CD371-positive pediatric B-cell acute lymphoblastic leukemia: propensity to lineage switch and slow early response to treatment

Article title

CD371+ pediatric B-cell acute lymphoblastic leukemia: propensity to lineage switch and slow early response to treatment

Running title

CD371 in pediatric B-cell ALL~~acute lymphoblastic leukemia~~

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Key points

- Pediatric CD371-positive B-precursor acute lymphoblastic leukemia shows transient lineage switch and slow early response to treatment.
- Accurate immunophenotypic identification of lineage switch is mandatory to properly assess MRD by flow-cytometry.

Abstract

In the effort to improve immunophenotyping and minimal residual disease (MRD) assessment in acute lymphoblastic leukemia (ALL), the international Berlin-Frankfurt-Münster (iBFM) Flow Network introduced the myelomonocytic marker CD371 in 2014, for a large prospective characterization with a long follow-up. In the present study, we aimed to investigate the clinical and biological features of CD371-positive (CD371^{pos}) pediatric BCP-ALL. From June 2014 to February 2017, 1812 pediatric patients with newly diagnosed BCP-ALLs enrolled in trial AIEOP-BFM ALL 2009 were evaluated as either screening (n=843, Italian centers) or validation cohort (n=969, other iBFM centers). Laboratory assessment at diagnosis consisted of morphological, immunophenotypic, and genetic analysis on bone marrow ~~or~~ peripheral blood ~~or bone marrow~~ samples. Response assessment relied on morphology, multiparametric flow-cytometry (MFC), and PCR-MRD. Overall, 160/1812 (8.8%) BCP-ALLs were CD371^{pos} at diagnosis. T~~his findings and~~ correlated with older age (p<0.001), lower *ETV6::RUNX1* frequency (p<0.001), immunophenotypic immaturity (p<0.001), strong expression of CD34~~,~~ and of CD45 (p<0.05). During induction therapy, CD371^{pos} BCP-ALLs showed a transient myelomonocytic switch (mm-SW: up to 65.4% of samples at Day 15) and frequently an inferior response to chemotherapy [Slow Early Response by PCR-MRD, p<0.001]. However, ~~T~~the 5-year event-free survival was 88.3%. Among 420 patients from the validation cohort, 27/28 (96.4%) cases positive for *DUX4*~~-~~ fusions were CD371^{pos}. In conclusion, we comprehensively characterized CD371^{pos} BCP-ALL in the largest pediatric cohort. CD371 is the most sensitive marker of transient mm-SW, whose recognition is essential for proper MFC-MRD assessment. CD371^{pos} is associated to poor early-treatment response, although a good outcome can be reached after MRD-based ALL-related therapies.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer, accounting for about 20% of all malignancies under 20 years of age.[1,2] In the last decades, remarkable progress has been achieved in the outcome of children with ALL, the current survival rate being around 90%.[3-5] Nonetheless, relapse still occurs in a significant proportion of ALL patients and is associated with a poor prognosis.[6] Therefore, it is mandatory to appropriately stratify patients and treat them with a tailored therapeutic regimen.

Multiparametric flow-cytometry (MFC) is fundamental in the diagnosis and monitoring of ALL treatment response.[7-9] Immunophenotypic characterization provides the diagnostic basis to identify and assign blasts~~ cells~~ to their specific ~~hematopoietic~~ B-, T-, or myeloid lineage.[7] Nowadays, MFC is used in pediatric ALL therapeutic protocols to evaluate the response to therapy and stratify patients into risk groups based on minimal residual disease (MRD) assessment on day 15 (D15) of induction therapy (induction).

Several new markers have been recently introduced in the~~ continuous~~ effort to improve ~~both the~~ ALL immunophenotype characterization at diagnosis and ~~the~~ MFC-MRD accuracy. Among them, CD371 (aliases CLL-1, CLEC12A, MICL, KLRL1, or DCAL-2) is a 30kD type II transmembrane glycoprotein with extracellular C-type lectin domains, belonging to the C-type lectin family.[10] It is expressed on normal monocytes, granulocytes, basophils, and most of ~~the~~ acute myeloid leukemia (AML) blasts and leukemic stem cells, being recently indicated as a promising target for AML immunotherapy~~the immunotherapy of AML~~.[10-14]

In 2014, several national MFC-reference laboratories of the international Berlin-Frankfurt-Münster ~~(iBFM)~~ Flow Network (iBFM-FN), including the *Associazione Italiana Ematologia Oncologia Pediatrica* (AIEOP) reference laboratory in Padova (Padova Lab), introduced CD371 in the antibody panel for ALL immunophenotyping at diagnosis. This allowed us characterizing a subset of pediatric B-cell precursor ALL (BCP-ALL) with aberrant expression of CD371 at diagnosis, recently described as associated with *DUX4* rearrangement (*DUX4*^{pos}).[15] We also found that some of these CD371-positive (CD371^{pos}) BCP-ALL cases showed monocytic population early during induction~~ therapy~~. A similar phenomenon was previously observed in BCP-ALLs with aberrant expression of CD2 antigen and the absence of *KMT2A*

rearrangements; it was interpreted as a transient switch (SW) to the monocytic lineage.[16,17] Generally, SW is defined as a lineage variation of blast immunophenotype during first-line therapy[16,18] or at relapse,[19-21] ~~being~~ observed in adults and children.[19,20,22] SW is potentially associated with a poor prognosis[23] and frequently ~~associated~~ with *KMT2A* rearrangements[18,24,25] or *BCR::ABL1*.[26] Notably, the SW phenomenon during induction ~~phase~~ carries a risk of misdiagnosis and/or erroneous interpretation of MFC-MRD findings.

These observations prompted us to investigate the clinical and biological features of CD371^{pos} pediatric BCP-ALLs on a consecutive screening cohort from AIEOP and on a validation cohort collected from other ~~iBFM Flow Network~~ iBFM-FN centers.

Methods

a. Study population and diagnostic workup – screening cohort

From June 2014 to February 2017, 883 children aged 1 to less than 18 years with newly diagnosed *BCR::ABL1*-negative BCP-ALL, were consecutively enrolled in the AIEOP-BFM ALL 2009 study (EudraCT Number: 2007-004270-43) in AIEOP centers.[27]

According to the protocol, the diagnosis of BCP-ALL was based on morphologic, cytochemical, immunophenotypic, and genetic analysis [karyotype, DNA index, presence of fusion genes *ETV6::RUNX1*, *TCF3::PBX1*, *KMT2A::AFF1].*[27] Response to therapy was assessed in peripheral blood (PB) on day 8 (D8, morphology), and bone marrow (BM) samples on D15 (MFC-MRD), day 33 (D33, BM morphology and PCR-MRD) and day 78 (D78, BM PCR-MRD).[27] PB and BM specimens were collected and centralized to the reference laboratories for morphology, immunophenotyping, MFC-MRD, molecular diagnosis, screening of IG/TR rearrangements, and PCR-MRD analysis.[27] Risk group stratification included three subgroups (standard, intermediate, high) relying on biological features (hypodiploidy, *KMT2A::AFF1)* and response to therapy on D8, D15, D33, and D78 (Supplemental Table 1).[27] ~~Risk group stratification according to protocolis summarized in Supplemental Table 1.~~

Local institutional ethical committees approved PB and BM samplings along with the international protocol. In compliance with the Declaration of Helsinki, written

informed consent to use excess diagnostic material for research purposes was obtained from parents or guardians.

b. Multiparametric flow-cytometry analysis

PB and BM samples were processed and analyzed in the Padova Lab, according to previously described standard operating procedures.[7,28,29] We evaluated CD371 expression using CD371-PE (clone 50C1, Becton Dickinson, Franklin Lakes, New Jersey, USA) for diagnostic immunophenotyping and CD371-PC5.5 (clone 50C1, BioLegend, San Diego California, USA) included in the dry 10-color preformulated DuraClone 10 Color Custom Mix (Beckman Coulter, Inc., Brea, CA, USA) for MFC-MRD monitoring.

Briefly, we performed immunophenotyping at diagnosis on erythrocyte-lysed whole BM samples, as reported.[28] BM samples were sent from AIEOP Centers at ambient temperature and processed within 24 hours from collection (for additional details, see "Supplemental data"). We graded antigen expression in negative, weak-positive, strong-positive, and partial-positive, as per the *AIEOP-BFM Consensus Guidelines 2016 for ALL immunophenotyping*, comparing the fluorescence shift and distribution pattern of the blasts ~~cells~~ to the appropriate negative control.[7,28,30-32]

We defined BCP-ALL by the presence of a leukemiac ~~blast~~ population with a strong positivity of at least two antigens among CD19, CD10, iCD22, iCD79a.[28,30,32,33] Four immunophenotypic subtypes of BCP-ALL were identified according to EGIL classification (B-I, B-II, B-III, B-IV).[34] We excluded mature B-ALL (B-IV) with L3 morphology and "Burkitt type" *MYC* rearrangement as per therapeutic protocol.[27]

MFC-MRD was performed as previously described[29] (see "Supplemental data") on D15 (BM) according to the therapeutic protocol[27] and experimentally on D8 (PB), D33 (BM), and D78 (BM) using leftover material whenever available. We defined MRD positivity as a cluster of at least 10 events with lymphoid-scattering properties and leukemia-associated immunophenotypic characteristics as previously reported.[29]

To measure DNA-ploidy by MFC (DNA-index), 500,000 mononuclear cells were stained by propidium iodide and analyzed by flow-cytometry, as previously described.[8]

c. PCR-MRD evaluation

IG/TR gene rearrangements were identified by PCR and used as markers to monitor MRD by real-time quantitative PCR (RQ-PCR), as previously described.[35,36]

Briefly, DNA samples obtained at diagnosis were screened for IG/TR rearrangements. We designed allele-specific oligonucleotide primers to complement the junctional region sequence of each target. We tested MRD-PCR targets and selected two of them for each patient. A reproducible sensitivity of at least 10^{-4} was required for at least one marker.[35,36] We performed and interpreted RQ-PCR analyses according to the *European Study Group for MRD detection in ALL* (EuroMRD ALL) guidelines.[36]

d. Remission induction treatment

Induction IA ~~therapy~~ consisted of a 7-day monotherapy with prednisone and one dose of intrathecal methotrexate, then associated with vincristine (4 doses), daunorubicin (4 doses; in non-high risk patients either *ETV6::RUNX1*-positive or MFC-MRD on induction D15 <0.1%: randomized 2 versus 4 doses), PEG-asparaginase (2 doses) and intrathecal methotrexate (2 doses for CNS1, ~~patients;~~ 4 doses in CNS2 and CNS3 patients. ~~Please see~~See "Supplemental data" for the definition of CNS involvement). Remission induction was followed by consolidation phase IB (consolidation).

e. Validation cohort from the iBFM Centers

After defining the main features of pediatric CD371^{pos} BCP-ALL in the AIEOP cohort, we built up a datasheet to retrospectively collect data on pediatric BCP-ALL from those iBFM-FN ~~Flow Network~~ ~~C~~centers which also had integrated CD371 assessment into the diagnostic work-up (Vienna, n=269; Kiel, n=537, Prague, n=163). The inclusion criteria were (1) enrolment in the AIEOP-BFM ALL 2009 protocol, and (2) use of CD371 monoclonal antibody (MoAb) in the panel for immunophenotyping at diagnosis. Datasheets were compiled by each iBFM center according to the sample analysis performed on site and centralized to the Padova Lab for data curation and elaboration. Additionally, within the validation cohort, B-other ALLs from the Austrian and Czech cohorts were screened for *DUX4*^{pos} ~~rearrangements~~ by RNA-sequencing[15] or *DUX4* positivity was excluded (*DUX*^{neg}) by the presence of other subtype-specific genetic alterations detected by fluorescence *in situ* hybridization (FISH), SNP array analysis, RT-PCR or mutation screening.

f. Statistical analysis

Descriptive methods were applied to present data, with frequencies and percentages for dichotomous and categorical variables (with categories defined according to standard criteria) and median, range, mean, and standard deviation, as appropriate, for continuous variables. The Chi-square or Fisher exact tests for group-wise comparisons of categorical variables were applied (depending on expected cell values above or below five), respectively. Two-sided p-values lower than 0.05 were considered to beregarded as statistically significant.

Outcome analysis was based on CD371 status at diagnosis [$CD371^{pos}$ vs. CD371 negative ($CD371^{neg}$)] and performed on the entire group of patients included in the screening and validation cohorts. Event-free survival (EFS) was defined as the time interval to from diagnosis until treatment failure due to any cause (death before complete remission, resistanceinduction failure, relapse, death in remission, or second malignancy) whichever occurred first or until the date of to the last contact, if failure-free. Cumulative incidence of relapse refers to time until relapse, considering all other events as competing ones.Cumulative incidence of relapsed/refractory disease (CIR) refers to the time to any ALL relapse/refractory disease. Death in remission was considered the competing event.

The statistical analysis was performed using SAS v 9.4 software (SAS Institute Inc., Cary, NC, USA).

g. Data sharing statement

For original data, please contact barbara.buldini@unipd.it

Results

a. Features of $CD371^{pos}$ BCP-ALL in the AIEOP cohort at diagnosis

Out of 883 BCP-ALL patients enrolled in the study period, 843 (95.5%) were evaluated for CD371 expression at diagnosis. The CD371 antigen was positive in 76 patients (9.0%) of which 69 were strong positive, 5 weak positive, and 2 only partially positive. $CD371^{pos}$ positivity was significantly more frequent among older children (≥10 years, p<0.001), and in the presence of DNA-index =1.00 (p<0.001).diploidy (DNA index=1, p<0.001). No significant difference in CD371

expression was found according to gender, PBperipheral white blood cell count at diagnosis, and the presence of a *KMT2A::AFF1* rearrangement, while an *ETV6::RUNX1* fusion gene was detected less frequently in CD371^{pos} than in CD371^{neg} BCP-ALL (p<0.001). *TCF3::PBX1* rearrangement was not identified in the analyzed CD371^{pos} BCP-ALL cohort (Table 1).

In CD371^{pos} BCP-ALL, the immunophenotype at diagnosis showed an earlier stage of differentiation, as per EGIL classification (p<0.001) (Table 1). Moreover, CD371 positivity was associated with strong expression of CD34 (p=0.013), CD45 (p<0.001), and CD58 (p=0.014), and the aberrant expression of at least one myeloid marker out of CD11b, CD14, CD15, CD33, CD64, CD65, iMPO, iLysozyme, CD13, and CD117 (p<0.001) (Table 2). No differences were observed in the distribution of 7 cases of biphenotypic acute leukemia (BAL) according to EGIL classification and mixed-phenotype acute leukemia (MPAL) by WHO definition (Table 1).between CD371^{pos} and CD371^{neg} BCP-ALL cases, as well as of 24 cases of mixed-phenotype acute leukemia (MPAL) by WHO definition (Table 1). Finally, we found a significant association between CD371^{pos} BCP-ALL and the aberrant expression of the CD2 antigen (p<0.001) (Table 2).

b. MFC-MRD evaluation of CD371^{pos} BCP-ALL in the AIEOP cohort

Of the 76 CD371^{pos} BCP--ALLs at diagnosis, 73 samples (96.1%) were evaluable for MFC-MRD on D15 of induction therapy; 3 samples did not reach the minimum of events (acquired nucleated cells) required to obtain an MRD sensitivity threshold of 1 x 10^{-4} and were thus excluded from the final analysis. Additionally, we performed MFC-MRD during induction therapy in 43 (58.9%) PB samples on D8, and in 40 (54.8%) and 54 (74.0%) BM samples on D33 and D78, respectively. When assessed, CD371 expression was always present with no downregulation in those samples still positive for blasts cells during induction therapy (39 samples), confirming CD371 asto be a useful marker for MRD detection in this subtype of CD371^{pos} BCP-ALL (Supplemental Figure 1).

During the first 15 days of induction therapy, we observed in the PB/BM of CD371^{pos} samples the appearance of a population characterized by a strong expression of CD34, CD58 and CD45, downregulation of CD19, and increased SSC signal. This phenomenon was interpreted as a myelomonocytic switch (mm-SW). It displayed two different patterns: (1) a single population of blasts with heterogeneous

expression of CD19 (strong to weak/negative) (Figure 1A); (2) two distinct populations of blast cells, with the first keeping the immunophenotype of diagnosis (unchanged lymphoblasts), and the second showing a downregulation of CD19 and CD34, an upregulation of CD45 and an increase of SSC (SW-blasts) (Figure 1B). Importantly, in the same samples, morphology assessment always revealed the presence of monocytes at different maturation stages (from monoblasts to mature monocytes) with no clear ~~malignant~~aberrant morphological features (Figure 2). Moreover, we tested two cases of the two-population pattern SW samples with an extensive panel including myeloid and monocytic markers: only SW-blasts showed positivity of monocytic antigens CD14, CD11b, CD33, all~~that were~~ negative at diagnosis (Supplemental Figure 2).

Additionally, both unchanged lymphoblasts and SW-blasts, when selected by cell-sorting, shared identical IG/TR rearrangements with blast cells detected at diagnosis (Supplemental Figure 3).

With a detection limit of 1 x 10^{-4}, we identified a mm-SW (mm-SW^{pos}) in 26 of 43 samples (60.5%) on D8 (PB), 51 of 73 samples (69.9%) on D15 (BM), and 1 of 40 samples (2.5%) on D33 (BM). On the 156 samples analyzed by MFC-MRD on Day 8, 15, and 33, 73 and 5 displayed the two-population or one-population mm-SW, respectively (see "Supplemental data" and Supplemental Table 2 for details). In our cohort, no switch (mm-SW^{neg}) was observed on D78 (BM) (Table 3). Of note, chemotherapy was always administered as per AIEOP-BFM ALL 2009 protocol ~~in all the patients~~. Of the 26 mm-SW^{pos} samples on D8, 25 (96.2%) were still mm-SW^{pos} on D15, and 1 also on D33. Of the 17 mm-SW^{neg} samples on D8, 6 (35.3%)~~5 (29.4%)~~ turned into mm-SW^{pos} on D15 (Figure 3).

A transient mm-SW was observed also in 4 of 767 (0.5%) CD371^{neg} BCP-ALLs, all showing an immunophenotype only partially overlapping (B-II ALL; absence of CD371 and CD2; weaker expression of CD45) with CD371^{pos} samples.

c. CD371 and CD2 positivity at diagnosis predicts myelomonocytic switch

Although there was a significant association between CD371 and CD2 expression at diagnosis ($p<0.001$), in our cohort CD2 was detected in 50.0% of the CD371^{pos} samples (Table 2).

We analyzed CD371 and CD2 independently to understand which antigen at diagnosis could better predict the mm-SW (Supplemental Table 3~~2~~). Thirty-one of

the 41 (75.6%) CD2^{pos} BCP-ALLs at diagnosis showed a mm-SW on D15: all of them were CD371^{pos}. Twenty-four of 789 (3.0%) CD2^{neg} samples at diagnosis presented a mm-SW on D15, and these were all CD371^{pos}.

Only 4 of the 757 (0.5%) samples from CD371^{neg} BCP-ALL at diagnosis showed a mm-SW on D15; and notably all of them were CD2^{neg}.

Consequently, in the AIEOP cohort, CD371 was more sensitive than CD2 (sensitivity 0.93 vs. 0.56) in predicting a mm-SW on D15 with a similar specificity (0.97 vs. 0.99).

d. Response to the induction and consolidation therapy and final risk grouping

CD371^{pos} BCP-ALL exhibited a worse early response to chemotherapy according to AIEOP-BFM ALL 2009 protocol as compared to CD371^{neg} BCP-ALL, based on MRD evaluation on D15, D33 (TP1), and D78 (TP2) (Table 4). A significantly higher proportion of CD371^{pos} *versus* CD371^{neg} patients were enrolled in the final high-risk therapeutic arm (p<0.001), mainly due to a significantly higher proportion of patients classified as slow early responders (MRD-SER) (p<0.001) (Table 4) (see "Supplemental data" and Supplemental Table 4 for details). ~~Additional details on high-risk features in the AIEOP cohort are summarized in Supplemental Table 3.~~

e. Validation cohort from the iBFM Flow Network

A total of 969 pediatric patients with newly diagnosed BCP-ALL (median age 5.1 years, range 1-<18 years) were enrolled in the present study from the other iBFM-FN-~~Flow Network~~ centers. Of these, 84 (8.7%) were CD371^{pos} and 885 (91.3%) were CD371^{neg} at diagnosis. As in the screening cohort, CD371^{pos} at diagnosis was associated with older age, a peculiar immunophenotype, absence of *TCF3::PBX1* and *ETV6::RUNX1* rearrangements, and no differences in the distribution of gender and *KMT2A::AFF1* rearrangement compared to CD371^{neg} ALLs (Tables 2, 4, and 5). ~~As already shown in the AIEOP BCP-ALL cohort, CD371^{pos} at diagnosis was associated with older age (median age 9.38 years in CD371^{pos} patients vs. 4.64 years in CD371^{neg} patients). Analogously to the AIEOP cohort, no difference was found in the distribution of gender and *KMT2A::FF1* rearrangement between CD371^{pos} and CD371^{neg} patients. iBFM data confirmed a low frequency of the *ETV6::RUNX1* rearrangement (no cases in the validation cohort, p<0.001) and the absence of *TCF3::PBX1* in the CD371^{pos} group (Table 5).~~

~~Similarly to the AIEOP cohort, in the iBFM CD371^{pos} BCP-ALLs the immunophenotype at diagnosis showed the following features: (1) an earlier stage of differentiation as defined by EGIL classification (p<0.001) (Table 4), (2) a strong expression of CD34 (p<0.001) and CD45 (p<0.001), (3) the aberrant expression of at least one of the myeloid markers CD11b, CD14, CD15, CD33, CD64, CD65, iMPO, iLysozyme, CD13, CD117 (p<0.001), and (4) the aberrant expression of the CD2 antigen (p<0.001) (Table 2). In the iBFM cohort, CD371 expression at diagnosis was associated with BAL as per EGIL classification (p=0.006), but not with MPAL status by WHO definition (Table 5).~~

During induction and consolidation~~ therapy~~, MFC-MRD was assessed in 165 samples collected at different re-evaluation time-points from CD371^{pos} BCP-ALL patients at diagnosis, and 68 of them (41.2%) showed mm-SW [D8: 16 of 21 samples (76.2%); D15: 49 of 80 samples (61.3%); D33: 3 of 32 samples (9.4%)]. No switch was detected on D78 (Table 3).~~Specifically, mm-SW was observed in 16 of 21 samples (76.2%) on D8, 49 of 80 samples (61.3%) on D15, and only in 3 of 32 samples (9.4%) on D33. No switch was detected on D78 (Table 3).~~

~~In the~~The iBFM-FN cohort~~, we also~~ confirmed the association between CD371^{pos} BCP-ALL and a worse response to induction and consolidation~~ therapy [final therapeutic high-risk group: 44 of 84 (52.4%) CD371^{pos} BCP-ALL vs. 172 of 877 (19.6%) available CD371^{neg} BCP-ALL, p<0.001]~~ (Table 4). Of note, the iBFM-FN CD371^{pos} BCP-ALLs showed a higher proportion of high-risk by morphology on D8, MFC-MRD on D15, and PCR-MRD on D33/D78 than AIEOP samples. Since the two cohorts included patients consecutively enrolled in the same therapeutic protocol with no selection biases, these differences, not present in the CD371^{neg} subgroup, could reasonably depend on the small number of samples of the CD371^{pos} subgroups.

~~Finally, we investigated the presence of *DUX4* gene rearrangements (*DUX4*^{pos}) or excluded the presence of *DUX4* rearrangement (*DUX4*^{neg}) by its mutual exclusivity from other subtype-specific genetic alterations in 420 samples at diagnosis from the Austrian and Czech cohorts. Among those,~~ Finally, on a total of 420 samples, 27 of 28 *DUX4*^{pos} vs. 7 of 387 *DUX4*^{neg} samples were CD371^{pos}, confirming CD371 as a sensitive and specific surrogate immunophenotype marker of *DUX4*^{pos}~~ alterations~~ in BCP-ALLs (Supplemental Table 54).

f. Outcome analysis

In a total of 1796 patients (158 CD371^{pos} and 1638 CD371^{neg} at diagnosis) from the screening and validation cohorts, the 5-year EFS was 88.3% in CD371^{pos} ~~BCP-ALLs~~ vs. 82.4% in CD371^{neg} BCP-ALLs (p=0.07), with a 5-year CIR of 6.4% vs. 14.3%, respectively (p=0.006) (Figure 4; Supplemental Figure 4). Analysing separately patients enrolled in the final high-risk (390 patients) and non-high risk (standard and intermediate, 1406 patients) group, the 5-year EFS was always superior in CD371^{pos} BCP-ALLs but significantly so only in the high-risk group (Supplemental Figure 5).

Discussion

This is the first study that extensively describes the biological and clinical features of a large multi-center cohort of CD371^{pos} BCP-ALLs. CD371 is usually expressed on normal monocytes, granulocytes, basophils, most AML blasts, and leukemiac~~s~~ stem cells.[10-13]

In our cohort CD371 positivity at diagnosis identified a specific subtype of BCP-ALL, with peculiar clinical and biological features accounting for about 9.0% of pediatric BCP-ALL patients. First, CD371^{pos} BCP-ALL was not associated with any of the traditional high-risk features of BCP-ALL at diagnosis, including hypodiploidy, *KMT2A::AFF1* rearrangement, or hyperleukocytosis. ~~Of note, we~~We did not analyse a potential association between CD371 positivity at diagnosis and *BCR::ABL1* ~~BCP-ALL~~, this fusion transcript being an exclusion criterion from the AIEOP-BFM ALL 2009 protocol. CD371^{pos} BCP-ALL was more frequent in children with age ≥10 years, which is not a high-risk criterion in AIEOP-BFM ALL 2009 protocol.

The immunophenotype at diagnosis showed some peculiarities. A considerable proportion of CD371^{pos} BCP-ALL (13.2%) belonged to the immature group B-I ALL as per EGIL classification and there was a trend toward weaker CD10 expression among EGIL B-II cases (data not shown). Yet, CD371^{pos} BCP-ALL showed a brighter intensity of CD34, CD45, and CD58 at diagnosis as compared to CD371^{neg} BCP-ALL cases. Altogether, these peculiar features suggest more immaturity and ~~thus~~ a potentially higher plasticity of CD371^{pos} BCP-ALL, with a propensity to mm-SW under therapeutic pressure.

In our study, with all AIEOP and other iBFM-FN cases cumulated, we observed a mm-SW in 42/64 (65.6%) of the available CD371^{pos} samples on D8, 100/153 (65.4%)

of samples on D15, and 4/72 (5.6%) of samples on D33, whereas we did not detect it on D78 (Table 3). The mm-SW was characterized by downregulation of CD19 and CD34, further upregulation of CD45, an increase of SSC, and strong expression of CD58. These changes appear to be related to the more general phenomenon of antigen expression modulation induced by ALL therapy, ~~which has been~~ described previously.[37-40] Considering its potential impact on MCF-MRD analysis and the resulting risk group assignment, we established a systematic approach to optimize switch detection and increase reproducibility. Indeed, the assessment of mm-SW during MRD monitoring is challenging. We classified the mm-SW in two distinct patterns: a one-population ~~pattern~~ and a two-populations pattern. When one-population was detected, it shared antigens of BCP- and myelomonocytic lineages, with a change in lymphoid antigen expression levels (SW-blasts only). More challenging was the detection of the two-populations pattern (consisting of SW-blasts and unchanged lymphoblasts) and its impact on MFC-MRD quantification on D15.

These observations led to two clinically relevant questions. First, whether the SW-blast population (CD19 downregulated, CD45 bright) should be considered as part of blasts~~ cells~~ and included in MFC-MRD quantification. Based on our findings, we recommend including it in the MFC-MRD final blast count on D15. This choice is due to the consideration that the mm-SW immunophenotype suggested a direct link with lymphoblasts, keeping, although downregulated, CD19 positivity. Additionally, we demonstrated that SW-blasts and unchanged lymphoblasts shared the same IG/TR rearrangements. Since the mm-SW blast population proportion varied widely, its appearance may deeply influence the final extent of MFC-MRD on D15 and consequently risk group definition. In addition, standard morphology (e.g., at D8) may underestimate the amount of blasts~~ cells~~ in the presence of a population of monocytes at different maturation stages, lacking clear malignant morphological features~~ of blast cells~~ (Figure 2).

The second question is whether in the presence of a preponderant mm-SW blast population chemotherapy should be changed to a myeloid leukemia schema. This option was never considered neither in the AIEOP nor in the iBFM-FN groups, attributing the origin of the SW-blast population to lymphoblastic cells. This choice is supported by Hrusak et al.[41] treatment strategies for ambiguous lineage leukemias.

Importantly, the mm-SW does not constitute a permanent and complete switch to the myeloid lineage as in *KMT2A*-rearranged ALLs but is always transient, characterized

by the co-expression of B- and MM-lineage immunophenotypic features, and limited to the first phase of induction. ~~therapy~~ Therefore, ~~, when~~ chemotherapy was always continued according to ALL treatment with no shift to AML protocol, differently from what is generally considered for *KMT2A*-positive-SW cases.[18,24,25] ~~Of note, h~~However, CD371^{pos} BCP-ALLs showed a slower response to the induction ~~therapy~~ in comparison to CD371^{neg} BCP-ALL. The significantly higher rate of patients enrolled in the final high-risk therapeutic arm well described this phenomenon. Furthermore, the high incidence of MRD-SER in the evaluation of PCR-MRD on D33 and D78 was a special feature of CD371^{pos} BCP-ALLs. These findings suggest both a reduced sensitivity of CD371^{pos} blasts ~~cells~~ to the drug combination administered during the first phase of ~~chemotherapy~~treatment and ~~hypothesize~~ a driving role of steroid therapy in the mm-SW.

Notably, CD371 expression in BCP-ALL was found very recently to be strongly associated with *DUX4*^{pos} ~~gene rearrangements~~,[15] and ~~associated with~~ a good long-term outcome.[42-44] Our study clearly confirmed the association between CD371 strong positivity and the *DUX4*^{pos} subtype whereas a weak expression of CD371 was observed in a few cases with other ~~in different~~ genotypes. ~~.~~ Additionally, our survival analysis showed a good prognosis in the CD371^{pos} subgroup, even in the high-risk group, always continuing on ALL-directed therapy without any changes to a myeloid-oriented treatment. Therefore, our study supports the hypothesis of a direct biological link between *DUX4*^{pos} and CD371 strong expression. This makes CD371~~could be~~ a suitable immunophenotype surrogate marker to identify blasts with a specific genetic lesion and biological behavior, potentially requiring a more intense treatment ~~in the first phase of chemotherapy~~ to reach a good long-term prognosis, similar to what has been described in early T-cell precursor (ETP) ALL.[45] Regarding the mm-SW, it was observed also in CD371 weak-positive BCP-ALLs in our cohorts as well as very rarely also in CD371-negative cases. Therefore, further studies are necessary to define the biological role of CD371 expression and *DUX4*^{pos} in mm-SW.

The inferior response to induction ~~therapy~~ highlights the importance of detecting accurate predictors of mm-SW. Slamova et al.[16] associated lineage switch with the aberrant expression of CD2 at diagnosis. Consequently, we investigated which marker, CD2 or CD371, might better predict this phenomenon. In the AIEOP cohort, despite both antigens showing high accuracy, CD371 was a more sensitive predictor of mm-SW than CD2. Therefore, we believe that detecting CD371^{pos} BCP-ALL at

diagnosis and accurately monitoring MFC-MRD on D15 is still critical for identifying precociously a subgroup of pediatric leukemia with the tendency to an inferior response to induction and consolidation and providing useful information for a more accurate prognostic communication to patients' caregivers. This said, considering the overall favourable outcome of pediatric CD371^{pos} BCP-ALL, further studies are necessary to establish whether the advancement in genomics and MRD techniques will confirm the role of D15 time-point for risk group stratification in this leukemia subtype and which leukemia-derived cell types must be monitored in particular.

Our study had some limitations. We used different MoAb panels for MFC-MRD detection in different time periods. Moreover, MFC-MRD MoAb panels did not include monocyte-lineage markers like CD14, CD15, CD64, CD11b/c. The introduction of some of these markers may help ~~to~~ discriminate the SW-blast population from normal monocytic counterparts.

In conclusion, in the largest pediatric cohort described so far, we comprehensively described the biological and clinical features of CD371^{pos} BCP-ALLs characterized by a potential mm-SW during the first phase of induction ~~therapy~~. Accurate identification of mm-SW is mandatory to properly assess MFC-MRD on D15 in CD371^{pos} patients. This is of particular importance since, in our cohort, CD371^{pos} BCP-ALLs showed a slower response to induction ~~chemotherapy~~ and led to a higher rate of patients included in the high-risk therapeutic group compared to CD371^{neg} BCP-ALLs. Expression of CD371 antigen is an accurate predictor of mm-SW in BCP-ALL, with high sensitivity and specificity. Chemotherapy should be continued according to an ALL-therapeutic protocol even in the presence of a prevalent SW-blast population during the mm-SW, a statement supported by the favourable outcome of CD371^{pos} BCP-ALL ~~as~~ shown herein.

Finally, based on our findings, we suggest the introduction of CD371 assessment in both BCP-ALL immunophenotyping at diagnosis and during MFC-MRD monitoring. ~~induction therapy.~~

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Authorship

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Tables

Table 1. Patients and leukemia main features at diagnosis in the screening cohort.

Variable	Total pts		CD371^{pos} pts		CD371^{neg} pts		P-value
	n°	%	n°	%	n°	%	
Total	843	100	76	9.0	767	91.0	
Age							**< 0.001**
1-9 years	676	80.2	41	54.0	635	82.8	
10-17 years	167	19.8	35	46.0	132	17.2	
Gender							0.770
Male	457	54.2	40	52.6	417	54.4	
Female	386	45.8	36	47.4	350	45.6	
~~**WBC count**~~							~~0.240~~
~~< 20 x 10⁹/L~~	~~611~~	~~72.6~~	~~61~~	~~80.3~~	~~550~~	~~71.8~~	
~~20-99 x 10⁹/L~~	~~186~~	~~22.1~~	~~11~~	~~14.5~~	~~175~~	~~22.8~~	
~~≥ 100 x 10⁹/L~~	~~45~~	~~5.3~~	~~4~~	~~5.2~~	~~41~~	~~5.4~~	
~~**DNA index**~~							~~**< 0.001**~~
~~= 1.00~~	~~511~~	~~61.2~~	~~66~~	~~88.0~~	~~445~~	~~58.6~~	
~~≠ 1.00~~	~~324~~	~~38.8~~	~~9~~	~~12.0~~	~~315~~	~~41.4~~	
WBC count							0.080
< 50 x 10⁹/L	734	87.1	71	93.4	663	86.5	
50-99 x 10⁹/L	63	7.5	1	1.3	62	8.1	
≥ 100 x 10⁹/L	45	5.3	4	5.3	41	5.4	
DNA index							**<0.001**
<0.8	7	0.8	0		7	0.9	
≥0.8 <1	0		0		0		
= 1.00	511	61.2	66	88.0	445	58.6	
>1 <1.16	111	13.3	2	2.7	109	14.3	
≥1.16	206	24.7	7	9.3	199	26.2	
Fusion genes							
ETV6::RUNX1	191	23.1	1	1.4	190	25.2	**< 0.001**
TCF3::PBX1	30	3.7	0	0.0	30	4.1	0.100
KMT2A::AFF1	6	0.7	1	1.4	5	0.7	0.430
Immunophenotype at diagnosis (EGIL)							**<0.001**
BI-ALL	22	2.6	10	13.2	12	1.6	

BII-ALL	635	75.3	66	86.8	569	74.2	
BIII-ALL	186	22.1	0	0.0	186	24.2	
Ambiguous leukemia							
BAL (EGIL)	7	0.8	1	1.3	6	0.8	0.490
MPAL (WHO)	24	2.8	3	4.0	21	2.7	0.540

Data not available: WBC count: 1 patient; DNA index: 8 patients; BAL: 2 patients
Abbreviations: ALL: acute lymphoblastic leukemia; BAL: biphenotypic leukemia; MPAL: mixed-phenotype leukemia; n°: number; neg: negative; pos: positive; pts: patients; WBC: white blood cell; %: percentage.

Table 2. Immunophenotype at diagnosis: distribution of antigen expression intensity in CD371+ and CD371- BCP-ALL.

Variable	Screening cohort						Validation cohort						P*
	Total	CD371^{pos}		CD371^{neg}		*P*	Total	CD371^{pos}		CD371^{neg}		P	
	n°	n°	%	n°	%		n°	n°	%	n°	%		
Total	843	76	9.0	767	91.0	-	969	84	8.7	885	91.3	-	0.795
CD45						**<0.001**						**<0.001**	
STRONG	258	50	65.8	208	27.2		169	30	85.7	139	35.0		0.674
WEAK	457	24	31.6	433	56.6		200	5	14.3	195	49.1		0.114
PP	2	1	1.3	1	0.1		-						
NEG	124	1	1.3	123	16.1		63	0		63	15.9		1.000
NOT DONE	2	0		2			537	49		488			
CD19						0.099						0.199	
STRONG	819	74	97.4	745	97.1		963	83	98.8	880	99.4		0.757
WEAK	23	1	1.3	22	2.9		4	0		4	0.5		1.000
PP	1	1	1.3	0			-						
NEG	0						2	1	1.2	1	0.1		
CD10						**<0.001**						**<0.001**	
STRONG	786	55	72.4	731	95.3		909	63	75.0	846	95.5		0.211
WEAK	22	9	11.8	13	1.7		34	14	16.7	20	2.3		0.984
PP	13	2	2.6	11	1.4		-						
NEG	22	10	13.2	12	1.6		26	7	8.3	19	2.2		0.186
CD58						**0.014**						0.380	
STRONG	749	73	96.1	676	88.5		687	64	97.0	623	98.3		0.077
WEAK	82	1	1.3	81	10.6		12	2	3.0	10	1.5		**0.042**
PP	1	1	1.3	0			-						
NEG	8	1	1.3	7	0.9		1	0		1	0.2		1.000
NOT DONE	3	0		3			270	18		251			
CD34						**0.013**						**<0.001**	
STRONG	645	68	89.5	577	75.9		659	78	92.9	581	65.7		0.459
WEAK	67	0		67	8.8		150	4	4.8	146	16.4		0.314
PP	45	6	7.9	39	5.1		-						
NEG	79	2	2.6	77	10.1		160	2	2.4	158	17.9		0.601
NOT DONE	7	0		7			-						
CD2						**<0.001**						**<0.001**	
STRONG	16	14	18.4	2	0.3		30	27	32.1	3	0.3		1.000
WEAK	17	16	21.1	1	0.1		84	20	23.8	64	7.2		**<0.001**
PP	8	8	10.5	0			-						
NEG	800	38	50.0	762	99.6		855	37	44.1	818	92.4		0.680
NOT DONE	2	0		2			-						
CD56						**<0.001**						**<0.001**	
STRONG	7	4	5.3	3	0.4		13	4	4.8	9	1.0		0.356
WEAK	13	7	9.2	6	0.8		67	14	16.7	53	6.0		**0.014**
PP	1	1	1.3	0			-						
NEG	811	64	84.2	747	98.8		889	66	78.5	823	93.0		0.717
NOT DONE	11	0		11			-						
MYELOID ANTIGENTS**						**<0.001**						**<0.001**	
YES	289	42	55.3	247	32.2		636	70	83.3	566	63.9		0.128
NO	554	34	44.7	520	67.8		333	14	16.7	319	36.0		0.218

Abbreviations: NEG: negative; NO: negativity of all myeloid antigens; POS: positive; PP: partially positivity; STRONG: strong positivity; WEAK: weak positivity; YES: any positivity (weak, strong, partial positive).

P*: comparison of variables distribution between screening (AIEOP) and validation (iBFM Flow Network) cohorts

****Any myeloid antigen of CD11b, CD14, CD15, CD33, CD64, CD65, MPO, LYSO, CD13, CD117

Table 3. Distribution of myelomonocytic switch at different disease re-evaluation time points in CD371 positive BCP-ALL.

	Total		Screening cohort		Validation cohort		*P*
	n°	**%**	**n°**	**%**	**n°**	**%**	
Total CD371^{pos} at diagnosis	157		73	46.5	84	53.5	
Switch on Day 8							0.217
Yes	42	65.6	26	60.5	16	76.2	
No	22	34.4	17	39.5	5	23.8	
NA	93		30		63		
Switch on Day 15							0.265
Yes	100	65.4	51	69.9	49	61.3	
No	53	34.6	22	30.1	31	38.7	
NA	4		0		4		
Switch on Day 33							0.317
Yes	4	5.6	1	2.5	3	9.4	
No	68	94.4	39	97.5	29	90.6	
NA	85		33		52		
Switch on Day 78							
Yes	0	0.0	0	0.0	0	0.0	
No	86	100.0	54	100.0	32	100.0	
NA	71		19		52		

Abbreviations: n°: number of samples; pos: positive; %: percentage.

Table 4. Response to induction and consolidation therapy and risk group stratification according to AIEOP-BFM ALL 2009 protocol criteria.

Variable	Screening cohort						Validation cohort						*P**
	Total	CD371^{pos}		CD371^{neg}		*P*	Total	CD371^{pos}		CD371^{neg}		*P*	
	n°	n°	%	n°	%		n°	n°	%	n°	%		
Total	843	76	9.0	767	91.0		969	84	8.7	885	91.3		0.795
Prednisone response						0.470						**<0.001**	
Good (PGR)	787	73	96.1	714	93.2		904	69	82.1	835	95.3		0.225
Poor (PPR)	55	3	3.9	52	6.8		56	15	17.9	41	4.7		**0.004**
NA	1	0		1			9	0		9			
MCF-MRD Day 15						**0.0060.008**						**<0.001**	
<0.1%	308	15	20.5	293	38.9		284	15	19.2	269	32.6		0.820
≥0.1-<10%	431	4647	63.06 4.4	384	50.9		515	42	53.8	473	57.4		0.149
≥10%	88	1211	16.51 5.1	77	10.2		103	21	26.9	82	10.0		0.147
NA	16	3		13			67	6		61			
Response to induction IA						1.000						0.087	
CR Day 33	825	75	100.0	750	98.7		950	80	97.6	870	99.5		0.618
Non-CR Day 33	10	0		10	1.3		6	2	2.4	4	0.5		0.125
NA	8	1		7			13	2		11			
PCR-MRD final						**< 0.001**						**<0.001**	
Standard	273	11	14.9	262	35.7		337	6	7.3	331	38.5		0.094
Medium	443	41	55.4	402	54.8		487	38	46.3	449	52.3		0.428
MRD-SER	66	18	24.3	48	6.5		88	27	33.0	61	7.1		0.646
High	25	4	5.4	21	3.0		29	11	13.4	18	2.1		0.126
NA	36	2		34			28	2		26			
Final risk group						**< 0.001**						**<0.001**	
Standard	277	12	15.8	265	34.6		314	6	7.1	308	35.1		0.088
Medium	390	37	48.7	353	46.0		431	34	40.5	397	45.3		0.416
High	176	27	35.5	149	19.4		216	44	52.4	172	19.6		0.200
NA	0						8	0		8			

Abbreviations: CD371^{pos}: any positivity (strong, weak, partially positive) of CD371 antigen at diagnosis; CD371^{neg}: negativity of CD371 antigen at diagnosis; MFC: multiparametric flow cytometry; MRD: minimal residual disease; PCR: polymerase chain reaction; SER: slow early response.
***P**:** comparison of variables distribution between screening (AIEOP) and validation (iBFM Flow Network) cohorts

Table 5. Patients and leukemia features at diagnosis in the validation (iBFM Flow Network) cohort.

Variable	Validation cohort					
	Total	CD371^{pos}		CD371^{neg}		*P*
	n°	n°	%	n°	%	
Total	969	84	8.7	885	91.3	
Age						
Median	5.1	9.2		4.6		
Gender						0.286
Male	516	51	60.7	465	54.6	
Female	419	33	39.3	386	45.4	
NA	34	0		34	-	
Fusion genes						
ETV6::RUNX1	208	0	0.0	208	23.5	**<0.001**
TCF3::PBX1	27	0	0.0	27	2.7	0.152
KMT2A::AFF1	5	2	2.4	3	0.4	0.063
Immunophenotype at diagnosis (EGIL)						**<0.001**
BI-ALL	23	7	8.4	16	1.8	
BII-ALL	703	64	77.1	639	72.6	
BIII-ALL	232	12	14.5	220	25.0	
B-IV(non Burkitt)	5	0		5	0.6	
NA	6	1	-	5	-	
Ambiguous leukemia						
BAL (EGIL)	27	6	7.1	21	2.4	**0.006**
MPAL (WHO)	32	4	4.6	28	3.2	0.514

Abbreviations: ALL: acute lymphoblastic leukemia; BAL: biphenotypic acute leukemia; MPAL: mixed-phenotype acute leukemia; n°: number; pts: patients; %: percentage.

Figure legends

Figure 1. Myelomonocytic switch in CD371 positive BCP-ALL by flow cytometry. With the beginning of steroid treatment as per AIEOP-BFM ALL 2009 protocol, residual lymphoblastic cells showed a myelomonocytic switch. It displayed two different patterns: (A) single population of blast cells with heterogeneous expression of CD19 (single population pattern: orange population); (B) a two-populations pattern: the first population keeping the immunophenotype of the diagnosis (blue population); the second population showing a downregulation of CD19 and CD34, an upregulation of CD45, and an increase of SSC (orange population). Technical information: fluorochromes: CD19-PC7, CD10-APC/ALEXA700, CD20- V450, CD34-APC, CD58-FITC, CD371-PC5.5, CD45-V500; sample preparation and acquisition at ambient temperature; sample acquisition: Navios Flow Cytometer (Beckman Coulter, Inc., Brea, CA, USA); sample analysis: Software Kaluza 2.1 (Beckman Coulter, Inc., Brea, CA, USA). Please see "Supplemental data" for additional information.

Figure 2. CD371 positive BCP-ALL: standard morphology evaluation of a peripheral blood sample on Day 8 of induction therapy. Standard morphology of a CD371-positive BCP-ALL peripheral blood sample collected on Day 8 of induction therapy revealed a population of monocytes at different maturation stages. Technical information: sample preparation and acquisition at ambient temperature; cytochemical staining: May Grünwald Giemsa; microscope model: LEICA DM2000 LED (Leica Microsystem Srl, Buccinasco, MI, Italy); objective: Leica HC PL Fluotar 40x Objective (Leica Microsystem Srl, Buccinasco, MI, Italy); camera model: LEICA DMC4500 (Leica Microsystem Srl, Buccinasco, MI, Italy); picture acquisition software: LAS (Leica Application Suite) V4.13 (Leica Microsystem Srl, Buccinasco, MI, Italy)

Figure 3. Myelomonocytic switch evolution during induction and consolidation therapy as per AIEOP-BFM ALL 2009 protocol.
On a total of 73 CD371 positive BCP-ALL samples evaluable for MFC-MRD from the screening cohort (AIEOP), the myelomonocytic switch was observed (detection limit

1 x 10^{-4}) since the first re-evaluation time point on Day 8 or later on Day 15 and Day 33. It was never detected on Day 78 in the screening cohort.

Figure 4. Outcome analysis of CD371 positive vs. CD371 negative BCP-ALL. In a total of 1796 patients (158 CD371 positive and 1638 CD371 negative at diagnosis) from the screening and validation cohorts, the 5-year EFS was 88.3% in CD371 positive BCP-ALLs vs. 82.4% in CD371 negative BCP-ALLs (p=0.07).