account that because the complete immunophenotyping was not prospectively performed, especially regarding ETP-ALL identification. The exclusion of cases as probable ETP-ALL (CD1a not available, CD5<sup>weak</sup>, CD4-/CD8- with myeloid antigens expression) could explain the lower ETP-ALL case in our T-ALL cohort. Another point is that the high frequency of ETP-ALL reported internationally was observed in children and adults up to 25 years old,<sup>4,20</sup> whereas our T-ALL cohort is predominantly of children under 18 years of age. It has been demonstrated that the first identification of ETP-ALL based on Coustan-Smith criteria could significantly underestimate ETP-ALL patients with leukemia with ambiguous phenotype.<sup>9,22,23</sup> Applying the scoring system proposed by Inukai et al,<sup>10</sup> we were able to characterize nine ETP-ALL cases, adding them to the previous cases identified by Coustan-Smith et al, criteria<sup>9</sup> and 10 T/M-MPAL newly diagnosed cases.

Because ETP-ALL and T/M-MPAL share cell antigens which characterize T- and myeloid phenotypes, we wanted to test if molecular aberrations frequently found in T-ALL and AML would help in the distinction of ETP-ALL from T/M-MPAL in our cohort. We observed that *STIL-TAL1*, *FBXW7* mutation and *CDKN2A/B* deletion were found only in ETP-ALL, while *NOTCH1*, *FLT3*, *N/KRAS* mutations, *TLX3-r*, and *KMT2A-r* were present in both T/M-MPAL and ETP-ALL cases. The frequencies of *NOTCH1* mutations in ETP-ALL vary worldwide according to series of cases. For instance, Zhang et al, (2012)<sup>6</sup> found a lower frequency of *NOTCH1* mutations (16%) compared to our series. On the other hand, Zuurbier et al, (2014)<sup>23</sup> found very high frequencies of *NOTCH1* mutations among ETP-ALL (69%). Alexander et al, (2018)<sup>19</sup> found 13 out of 19 ETP-ALL with *NOTCH1* mutations (68%). In addition, mutations in *NOTCH1* were described frequently in T/M-MPAL.<sup>7,19,24</sup> The similarities between T/M-MPAL and ETP-ALL cases regarding *KMT2A-r*, *FLT3*, *N/KRAS*, and *NOTCH1* mutations seem to share genomic aberrations with AML.<sup>7,24–26</sup> Kotrova et al, have demonstrated in ambiguous lineage



**Figure 2** Five-year survival of early T-cell precursor ALL and T/M Mixed phenotype acute leukemia. **(A)** Overall survival of ETP-ALL and M/T-MPAL. **(B)** Overall survival of ETP-ALL and T/M-MPAL according to *NOTCH1* status.

**Abbreviations:** ETP-ALL, early T-cell precursor acute lymphoblastic leukemia; T/M-MPAL, T-lymphoid/myeloid mixed phenotype acute leukemia; Mut, Mutated; WT, Wild Type; NE, number of events; TN, Total number; OS, overall survival; CI, confidence interval.

leukemia with distinct T and M-blast populations the presence of typical T-ALL mutations that activate the *NOTCH1* signaling pathway.<sup>27</sup> In mice, aberrations such as *KMT2A-ENL* and *KRASG12D* cooperate to the rise of both AML and T-ALL.<sup>28</sup> We have found one T/M-MPAL case with *KMT2A-ENL*, *KRAS* and *NOTCH1* mutation that may be more consistent with Ugale's animal model.<sup>28</sup>

The genomic landscape of ETP-ALL has already been described, but only very recently have the genetic basis and differences between T/M-MPAL been deeply explored with the distinct profiles of ambiguous lineage leukemia.<sup>6,19,29</sup> Authors showed that ETP-ALL and T/M-MPAL are genomically similar, characterized by mutations in regulators of hematopoietic stem cell, although, variation in mutations within the transcription factor gene have been observed. For example, the aberrations of core transcription factors that drive T-cell (eg, *TALI*, *TLX1*) were less frequent in T/M-MPAL.<sup>19</sup> Our findings validate this previous study, regarding *NOTCH1* and *RAS* mutations found in T/M-MPAL.<sup>19</sup> *FLT3* mutated were not found at similar frequencies in T/M MPAL and ETP-ALL.

Recently, in an international study of leukemia with B/M-MPAL, the event-free survival of patients treated with ALL-type therapy was demonstrated to be superior to those who received AML-type, or with combined-type treatment.<sup>30,31</sup> Our data regarding T/M-MPAL are aligned with the findings of Hrusak et al<sup>31</sup>.

In our cohort, five patients were treated as AML with a dismal outcome as opposed to cases that were treated

based on ALL-therapy [(56.4% (31.7–49.7)]. The overall survival of patients with ETP-ALL and T/M-MPAL in our cohort was similar to the results obtained in ETP-ALL patients treated according to AIEOP-BFM 2009 [(55.6% (28.6–75.9)].<sup>21</sup> In the AIEOP centers with AIEOP-BFM protocols, the accuracy of the ETP-ALL diagnosis was confirmed by the score proposed by Inukai et al<sup>10</sup>. Conversely, the ETP-ALL enrolled into the MRC UK-ALL 2003 trial had an OS [82.4% (69.7–95.1%)] in a 5-year followup.<sup>20</sup>

A noteworthy limitation of our study is the sample size, which is to be expected with a rare disease, but which limits the interpretation of the data, particularly the outcome between ETP-ALL and T/M-MPAL compared with other determinants, such as age, sex, WBC, minimal residual disease and *NOTCH1*, *RAS*, *FLT3*, and *KMT2A-r*. The outcome in patients treated either with chemotherapy alone or with both chemotherapy and alloHSCT was not performed due to the sample size.

One strong finding in our study was to demonstrate that ETP-ALL and T/M-MPAL, irrespective of similarities or differences, have *NOTCH1* mutations as a good predictive responsive treatment in both ambiguous leukemia subtypes. The presence of *NOTCH1* mutations was associated with significantly better OS.

## Conclusion

In conclusion, we demonstrated that ETP-ALL and T/M-MPAL are closely related in terms of

immunophenotyping, genetic aberrations, and outcomes. In both diseases, genetic mutations were in *NOTCH1*, *FLT3*, *RAS*, and *KMT2A-r*, which should be taken into account for potential innovative therapies in front-line treatments. This is especially true regarding *NOTCH1* mutations for prognostic assessment and risk-stratification of these cases.

## Ethics approval and informed consent

Patients were treated in accordance with the Declaration of Helsinki medical ethical standards. The Research Ethical Committee of the Instituto Nacional de Cancer, Rio de Janeiro, Brazil approved the study (CEP/INCA#117/12; CEP-CONEP: PB #888.277). A parent or legal guardian of all children and adolescent have provided the written informed consent for samples tests.

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## Author contributions

All authors contributed toward data analysis, drafting and revising the paper, gave final approval of the version to be

published and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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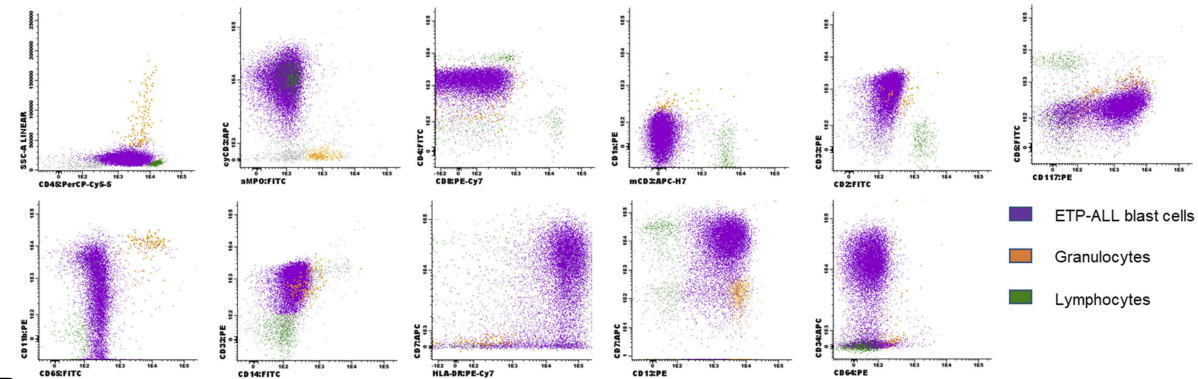
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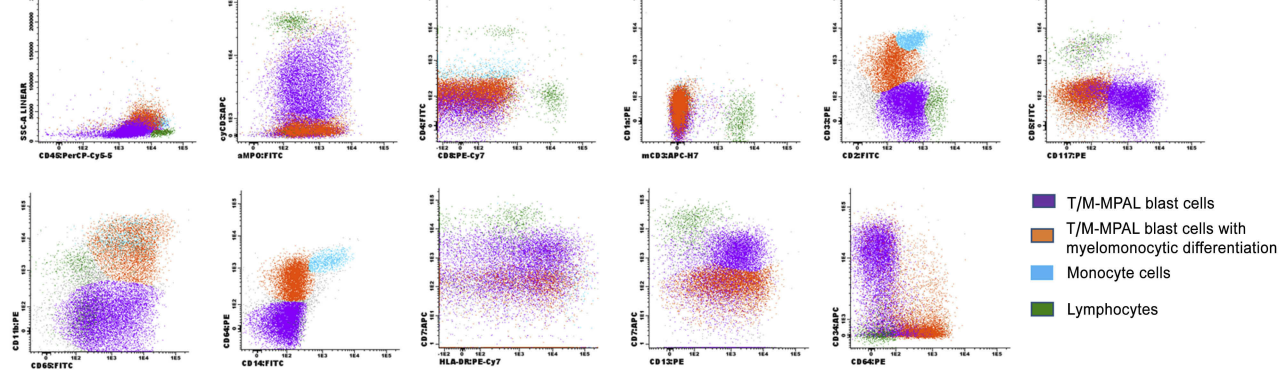
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# Supplementary Materials

**A**



**B**



**Figure S1** Immunophenotyping of Early T-cell precursor ALL (ETP-ALL) and T-lymphoid/myeloid mixed phenotype acute leukemia (T/M-MPAL). **(A)** ETP-ALL with blast cells positive for cyCD3, CD4, CD33, CD117, CD11b, CD33, CD7, HLA-DR, CD34, CD13 and negative for aMPO, CD8, CD1a, mCD3, CD2, CD5, CD64. **(B)** T/M-MPAL with two distinct blast populations, one with positivity to aMPO, cyCD3, CD2, CD33, CD117, CD65, CD11b, CD64, HLA-DR, CD7, CD13, CD34; a second blast population negative for cyCD3, CD5, CD1a, mCD3, CD4, and CD8.

**Abbreviations:** Cy, cytoplasmic; m, membrane.

**Table S1** Panel of monoclonal antibodies and fluorochromes used and revisited in the diagnostic panel

	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7
First round MoAb	aMPO	cyCD22/CD79a	CD45	CD19	cyCD3	
Complementary MoAb	CD7	mCD3	CD45	CD8	CD34	mCD3
	CD2	CD1a		CD117	CD7	
	CD4	CD7		CD56	CD11b	
	CD5	CD10		CD10	CD34	
	CD14	CD11b		HLA-DR		
	CD15	CD13				
	CD16	CD19				
	CD42	CD33				
	CD65	CD34				
	CD61	CD41				
	TCR $\gamma\delta$	CD64				
	HLA-DR	TCR $\alpha\beta$				
	TdT					

**Abbreviations:** MoAb, monoclonal antibodies; cy, cytoplasmic; m, membrane.

**Table S2** Summary of the immunophenotypic profile of acute leukemia with aberrant phenotypes, Brazil, 2005–2017

	<b>Total Np/Nt (%)</b>	<b>T/M-MPAL Np/Nt (%)</b>	<b>ETP-ALL Np/Nt (%)</b>	<b>AML-M0 Np/Nt (%)</b>	<b>p-value*</b>
CD34	35/43 (81.4)	6/9 (66.7)	22/26 (84.6)	7/8 (87.5)	0.435
HLA-DR	23/40 (57.5)	8/9 (88.9)	8/23 (34.8)	7/8 (87.5)	<0.01
CD117	26/38 (68.4)	5/9 (55.6)	15/21 (71.4)	6/8 (75)	0.626
TdT	24/34 (70.6)	6/7 (85.7)	15/21 (71.4)	3/6 (50)	0.367
CD10	7/41 (17.1)	1/7 (14.3)	5/26 (19.2)	1/8 (12.5)	0.886
aMPO	9/44 (20.5)	8/10 (80)	0/26 (0)	1/8 (12.5)	<0.001
CD11b	19/38 (50)	7/10 (70)	11/22 (50)	1/6 (16.7)	0.118
CD13/CD33	39/42 (92.9)	10/10 (100)	21/24 (87.5)	8/8 (100)	0.298
CD14	1/30 (3.3)	1/9 (11.1)	0/13 (0)	0/8 (0)	0.299
CD15	4/32 (12.5)	1/7 (14.3)	1/17 (5.9)	2/8 (25)	0.398
CD56	6/33 (18.2)	2/9 (22.2)	2/17 (11.8)	2/7 (28.6)	0.678
T-cell markers					
cyCD3	36/44 (81.8)	10/10 (100)	26/26 (100)	0/8 (0)	<0.001
CD7	43/44 (97.7)	10/10 (100)	23/24 (95.8)	8/8 (100)	0.702
CD1a	1/38 (2.6)	1/10 (10)	0/26 (0)	0/2 (0)	0.263
CD2	17/38 (44.7)	9/10 (90)	8/26 (29.2)	0/2 (0)	<0.01
CD5	21/38 (55.3)	5/10 (50)	16/26 (61.5)	0/2 (0)	0.223
mCD3	4/44 (9.1)	3/10 (30)	1/26 (3.8)	0/8 (0)	0.031
CD4	5/41 (12.2)	3/10 (30)	1/26 (3.8)	1/5 (20)	0.085
CD8	7/41 (17.1)	4/10 (40)	3/26 (11.5)	0/5 (0)	0.070

**Notes:** Number of cases positive/number of total cases tested. Cases were considered positive when expressed  $\geq 20\%$  in blast cells. \*p-value calculated by Exact Fisher test comparing T/M- MPAL, ETP-ALL, and AML-M0.

**Abbreviations:** T/M-MPAL, T-lymphoid/myeloid mixed phenotype acute leukemia; ETP-ALL, early T-cell precursor acute lymphoblastic leukemia; AML-M0, minimally differentiated acute myeloid leukemia; Np, number of positive cases; Nt, number of total tested; cy, cytoplasmatic; m, membrane.

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