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Transient Switch to myeloid lineage in Acute Lymphoblastic Leukemia in Induction: role of CD371 expression and implication for Minimal Residual Disease detection

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MAIN PROJECT

Transient Switch to myeloid lineage in Acute Lymphoblastic Leukemia in Induction: role of CD371 expression and implication for Minimal Residual Disease detection

Summary

Background: ImmunoPhenotyping (IP) by multi-colour flow cytometry (FCM) is a cornerstone of pediatric B-lineage acute lymphoblastic leukemia (BCP-ALL) diagnosis and is gaining ever greater prognostic role in minimal residual disease (MRD) monitoring. It allows also to detect lineage switch (SW), defined as any variation of blast IP during therapy. SWs to myeloid lineage were described in ALLs with KMT2A rearrangements and BCP-ALL expressing antigen CD2. We observed a transient SW to myelomocitic lineage during the first phase of AIEOP-BFM ALL 2009 protocol (Induction IA-steroid phase) in a subset of BCP-ALL. This behaviour was seen in association with the aberrant expression of myeloid antigen CD371 at diagnosis. We retrospectively compared CD371-positive (CD371+) to CD371-negative (CD371-) BCP-ALLs. Aims of our study: to verify the association between CD371 expression at diagnosis and transient SW during Induction phase; to define SW blasts immunophenotypic changes and redefine a standardized approach to accurate FCM-MRD analysis on Day (D) 15

Materials and Methods: Inclusion criteria: new diagnosis of BCP-ALL, except BIV-ALL according to EGIL classification; treatment regimen: AIEOP BFM ALL 2009 protocol; enrolment period: 01/06/2014 – 31/01/2017. Eight-hundred-twenty-three paediatric patients (pts) were included in our study (age: 1-17 years; male/female 446/377 pts). Peripheral and bone marrow samples were centralized from all Italian AIEOP Centres to the Laboratory of Diagnosis and Research of Pediatric Hematology Oncology, University of Padua. Samples were processed and analysed according to standardized operating protocols designed by the AIEOP-BFM Flow Network. Nine combinations of 8 monoclonal antibodies were used for IP at diagnosis, 2 for FCM-MRD from June 2014 to May 2016; subsequently dry 10 colours pre-formulated DuraClone 10 Conj Custom Mix, Per Test, 2500 Test per Yr (Beckman Coulter) was adopted. BD FACS Canto II (Becton Dickinson) and Navios (Beckman Coulter) cytometers were used for samples acquisition and analysis.

Results: CD371+ was detected in 75/823 pts (9.1%) at diagnosis. CD371+ was associated with older age (>9 years: 34/75 vs 130/748 pts, p<.001); DNA index=1.0 (65/75 vs 437/748 pts, p<.001); immature immunophenotype according to EGIL classification (BI-ALL 10/75 vs 12/748 pts; BII-ALL 65/75 vs 555/748 pts, BIII-ALL 0/75 vs 181/478 pts, p<.001); CD2 positivity (38/75 vs 3/748 pts, p<.001); other

myeloid antigen positivity (42/75 vs 240/748 pts, p<.001); worse response to Induction therapy (high risk group: 27/75 vs 147/748 pts, p<.001). Samples of 72/75 pts (96%) were available for FCM-MRD analysis. SW was defined as the appearance of a «monocytoid» population characterized by the following immunophenotype: CD34 strong; CD58 strong; CD19 dim positive/negative; increased CD45 expression and Side Scatter characteristic. SW was observed in 50/72 CD371+ pts vs 4/748 CD371- pts (sensitivity 0.93, 95%IC ±0.06; specificity 0.98, 95%IC ±0.005; PPV 0.82; NPV 0.99; accuracy 0.98). CD371+ pts: SW was detected in 26/42 evaluated pts (61.9%) on D8 and 50/72 pts (69.4%) on D15. No more SW was detectable on D33 and D78, despite chemotherapy regimen according to AIEOP-BFM ALL 2009 protocol had been carried on.

Conclusions: CD371 is an accurate marker for the detection of transient SW in BCP-ALL. CD371+ is associated with worse response to Induction therapy. It should suggest peculiar attention in FCM-MRD analysis on Day 15 in these pts.

Riassunto

Background: L'analisi immunofenotipica mediante citometria a flusso multiparametrica è una metodica fondamentale per la diagnosi delle leucemie linfoblastiche acute a precursori B (BCP-ALL) in età pediatrica. Essa sta acquisendo sempre maggiore rilevanza nel monitoraggio della malattia residua minima. Permette inoltre di identificare eventuali variazioni nell'immunofenotipo delle cellule blastiche in corso di chemioterapia, fenomeno definito come "switch" immunofenotipico. In passato è stato descritto uno SW a leucemia mieloide acuta in BCP-ALL associate a riarrangiamenti del gene KMT2A; più recentemente è stato descritto uno switch mieloide transitorio in un subset di BCP-ALL caratterizzate dall'espressione dell'antigene di superficie CD2, in assenza di riarrangiamenti del gene KMT2A.
Il Laboratorio di Diagnositica e di Ricerca di Padova è il centro di riferimento nazionale a cui vengono centralizzati i campioni per la diagnosi morfologica e la CMF dei pazienti con ALL provenienti da tutti i centri AIEOP. Qui e' stata osservata la possibile comparsa di blasti di linea mielo-moniciticitica in un subset di BCP-ALL apparentemente caratterizzato, alla diagnosi, dall'espressione dell'antigene mieloide CD371. Tale fenomeno è stato individuato solo nelle prime fasi della chemioterapia (terapia steroidea della fase di Induzione IA secondo protocollo internazionale AIEOP-BFM ALL 2009), per poi scomparire con la prosecuzione della terapia stessa. E' stato pertanto deciso di condurre un'analisi retrospettiva per confrontare le BCP-ALL con espressione, alla diagnosi, dell'antigene CD371(CD371+) con quelle negative per tale antigene (CD371-). Gli obiettivi del nostro studio sono i seguenti: verificare l'associazione tra espressione di CD371 alla diagnosi e lo SW mielomonicitico transitorio durante la terapia di Induzione; definire l'immunofenotipo delle cellule che presentano lo SW; ridefinire un approccio standardizzato per un'accurata analisi della CFM-MRM al giorno 15.
Materiali e metodi: Criteri di inclusione: nuova diagnosi di BCP-ALL, escluso il sottotipo B-IV secondo la classificazione EGIL; trattamento secondo protocollo internazionale AIEOP-BFM ALL 2009; periodo di arruolamento: dall' 01/06/2014 al 31/01/2017. 823 pz pediatrici sono stati inclusi nel nostro studio (età 1-17 anni, maschi/femmine 446/377 pz). I campioni di sangue periferico e midollare sono stati centralizzati al Laboratorio di Diagnostica e Ricerca di Padova per l'analisi dell'immunofenotipo alla diagnosi ed il monitoraggio della malattia residua minima

molecolare ai giorni 8, 15, 33 e 78, come previsto dal protocollo di cura. I campioni sono stati processati ed analizzati secondo protocolli operativi standardizzati, definiti dal gruppo di lavoro internazionale AIEOP-BFM Flow Network. Pannelli di 8 anticorpi monoclonali sono stati allestiti presso il nostro Laboratorio (di cui 9 sono stati utilizzati per l'IF alla diagnosi e 2 per lo studio della malattia residua minima molecolare); a partire dal mese di maggio 2016, lo studio della malattia residua minima molecolare è stato effettuato con l'utilizzo di un pannello di 10 anticorpi monoclonali cristallizzati (DuraClone 10 Conj Custom Mix, Per Test, 2500 Test per Yr, Beckman Coulter). I campioni sono stati acquisiti e analizzati con i citofluorimetri a flusso multiparametrici BD FACS Canto II (Becton Dickinson) e Navios (Beckam Coulter).

Risultati: 75/823 pz (9.1%) sono risultati caratterizzati dall'espressione di CD371+ alla diagnosi. CD371+ è risultato associato a: età maggiore alla diagnosi (> 9 anni: 34/75 vs 130/748 pz, p<.001); DNA index=1.0 (65/75 vs 437/748 pz, p<.001); immufenotipo più immaturo secondo la classificazione EGIL (B-I 10/75 vs 12/748 pz; B-II 65/75 vs 555/748 pz, B-III 0/75 vs 181/478 pz, p<.001); positività per CD2 (38/75 vs 3/748 pz, p<.001); positività per altri antigeni mieloidi (42/75 vs 240/748 pz, p<.001); peggiore risposta alla terapia di Induzione (gruppo terapeutico di rischio alto: 27/75 vs 147/748 pz, p<.001). 72/75 pz (96.0%) sono risultati valutabili per la malattia residua minima in citofluorimetria al giorno 15. Lo switch è stato definito come la comparsa di una popolazione "monocitoide" caratterizzata dal seguente immunofenotipo: CD34 positivo forte; CD58 positivo forte; riduzione dell'intensità di CD19 fino a completa negativizzazione; aumento dell'intensità di CD45; aumento del Side Scatter, parametro fisico che identifica la granulosità cellulare. Lo switch è stato osservato in 50/72 pz CD371+ vs 4/748 pz CD371- (sensibilità 0.93, 95%IC ±0.06; specificità 0.98, 95%IC ±0.005; VPP 0.82; VPN 0.99; accuratezza 0.98). Nei pz CD371+ lo SW è stato individuato in 26/42 pz valutati (61.9%) al giorno 8 e in 50/72 pz (69.4%) al giorno 15. Lo switch si è completamente negativizzato a partire dal giorno 33, proseguendo la terapia in atto come da protocollo per pB-LLA (AIEOP-BFM ALL 2009).

Conclusioni: il nostro studio ha permesso di identificare un nuovo sottotipo di BCP-ALL, caratterizzato dall'espressione aberrante di CD371 e potenziale switch mielo-monocitario durante la fase terapeutica di Induzione

1. Background

Acute lymphoblastic leukemia (ALL) is the most prevalent cancer in childhood, accounting for about 20% of all the malignancies under 20 years of age[1,2]. Remarkable progress in the outcome of pediatric ALL has been made in the last few decades, with a survival rate up to 90% in many developed countries[3-13]. Improved outcome can be attributed to the increased proficiency in the use of chemotherapy[14-17], the ability to control opportunistic infections[18,19], the use of innovative therapies[20,21], the improvement of biological knowledge of ALL with the identification of new subtypes[16,22-27], and a better evaluation of response to therapy by Minimal Residual Disease (MRD) assessment[28-31]. Regardless, ALL relapse still occurs in a significant proportion of patients and is associated with a severe outcome[32,33]. Therefore, it is mandatory to identify high-risk ALL subsets and address them to a more intense therapeutic regimen.

Multiparametric flow cytometry (MFC) is critical in the diagnosis and management of ALL, through immunophenotyping[34,35], DNA index measurement[36], and MRD analysis[31,37]. MFC plays a prominent role in the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues[38-40]. It is the most accurate approach for identifying, enumerating and characterizing leukemic cells. At diagnosis, MCF allows assigning blast cells to a specific hematopoietic lineage, the first fundamental step towards the proper management of ALL[41].

In the assessment of the response to therapy, MFC is the most reliable method, together with polymerase chain reaction (PCR), for the detection of MRD. MCF analysis of leukemia-associated immunophenotypes applies to almost 100% of patients and provides independent prognostic information, overriding the classical risk factors[31,37].

Different multicentric groups have proposed standardization strategies for MFC immunophenotyping[42,43] and MRD monitoring[44-45]. To improve the reproducibility and validation of MFC at diagnosis, the International Associazione Italiana Ematologia Oncologia Pediatrica and Berlin-Frankfurt-Munster Group (AIEOP-BFM) Flow Network published in 2017 *the AIEOP-BFM Consensus Guidelines 2016 for ALL immunophenotyping at diagnosis*. Dworzak et al. designed a panel of mandatory and optional monoclonal antibodies (MoAb) for the assignment of each leukemic

population to the proper hematopoietic lineage at diagnosis[23,24 43,46-49]. Moreover, they established the *AIEOP-BFM lineage assignment criteria,* adapted from the WHO classification[38-40], to define the dominant immunophenotype of blast cells at diagnosis. B-cell precursor ALL (BCP-ALL) was defined as the presence of at last two strong positivity of CD19, CD10, iCD22 or iCD79a antigens. The T-ALL subtype required a strong positivity of both iCD3 and CD7 antigens in the absence of iMPO expression; alternatively, it required a weak positivity of iCD3 and the simultaneous expression of CD2 or CD5 antigens. Finally, to belong to the myeloid lineage (AML), a strong positivity of two among iMPO, CD13, CD33, CD64, CD65, CD117 was necessary, in the absence of any criteria for BCP- or T-ALL. To evaluate antigen expression, the authors suggested a semiquantitative approach rather than reporting numerical values for each antibody. Indeed, qualitative description of antibody fluorescence distribution as “negative”, “positive”, “partially expressed” (compared to an appropriate negative control population), as well as antibody fluorescence intensity as “dim”, “bright”, “heterogeneous” (relative to the closest normal hemato-lymphoid population), provides a more precise description of the immunophenotypic deviation from normal cells[38-40,42,43,50]. Dworzak and colleagues’ efforts worked towards a standardization of that semiquantitative approach. They graded antigen expression comparing the fluorescence shift and distribution pattern of the blast cells to the appropriate negative control, as described before[34,43,51-53]. Antigen negativity was defined as the presence of less than 10% of positive blast cells compared to the control. The authors classified antigen positivity in two main categories: weak positivity when 10% to 49% of leukemic cells were positive for the analyzed antigen. Strong positivity was defined as a positive blast subset of at least 50%. In the presence of two distinct blast population, the authors named antigen expression as partial positive (PP)[34,42,43,51,53].

The aberrant expression of myeloid markers in B- and T-lineage ALL is a well-known phenomenon, observed both in adults and children[39,40,54-56]. When the degree of co-expression is so considerable that it is not possible to assign a blast population to any lineage with certainty, we may define it as mixed phenotype acute leukemia (MPAL). MPAL may contain two or more blast populations, belonging to different hematopoietic lineages (bilineage). Otherwise, a single population of leukemic cells may express multiple antigens of different lineages (biphenotypic). The WHO

Classification of Tumors of Haematopoietic and Lymphoid Tissues[38] allocates MPAL to the category of acute leukemia of ambiguous lineage, together with those leukemias expressing no lineage-specific antigens (acute undifferentiated leukemia). MPAL may show one combination of B/myeloid or T/myeloid antigens, irrespective of the number of blast populations seen. The association of an MPAL with t(9;22)(q34;q11.2);BCR-ABL1 and t(v;11q23);KMT2A rearranged, identifies two distinct subtypes, as per Who Classification. In the absence of those rearrangements, MPAL is classified as NOS (B/myeloid, NOS and T/myeloid, NOS). However, all those cases that may be assigned to any other category, either by genetic or clinical features, are excluded from MPAL definition. The diagnosis rests on MFC. The myeloid component may be identified in three ways: in the presence of two or more populations, one would meet immunophenotypic criteria for AML, always accounting for less than 20% of total nucleated cells. Secondly, a myeloid component is detected in the presence of a single ALL population co-expressing myeloperoxidase (iMPO). In this case, the myeloid lineage antigens CD13, CD33, CD117 are not specific enough to allow the identification of an MPAL. Finally, myeloid component is defined as the presence of a single ALL population with monoblastic differentiation (co-expression of non-specific esterase, CD11c, CD14, CD36, CD64, lysozyme). In some rare circumstances, leukemia may show combinations of markers that are not included in the previous definitions[38-40].

The aberrant expression of myeloid antigens is often associated with poor response to chemotherapy[54,57-59]. The association with peculiar genetic anomalies, such as t(v;11q23);KMT2A rearranged[60,61], or a specific biological subgroup, like early T cell precursor leukemia[62], may explain a worse outcome.

To notice, the expression of aberrant antigens may be the result of an adaptive mechanism by lineage switch, defined as any variation of blast immunophenotype over time or at relapse[54,57-59,63,64]. A lineage switch is classified as a rare subtype of MPAL[38,65], with a frequency between 6-9% of cases in relapse[63]. Usually, lineage switch disease is refractory to therapy and associated with a poor prognosis[66]. It has been observed in association with rearrangements involving KMT2A (MLL) gene on locus 11q23[54,59,61,67-74], as well with the translocation t(9;22)(q34;q11.2);BCR-ABL1[54,75,76], both in ALL and MPAL, at any age, including congenital and infant subtypes. Lineage switch may appear during the first-line chemotherapy[71,77], as well

as at relapse[59,63,65,78-80]. In the last few years, switch behavior has been observed following the administration of immunotherapy for relapsed/refractory leukemia [e.g., bispecific T-cell engager (BiTe) blinatumomab, CD19 chimeric antigen receptor (CAR) T cells], as a mechanism of immunological escape[58,72,81].

Lineage switch is usually permanent, but it was also observed as a transient phenomenon[77,78]. In 2014, Slamova et al. describe a new subset of BCP-ALL, characterized by the aberrant expression of the CD2 antigen, the absence of KMT2A rearrangements, and an early switch to the monocytic lineage during the steroid phase of Induction Therapy for ALL. Despite their phenotype, the monocytoid cells shared immunoreceptor gene rearrangements with BPC ALL blasts. The lineage switch was transient and disappeared proceeding with ALL chemotherapy[77].

AIEOP-BFM laboratories carried out many efforts to the standardization of MFC-MRD, working on a methodological harmonization in sample preparation, reagent selection, instrument set up, intra- and inter-laboratory quality monitoring, training of specific personnel focused on MFC data interpretation[44,50]. A positive MRD is defined as the detection of at least 10-20 clustered events with similar overall immunophenotypic features[50,82]. To reach a sensitivity threshold of 0.01%, the acquisition of a minimum amount of 100,000 nucleated cells is required. Moreover, the inclusion of Syto16/41 in the panel for MRD detection is recommended to include only nucleated cells in the analysis[50]. MCF-MRD is useful in evaluating early response to therapy in pediatric ALL and is a predictor of overall response. It allows a precocious identification of very high risk and very low-risk patients. In 2009, Basso and colleagues assessed the role of MFC-MRD role in BM on Day 15 of Induction Therapy in pediatric ALL patients. The authors identified three risk group: standard (<0.1% of blast cells), intermediate (0.1 to <10% of blast cells), and high (≥10% of blast cells), associated with a significantly different 5-years cumulative incidence of relapse (7.5%, 17.5%, and 47.2%, respectively). Nowadays, MFC-MRD on Day 15 is used to assign patients to the proper therapeutic risk groups, together with PCR-MRD at later time points. Moreover, it is the final criterium of risk group stratification, when PCR-MRD data are missing[28,83].

The improvement in technologies and, consequently, the introduction of 8-colors cytometers in the diagnostic field, have allowed the introduction of new markers for

immunophenotyping at diagnosis and MRD monitoring. Traditionally, the identification of new markers derived from the direct comparison between the immunophenotype of normal and leukemic cells[84]. Regardless, this approach was used recently and allowed the identification of CD81, CD49f, and CD11b as potential markers[85-87]. A different strategy is based on the comparison between the gene expression profiles of normal and leukemic cells. This approach led to the identification of CD58as a useful marker of MRD[88].

CD371, also known as 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[89]. It is expressed on normal monocytes, granulocytes, basophils, as well as most of the AML blasts and leukemic stem cells[90-96]. It has been recently indicated as a promising target of immunotherapy in AML[97-100]. The expression of CD371 in normal hematopoietic stem cells, T and B lymphocytes, and ALL blasts has not been previously described[89,92,95,97,99].

In the Laboratory of Diagnosis and Research of Pediatric Hematology Oncology, University of Padua, we also observed a transient switch to myelomonocytic lineage in a subset of BCP-ALL during the early phase of Induction Therapy Regardless, after the introduction of CD371 MoAb in the B-ALL panel for immunophenotyping at diagnosis, we noticed an apparent association between CD371 aberrant expression and the myelomonocytic switch. Consequently, we retrospectively analyzed BPC-ALL samples at diagnosis and during the response to therapy monitoring, to better define the clinical and biological features of CD371 positive BPC-ALL, with particular attention to switch behavior, as detected by MFC.

2. Objectives

The aims of this study are the following:

- to describe the frequency and clinical, biological and immunophenotypic characteristics at diagnosis of CD371 positive BPC-ALL in a cohort of pediatric patients uniformly treated.
- to evaluate the rate of the myelomonocytic switch in CD371 BCP-ALL and describe its immunophenotypic features and behavior.
- to provide a standardized approach to the detection of the myelomonocytic switch with MCF during MRD monitoring.
- to compare the accuracy of the aberrant expression of CD371 vs. CD2 antigen at diagnosis in BPC-ALL, as a marker of a myelomonocytic switch behavior.
- to analyze the early response to therapy of CD371 positive BPC-ALL
- to investigate the impact of the myelomonocytic switch on the detection of MCF-MRD on Day 15.
-to compare MFC-MRD vs. PCR-MRD results on Day 15 in CD371 positive BCP-ALL.

3. Materials and Methods

3.1 Study population and diagnostic workup

From June 2014 to January 2017, 862 children aged 1 to less than 18 years with newly diagnosed t(9;22)(q34.1;11.2);BCR-ABL1 negative BCP-ALL, were consecutively enrolled in the AIEOP BFM ALL 2009 study (NCT01117441) by AIEOP centers.

The diagnosis of BCP-ALL was based on standard morphologic, cytochemical, immunophenotypic, and genetic analysis [karyotype, DNA index, presence of primary chromosomal translocation t(12;21)(p13.2;q22.1);ETV6-RUNX1, t(1;19)(q23;p13.3);TCF3-PBX1, t(4;11)(q21;q23);KMT2A-AFF1], according to the therapeutic protocol[83]. Response to therapy was assessed on peripheral blood (PB) at Day 8 (standard morphology), and bone marrow (BM) samples at Days 15 (MFC-MRD), 33 (morphology and PCR-MRD) and 78 (PCR-MRD), as per AIEOP BFM ALL 2009 study[83]. PB and BM specimens were collected and centralized to the reference laboratories, respectively Padua for morphology, immunophenotyping, and MFC-MRD, and Monza for the molecular diagnosis, the screening of IGH rearrangements and PCR-MRD analysis[83]. When exceeding material was available, PCR-MRD and MCF-MRD were experimentally performed at each time point. Risk group stratification according to the therapeutic protocol is summarized in Table 1.
Local institutional ethical committees approved PB and BM sampling along with the international protocol. Each procedure was performed strictly according to informed consent guidelines.

3.2 Multiparametric Flow Cytometry analysis

PB and BM samples were processed and analyzed in the Laboratory of Diagnosis and Research of Pediatric Hematology Oncology, University of Padua, according to standardized operating protocols previously described[34,42,44].

Table S1: BCP-ALL Risk group stratification according to AIEOP-BFM ALL 2009 protocol

High risk group
Hypodiploidy (DNA index < 0.8)
t(9;22)(q34.1;11.2);BCR-ABL1
t(4;11)(q21;q23);KMT2A-AFF1
Day 8 (morphology): ≥ 1,000 blasts/uL on PB
Day 15 (MFC-MRD): ≥ 10% blasts on BM
Day 33 (morphology): non-complete remission (≥ 5% blasts in BM)
PCR-MRD on TP1(Day 33) and TP2 (Day 78)
PCR-MRD ≥ 10-3 on TP2
PCR-MRD Slow Early Response (SER): MRD $\geq 10^{-3}$ on TP1 and MRD pos $<10^{-3}$ on TP2
Standard risk group
t(12;21)(p13.2;q22.1);EVT6-RUNX1 in absence of any high risk criteria
Day 15: < 0.1% blasts in BM in absence of any high-risk criteria
Intermediate risk group
Any condition other than high and standard risk group

Abbreviations: PB: peripheral blood sample; MCF: multiparametric flow cytometry; MRD: minimal residual disease; PCR: polymerase chain reaction; BM: bone marrow sample; TP: time point

Briefly, we performed immunophenotyping at diagnosis on erythrocyte-lysed whole BM samples. We used a direct immunofluorescence technique with nine different 8-colors combinations of MoAbs, based on a CD45/CD19-backbone plus markers against stem cells, lymphoid and myeloid lineages (Table 2a). BM samples were delivered from the other AIEOP Centers at ambient temperature and processed within 24 hours of collection. We incubated 500,000 nucleated cells per analysis at room temperature for 15 min in the dark with the appropriate combination of MoAbs. Samples were then lysed using 3 ml of NH4Cl, washed in phosphate-buffered saline (PBS) and re-suspended in 0.5 ml of PBS.

We performed intracellular staining with a two-step fixation and permeabilization method, using a commercial kit (Valter Occhiena-Caltag Laboratories – Fix&Perm™, San Francisco, CA) according to manufacturer's instructions (Lanza, Kappelmayer).

We adopted the following monoclonal MoAbs: CD3-ACP-Cy7, CD4-FITC, CD8-PE, CD10-PE, CD11c-APC, CD15-FITC, CD16-PC5.5, CD19-V450, CD20-APC-Cy7,

CD22-PE, CD45-V500, CD66c-FITC, CD123-PC5.5, CD371-PE, kappa-FITC, lambda-PE, cyIg-PE (Becton Dickinson, Franklin Lakes, New Jersey, USA); cyCD3-APC, CD13-APC, CD65-FITC, HLA-DR-APC (Caltag Medsystem Ldt., Buckingham, UK); CD7-FITC, cyLYSO-FITC (Invitrogen, Carlsbad, CA, USA); CD2-PE, CD5-PC7, CD7-PC5.5, CD9-APC-Cy7, CD10-PC7, CD11b-APC, CD14-PC5.5, cyCD22-PC5.5, CD24-PC5.5, CD25-PC7, CD33-PC7, CD34-APC, CD34-APC-Cy7, CD38-PC5.5, CD38-PC7, CD56-PC7, CD58-FITC, CD64-APC-Cy7, cyCD79a-PC5.5, CD117-PC7, NG2-PE (Beckman Coulter, Inc., Brea, CA, USA); MPO-FITC (Sanquin, Amsterdam, The Netherlands); CD33-APC-Cy7, CD133-APC, CRLF2-PE (BioLegend, San Diego, CA, USA) 34,42,43,46, 77,102,103.

Cell acquisition was performed using a BD FACSCanto II cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA), equipped with three lasers: 488 nm blue, 633 nm red and 405 nm violet. Analyses were conducted using BD FACSDiva Software (Becton Dickinson, Franklin Lakes, New Jersey, USA). We acquired 30,000 events for each sample-MoAb combination (Johansson, Wood 2013). We routinely optimized the instrument set-up, analyzing Flow-CheckM Fluorospheres (Beckman Coulter, Inc., Brea, CA, USA) or similar products according to manufacturer's recommendations, and normal PB T lymphocytes stained with the anti-CD4-FITC/CD8-PE/CD45-ECD/CD7-Pe-Cy5/CD3-Pe-Cy7 five-colors combination, as previously indicated[34,42,52,104].

We graded antigen expression comparing the fluorescence shift and distribution pattern of the blast cells to the appropriate negative control, as described before (see Background) [34,42,43,51,53].

Consequently, we defined B-ALL dominant lineage as the presence of blast population with a strong positivity of at least two antigens among CD19, CD10, cyCD22, cyCd79a[42,43,49]. Three immunophenotypic subsets of BCP-ALL were identified according to EGIL classification (B-I, B-II, B-III). We excluded mature B-ALL (B-IV) according to the therapeutic protocol[46,83].

As concern MFC-MRD, we processed and analyzed the samples with the same procedure and instrument adopted at diagnosis, using 10,000,000 nucleated cells per analysis and acquiring at least 500,000 events[44]. MoAbs combinations included those markers previously shown as the most relevant for MRD detection in BCP-ALL[44,103,105]. Between June 2014 and May 2016, two 8-colors combinations were

adopted, including the following MoAbs: CD3-APC (Caltag Medsystem Ldt., Buckingham, UK); CD7-V450, CD10-PE, CD19-V450, CD20-APC-Cy7, CD22-PE, CD45-APC-Cy7, CD45-V550, CD123-PC5.5 (Becton Dickinson, Franklin Lakes, New Jersey, USA); CD19-PC7, CD34-APC, CD38-PC7, CD58-FITC (Beckman Coulter, Inc., Brea, CA, USA)[31,44] (Table 2b). Cell acquisition was performed using a BD FACSCanto II cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA). Analyses were conducted using BD FACSDiva Software (Becton Dickinson, Franklin Lakes, New Jersey, USA). Since June 2016, we adopted dry 10-colors pre-formulated DuraClone 10 Conj Custom Mix, Per Test, 2500 Test per Yr (Beckman Coulter, Inc., Brea, CA, USA), including the following MoAbs: CD3-APC, CD7-V450, CD9-PE, CD10-APC-Alexa700, CD19-PC7, CD20-V450, CD22-PE, CD34-APC, CD38-APC-Cy7, CD45-APC-Cy7, CD45-V500, CD58-FITC, CD123-ECD, CD371-PC5.5[31,106] (Table 2c). We acquired these latest samples with a Navios Flow Cytometer (Beckman Coulter, Inc., Brea, CA, USA), equipped with three lasers: 488 nm blue, 633 nm red and 405 nm violet. Software Kaluza 2.1 (Beckman Coulter, Inc., Brea, CA, USA) was used for samples analysis.

To identify dead cells and the efficiency of erythrocyte lysis, we adopted an additional staining combination of SYTO16-FITC (Molecular Probes, Leiden, The Netherlands), a live-cell-permeant nucleated-cell dye, and 7AAD-PC5.5 dye (Beckman Coulter, Inc., Brea, CA, USA)[44].

We defined MRD positivity as a cluster of at least 10 events with lymphoid-scattering properties and leukemia-associated immunophenotypic characteristics as previously reported[44].

Table 2: Antiboby panel used for B-precursor acute lymphoblastic leukemia immunophenotyping at diagnosis and MRD monitoring

a. Antibody panel used for immunophenotype at diagnosis

FITC	**PE**	**PC5.5**	**PC7**	**APC**	**APC-Cy7**	**V450**	**V500**
CD4	CD8	CD7	CD5	CD34	CD3	CD19	CD45
CD66C	NG2	CD7	CD33	CD13	CD34	CD19	CD45
CD58	CD10	CD123	CD38	CD34	CD20	CD19	CD45
CD7	CD2	CD16	CD56	cyCD3	CD3	CD19	CD45
CD15	CD22	CD24	CD117	HLA-DR	CD64	CD19	CD45
CD65	CD371	CD14	CD25	CD133	CD33	CD19	CD45
cyLYSO	CRFL2	CDcy79a	CD33	CD11C	CD9	CD19	CD45
MPO	cyIg	cyCD22	CD33	CD11B	CD20	CD19	CD45
Kappa	lambda	CD38	CD10	CD34	CD20	CD19	CD45

b. Antibody panel used for MFC-MRD from June 2014 to May 2016

FITC	**PE**	**PC5.5**	**PC7**	**APC**	**APC-Cy7**	**V450**	**V500**
CD58	CD10	CD123	CD38	CD34	CD20	CD19	CD45
SYTO16	CD22	7AAD	CD19	CD3	CD45	CD7	

c. Antibody panel used for MFC-MRD after May 2016 DuraClone 10 Conj Custom Mix, Per Test, 2500 Test per Yr (Beckman Coulter, Inc., Brea, CA, USA)

FITC	**PE**	**ECD**	**PC5.5**	**PC7**	**APC**	**APC-ALEXA700**	**V450**	**V500**
CD58	CD9	CD123	CD371	CD19	CD34	CD10	CD20	CD45
SYTO16	CD22		7AAD	CD19	CD3	CD45	CD7	

Abbreviations: FITC: fluorescein isothiocyanate; PE: phycoerythrin; PC5.5: R Phycoerythrin-Cyanin 5.5; PC7: R phycoerythrin cyanine 7; APC: allophycocyanin; APC-Cy7: allophycocyanin-cyanine 7; ECD: electron coupled dye

3.3 RT-PCR MRD evaluation

MRD markers were identified and the PCR assay was performed as previously described[107-109].

Briefly, DNA samples obtained at diagnosis were screened for *IGH* rearrangements. We designed allele-specific oligonucleotide (ASO) primers to complement the junctional region sequence of each target. We tested MRD-PCR targets and selected two of them for each patient. A sensitivity of at least 10^{-4} was required, with a quantitative range of at least 10^{-4} for the first target, and 5×10^{-4} for the second one[107-109].

We performed and interpreted real-time quantitative PCR analysis according to the *European Study Group for MRD detection in ALL* (EuroMRDALL) guidelines[109].

3.4 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), PEG-Asparaginase (2 doses) and intrathecal methotrexate (2 doses). Remission induction was followed by Induction IB phase (intravenous cyclophosphamide and cytarabine, intrathecal methotrexate, and oral mercaptopurine)[83].

3.5 Statistical analysis

We adopted descriptive methods to represent all the data of this study. We used absolute frequencies and percentages for dichotomous and categorical variables.

Continuous variables were expressed by median, range, mean, and standard deviation, except for age, white blood cells count, and DNA index, that were analyzed as categorial variables on established criteria. We applied the Chi-square or Fisher exact tests for group-wise comparisons of categorical variables, depending on expected cell values above or below five, respectively. Two-sided p values less than 0.05 were regarded as significant.

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

4. Results

4.1. CD371 positive BCP-ALL: features at diagnosis

In this study, we included 862 patients with newly diagnosis of BCP-ALL. Of those, 823 (95.5%) were evaluated for CD371 expression at diagnosis. CD371 antigen was positive (CD371+) in 75 (9.1%) patients, whereas 748 (90.9%) were negative (CD371-). At diagnosis, we found an association between CD371+ and older age [≥ 10 years: 34 of 75 (45.3%) CD371+ patients vs. 130 of 748 (17.4%) CD371- patients, $p < 0.001$], and euploidy [DNA index =1: 65 of 75 (86.7%) CD371+ patients vs. 437 of 748 (63.2%) CD371- patients, $p < 0.001$]. We did not observe any difference in the distribution of gender [males: 40 of 75 (53.3%) CD371+ patients vs. 406 of 748 (54.3%) CD371- patients, $p = 0.88$], peripheral white blood cells count (WBC) [WBC ≥ 100 $x10^9$/L: 4 of 75 (5.3%) CD371+ patients vs. 40 of 748 (5.3%) CD371- patients, $p = 0.28$], and t(1;19)(q23;p13.3);TCF3-PBX1 [0 of 75 (0%) CD371+ patients vs. 30 of 748 (4.0%) CD371- patients] or t(4;11)(q21;q23);KMT2A-AFF1 [1 of 75 (1.3%) CD371+ patients vs. 5 of 748 (0.7%) CD371- patients] between CD371 + and CD371- patients. The rearrangement t(12;21)(p13.2;q22.1);EVT6-RUNX1 was detected more frequently in CD371- BCP-ALL [1 of 75 (1.3%) CD371+ BCP-ALL vs. 187 of 748 (25%) CD371- BCP-ALL, $p < 0.001$] (Table 3).

The immunophenotyping at diagnosis showed an association between CD371+ BCP-ALL and a more immature immunophenotype, as defined by EGIL classification [BI-ALL: 10 of 75 (13.3%) CD371+ BCP-ALL vs. 12 of 748 (1.6%) CD371- BCP-ALL; BII-ALL: 65 of 75 (86.7%) CD371+ BCP-ALL vs. 555 of 748 (74.2%) CD371- BCP-ALL, BIII-ALL: 0 of 75 (0%) CD371+ BCP-ALL vs. 181 of 478 (24.2%) CD371- BCP-ALL, $p < 0.001$). (Table 3)

A strong expression of CD34 [73 of 75 (97.3%) CD371+ BCP-ALL vs. 56 of 741 (7.6%) CD371- BCP-ALL, $p = 0.007$], CD45 [49 of 75 (65.3%) CD371+ BCP-ALL vs. 208 of 746 (27.9%) CD371- BCP-ALL, $p < 0.001$], and CD58 [72 of 75 (96.0%) CD371+ BCP-ALL vs. 657 of 745 (87.8%) CD371- BCP-ALL, $p = 0.04$] antigens was observed in these patients. Moreover, CD371 positivity was associated with

Table 3: Patients and leukemia's main features at diagnosis

Variable	Total pts (n)	CD371+ pts (n)	CD371- pts (n)	*P*
Age				**< 0.001**
1-9 years	659	41	618	
10-17 years	164	34	130	
Gender				0.88
Male	446	40	406	
Female	377	35	342	
WBC count				0.28
< 20 x 10^9/L	598	60	538	
20-99 x 10^9/L	180	11	169	
≥ 100 x 10^9/L	44	4	40	
DNA index				**< 0.001**
< 0.8	7	0	7	
0.8-1.16	608	67	541	
1.16-1.6	187	4	183	
≥ 1.6	13	3	10	
DNA index = 1	502	65	437	**< 0.001**
Translocations				
t(12;21)(p13;q22)	188	1	187	**< 0.001**
t(1;19)(q23;p13)	30	0	30	0.10
t(4;11)(q21;q23)	6	1	5	0.43
Immunophenotype at diagnosis (EGIL)				**<0.001**
BI-ALL	22	10	12	
BII-ALL	620	65	555	
BIII-ALL	181	0	181	
Ambiguous leukemia				
BAL (EGIL)	6	1	5	0.46
MPAL (WHO)	23	3	20	0.44

Abbreviations: pts: patients; CD371+: CD371 positive; CD371-: CD371 negative; n: number; BAL: biphenotypic acute leukaemia; MPAL: mixed phenotype acute leukemia

the aberrant expression of at least one myeloid marker out of CD11b, CD14, CD15, CD33, CD64, CD65, MPO, LYSO, CD13, CD117 [42 of 75 (56.0%) CD371+ BCP-ALL vs. 240 of 748 (32.1%) CD371- BCP-ALL, $p < 0.001$] (Figure 1). Regardless, when we analyzed myeloid antigens one by one, we did not find any significant difference in their distribution between CD371+ and CD371- BCP-ALL (data not reported in the present manuscript). We did not observe any difference in the distribution of biphenotypic acute leukemia (BAL) according to EGIL classification [1 of 75 (1.3%) CD371+ BCP-ALL vs. 5 of 748 (0.7%) CD371- BCP-ALL, $p = 0.46$], as well as mixed phenotype acute leukemia (MPAL) by WHO definition [3 of 75 (4.0%) CD371+ BCP-ALL vs. 20 of 748 (2.7%) CD371- BCP-ALL, $p = 0.44$] between CD371+ and CD371- BCP-ALL (Table 3). Finally, our analysis showed a significant association between CD371+BCP-ALL and the aberrant expression of CD2 antigen [38 of 75 (50.7%) CD371+ BCP-ALL vs. 3 of 748 (0.4%) CD371- BCP-ALL, $p < 0.001$) (Figure 1). For a detailed description of the immunophenotype of CD371+ and CD371- BCP-ALL at diagnosis, see Table 4.

4.2 CD371 positive pB-ALL: MFC-MRD evaluation

We performed MFC-MRD analysis on a total of 207 samples. On Day 15 of Induction Therapy, we assessed MFC-MRD on BM in all the CD371+ patients, as per therapeutic protocol. Of those, 72 (96%) samples were evaluable for MCF-MRD detection: 3 samples did not reach the minimum number of events required to obtain a sensitivity threshold of 1×10^{-4} nucleated cells and were excluded from the final analysis. As an additional study, we performed MFC-MRD during Induction Therapy on PB in 42 (56%) CD371+ patients on Day 8, and on BM samples in 40 (53.3%) and 53 (70.7%) CD371+ BCP-ALL specimens on Day 33 and Day 78, respectively.

Altogether, we adopted the 8-colors combination in 138 of 207 (66.7%) samples [26 of 42 (61.9%) samples on Day 8, 44 of 72 (61.1%) samples on Day 15, 30 of 40 (75%) samples on Day 33, 38 of 53 (71.7%) samples on Day 78]. Whereas, we used the 10-colors DuraClone in 69 of 207 (33.3%) samples [16 of 42 (38.1%) samples on Day 8, 28 of 72 (38.9%) samples on Day 15, 10 of 40 (25%) samples on Day 33, 15 of 53 (28.3%) samples on Day 78].

During the first 15 days of Induction therapy, we observed an increased percentage of monoblastic cells in CD371+ PB and BM smears by optic microscopy (Figure 2). At the same time, 51 (68%) CD371+ samples showed the appearance of a monocytoid population, characterized by strong expression of CD34, CD58 and CD45, reduced expression of CD19, and high SSC. To notice, CD45 and SSC were both more intense than those seen in myeloid precursor population in the same samples.

This phenomenon, interpreted as a switch to the myelomonocytic lineage, displayed two different patterns: the presence of a single population of blast cells with heterogeneous expression of CD19 (strong to dim/negative) (Figure 3a). Alternatively, two distinct populations were detected: the first one keeping the immunophenotypic pattern of diagnosis; the second one showing a downregulation of CD19 and CD34 intensity, an intensification of CD45, and an increase of SSC (Figure 3b).

To standardize the approach to switch detection, we analyzed all the 207 available samples as follows: starting with a CD19/SSC dot plot, we selected all the events characterized by low SSC, independently from CD19 intensity (negative to strong). Of those, on a CD20/CD10 dot plot, we isolated all CD20 negative events. Then, we applied the FSC/SSC gate, based on physical parameters. Finally, we analyzed the selected events on CD34/CD58, CD19/SSC, and CD45/SSC gates at the same time, to detect the presence of switch and define its expression as the single or two-populations pattern (Figure 3).

We observed a myelomonocytic switch in 26 of 42 CD371+ samples (61.9%) on Day 8 and in 50 of 72 CD371+ samples (69.4%) on Day 15. Regardless, chemotherapy according to AIEOP BFM ALL 2009 protocol was carried on in all the patients. Finally, lineage switch disappeared utterly, as we could never detect it on Day 33 and Day 78 (Table 5). Of the 26 CD371+ BCP-ALLs that showed a lineage switch on Day 8, 25 (96.2%) were still positive on Day 15. Of the 16 CD371+ BCP-ALL negative for a switch on Day 8, 5 (31.3%) were positive at Day

Figure 1: Main antigen expression at diagnosis: comparison between CD371 positive and CD371 negative BCP-ALL

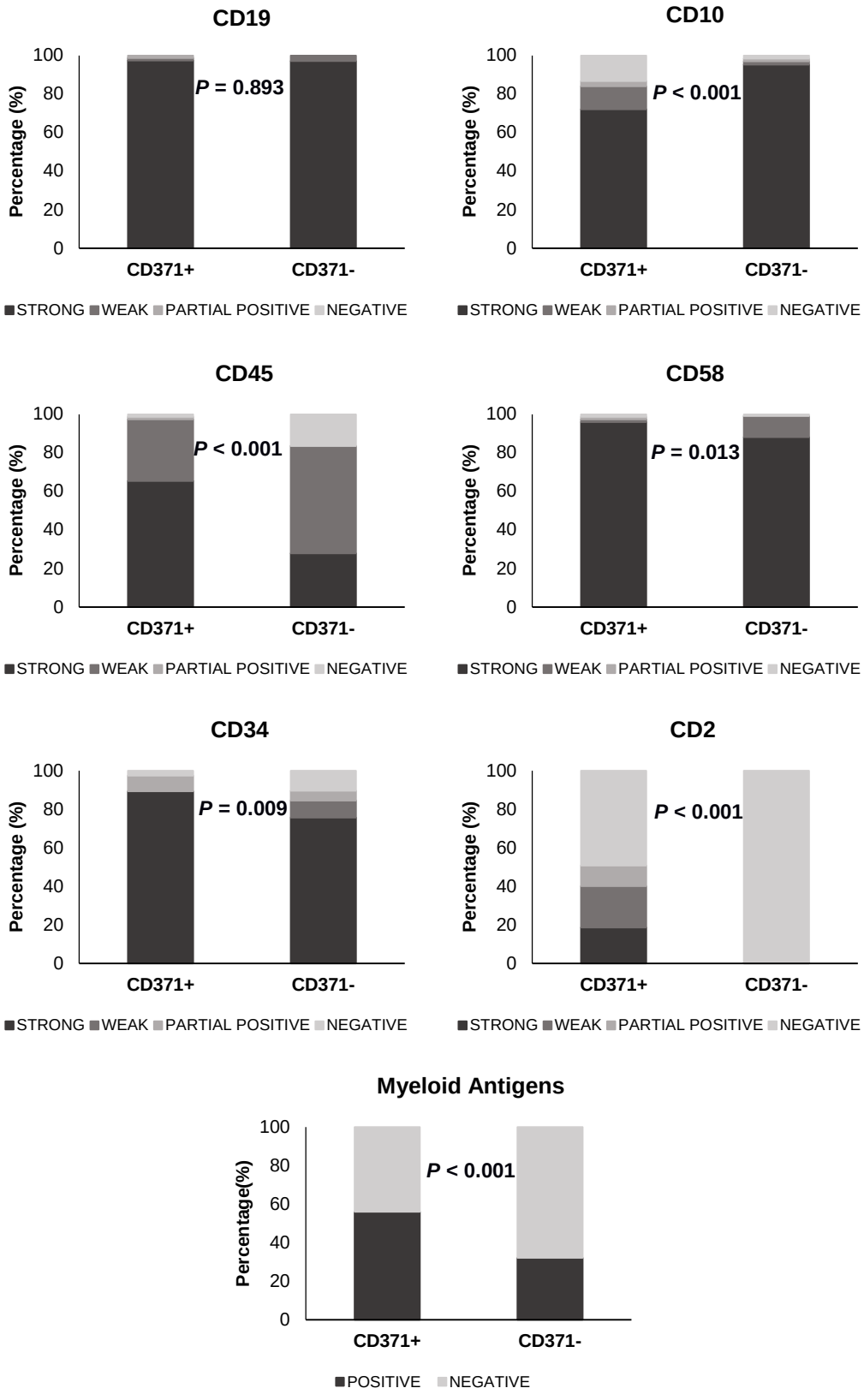


Table 4: Immunophenotype at diagnosis: antigen expression distribution in CD371 positive and CD371 negative pB-ALL

	CD371 POSITIVE		**CD371 NEGATIVE**		
	N	**%**	**N**	**%**	***P***
CD45					**< 0.001**
STRONG	49	65.4	208	27.9	
WEAK	24	32.0	415	55.6	
PP	1	1.3	1	0.1	
NEG	1	1.3	122	16.4	
NOT DONE	0	0.0	2	0.3	
CD19					0.893
STRONG	73	97.3	726	97.1	
WEAK	1	1.3	22	2.9	
PP	1	1.3	0	0.0	
NEG	0	0.0	0	0.0	
NOT DONE	0	0.0	0	0.0	
CD10					**< 0.001**
STRONG	54	72.0	712	95.2	
WEAK	9	12.0	13	1.7	
PP	2	2.7	11	1.5	
NEG	10	13.3	12	1.6	
NOT DONE	0	0.0	0	0.0	
CD58					**0.013**
STRONG	72	96.0	657	88.2	
WEAK	1	1.3	81	10.9	
PP	1	1.3	0	0.0	
NEG	1	1.3	7	0.9	
NOT DONE	0	0.0	3	0.4	
CD38					**< 0.001**
STRONG	70	93.3	679	92.0	
WEAK	0	0.0	53	7.2	
PP	2	2.7	0	0.0	
NEG	3	4.0	6	0.8	
NOT DONE	0	0.0	10	1.3	
	CD371 POSITIVE		**CD371 NEGATIVE**		

	N	**%**	N	%	***P***
CD34					**0.009**
STRONG	67	89.3	560	74.9	
WEAK	0	0.0	66	8.8	
PP	6	8.0	38	5.1	
NEG	2	2.7	77	10.3	
NOT DONE	0	0.0	7	0.9	
CD2					**< 0.001**
STRONG	14	18.2	1	0.1	
WEAK	16	21.3	2	0.3	
PP	8	10.7	0	0.0	
NEG	37	49.3	743	99.6	
NOT DONE	0	0.0	2	0.3	
CD7					0.809
STRONG	0	0.0	5	0.7	
WEAK	2	2.7	11	1.5	
PP	0	0.0	1	0.1	
NEG	73	97.3	730	97.7	
NOT DONE	0	0.0	1	0.1	
CD56					**< 0.001**
STRONG	4	5.3	3	0.4	
WEAK	7	9.3	6	0.8	
PP	1	1.3	0	0.0	
NEG	63	84.0	728	98.8	
NOT DONE	0	0.0	11	1.5	
MYELOID ANTIGENS*					**< 0.001**
YES	42	56.0	240	32.1	
NO	33	44.0	508	67.9	

*Any myeloid antigen of CD11b, CD14, CD15, CD33, CD64, CD65, MPO, LYSO, CD13, CD117
Abbreviations: STRONG: strong positivity; WEAK: weak positivity; PP: partial positivity; NEG: negativity; YES: any positivity (weak, strong, partial positive); NO: negativity of all myeloid antigens

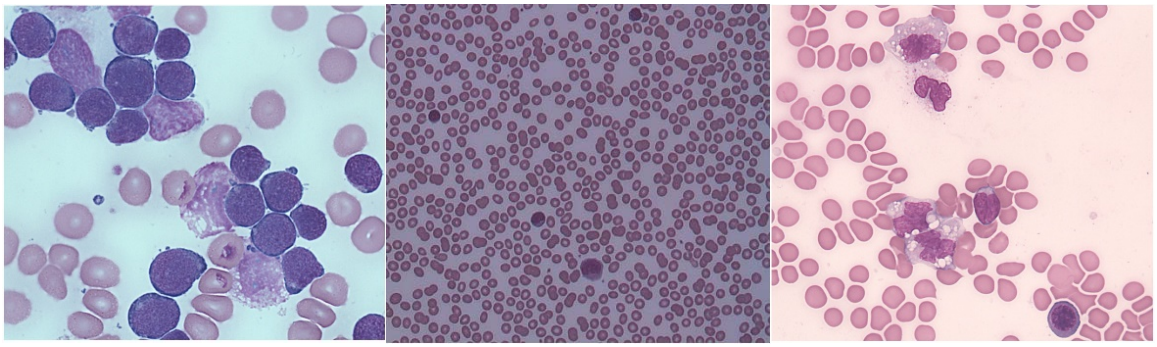
15. Among the 42 CD371+ patients with an available MFC MRD evaluation both on Day 8 and Day 15, 11 (15.3%) did not show any switch. Of those, 2 (18.2%) were negative or partial positive for CD34 at diagnosis, and consequently more difficult to be evaluated for MRD with MFC using the standard panel. Six of them (54.5%) showed an MRD less than 0.1%. To notice, we could not detect any lineage switch in our cohort when MRD was less than 0.1%, making it a potential sensitivity cut off (Figure 4).

As concern the switch pattern, we detected two distinct populations of blast cells in 71 of 207 (34.3%) samples, 24 on Day 8 and 47 on Day 15, respectively. Only 5 of 207 (2.4%) specimens showed the single-population switch pattern, 2 on Day 8 and 3 on Day 15, respectively. Of the 2 BCP-ALL with one blast cell population on Day 8, both changed their switch profile to the two-populations pattern on Day 15.

4.3 CD371 and CD2 antigens as markers of myelomonocytic lineage switch

We observed a significant association between CD371 positivity and CD2 expression at diagnosis [38 of 75 pts (50.6%) vs. 3 of 746 pts (0.4%), $p < 0.001$], even if 37 of 75 (49.3%) CD371+ samples were negative for CD2. To understand which antigen between CD371 and CD2 could better predict a transient switching behavior during Induction Therapy, we analyzed the two parameters independently (Table 6). Of the 37 BCP-ALLs expressing CD2 at diagnosis, 32 (86.4%) showed a switching behavior. Regardless, we found a lineage switch in 23 of 771 (3.0%) samples negative for CD2 at diagnosis, whereas we observed this phenomenon in just 4 of 748 (0.5%) CD371- patients at diagnosis. Consequently, CD371 antigen had a higher sensitivity for the detection of the myelomonocytic switch in our cohort.

Figure 2: CD371 positive BCP-ALL standard morphology at diagnosis and at different re-evaluation time points during Induction Therapy. **a-** At diagnosis, CD371 positive BCP-ALL showed an L1-ALL morphology, according to FAB classification, without any abnormal monocytoid population (bone marrow sample); **b-** On Day 8, after steroid therapy, a prevalence of monoblastic cells was observed on peripheral smear; **c-** On Day 15, a distinct population of monoblastic cells could be detected on bone marrow smear.



a **b** **c**

Figure 3: Switching behavior in CD371 positive BCP-ALL. With the beginning of steroid therapy according to AIEOP BFM ALL 2009 protocol, surface antigen expression of residual lymphoblastic cells could show a switch towards the myelomonocytic lineage. Switching behavior displayed two different patterns: **a-** a single population of blast cells with heterogeneous expression of CD19 (single population pattern: blue population); **b-** a two distinct populations pattern: the first population keeping the immunophenotypic pattern of the diagnosis (orange population); the second population showing a reduction of CD19 and CD34 intensity, an intensification of CD45, and an increase of SSC (blue population).

Figure 3a

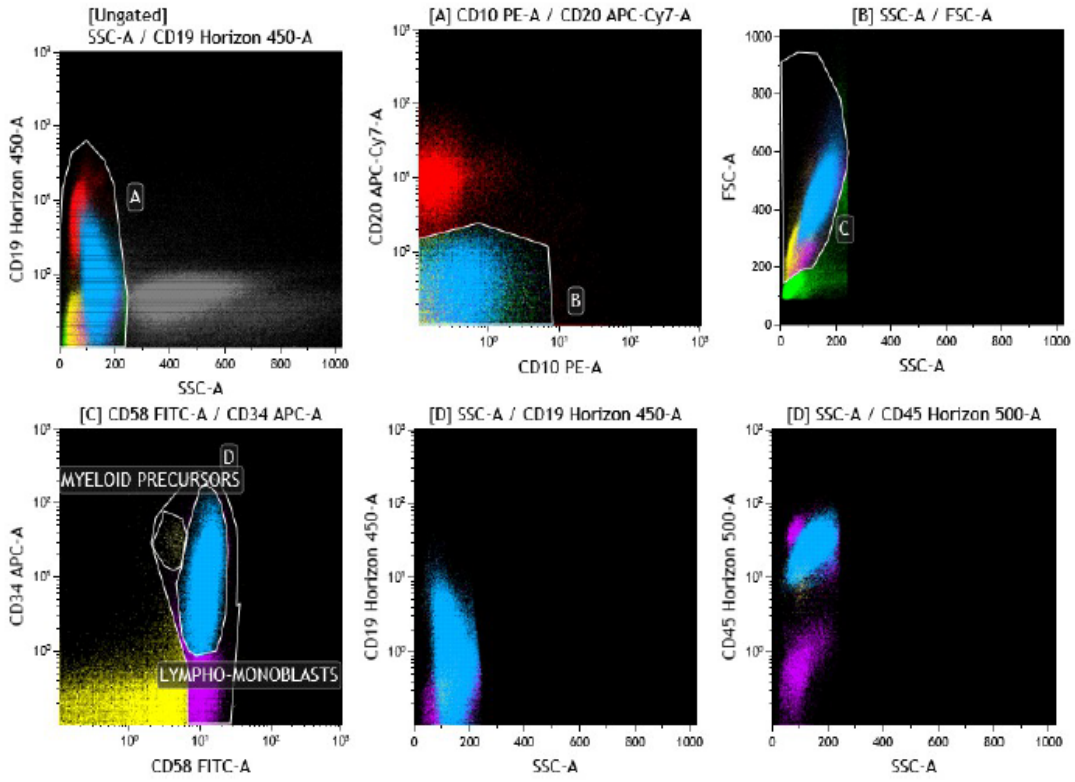


Figure 3b

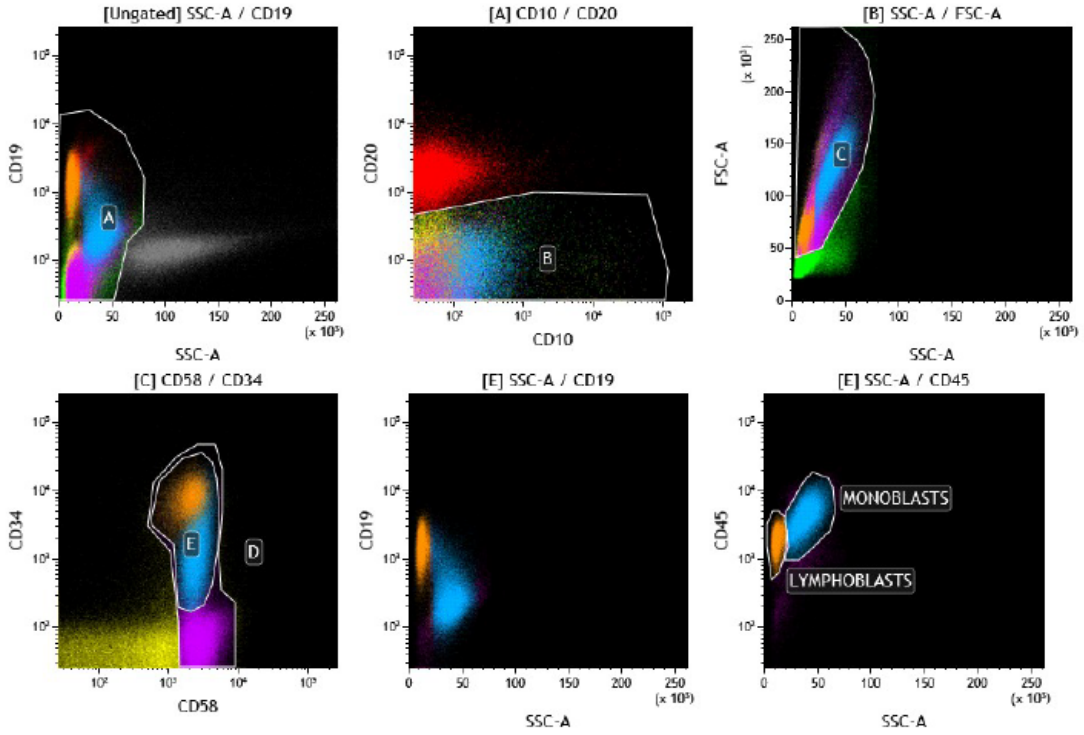
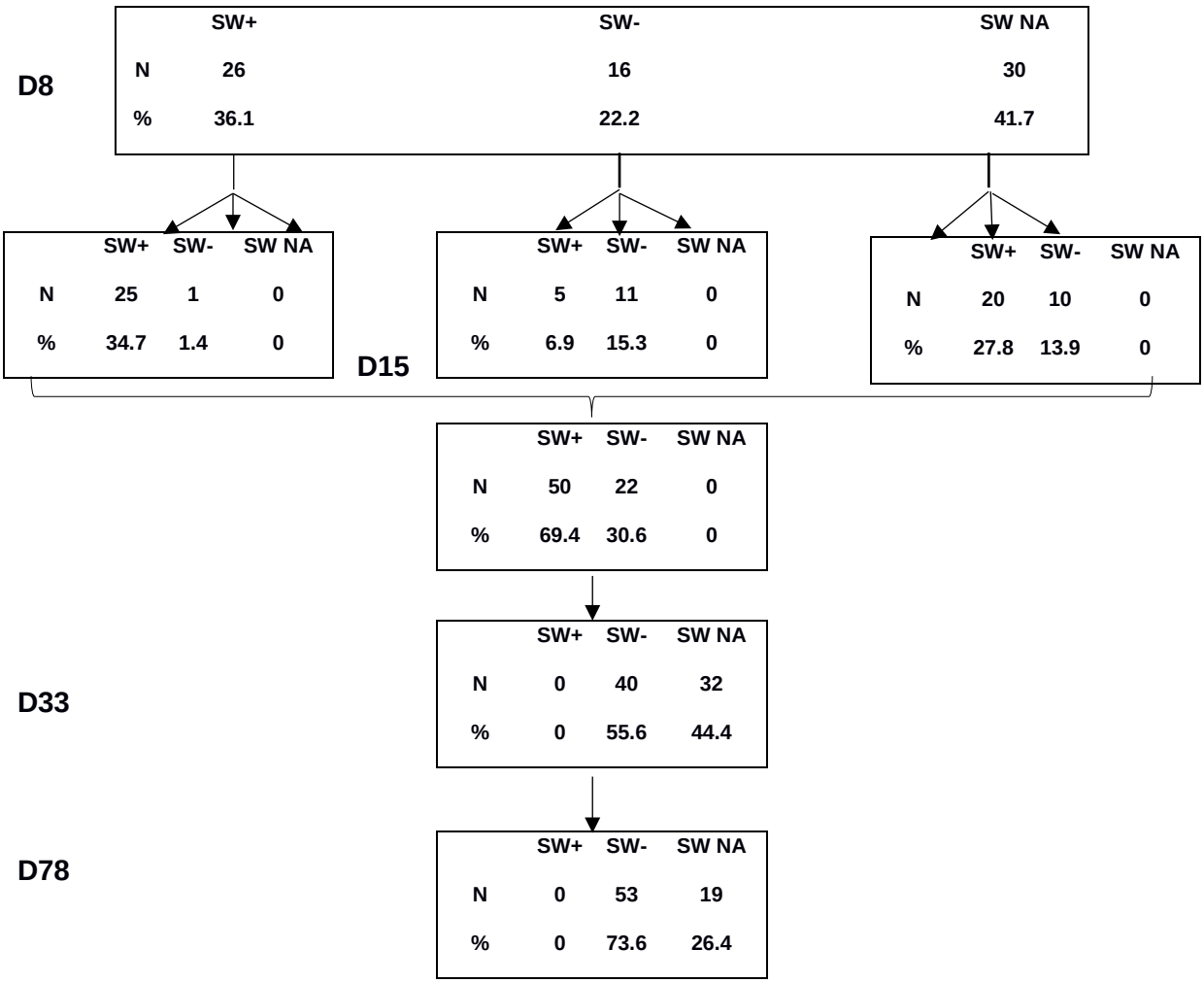


Table 5: Distribution of the lineage switch in CD371 positive BCP-ALL at diagnosis, on Day 8, Day 15, Day 33, and Day 78

	Diagnosis		**Day 8**		**Day 15**		**Day 33**		**Day 78**	
	n	**%**	**n**	**%**	**N**	**%**	**n**	**%**	**N**	**%**
Switch +	0	0.0	26	**36.1**	**50**	**69.4**	0	0.0	0	0.0
Switch -	72	100.0	16	22.2	22	30.6	40	55.6	53	73.6
Total	72	100.0	42	58.3	72	100.0	40	55.6	53	73.6
Not done	0	0.0	**30**	**41.7**	0	0.0	**32**	**44.4**	**19**	**26.4**

Abbreviations: n: number of samples; %: percentage; Switch +: presence of Switching behaviour; Switch -: absence of Switching behavior.

Figure 4: Lineage switch evolution in CD371 positive BCP-ALL during Induction Therapy according to AIEOP BFM ALL 2009 protocol. Lineage switch could be observed since Day 8 or later on Day 15. Lineage switch was never detected on Day 33 nor Day 78



Abbreviations: SW+: presence of lineage switch; SW-: absence of lineage switch; SW NA: MCF-MRD not available; N: number of samples; %: percentage; D: day

Table 6: The role of CD371 (a) and CD2 (b) antigens in predicting BCP-ALL switching behavior during Induction Therapy of AIEOP BFM ALL 2009 protocol. Patients without an MFC-MRD assessment on Day 8 were excluded from this analysis.

a

	SWITCH+	SWITCH-	TOTAL
CD371+	51	11	62
CD371-	4	744	748
TOTAL	55	755	810

Sensitivity = 0.93 (95% CI ± 0.06)

Specificity = 0.98 (95% CI ± 0.005)

Positive Predictive Value = 0.82

Negative Predictive Value = 0.99

Accuracy = 0.98

b

	SWITCH+	SWITCH-	TOTAL
CD2+	32	5	37
CD2-	23	748	771
TOTAL	55	753	808

Sensitivity = 0.58 (95% CI ± 0.06)

Specificity = 0.99 (95% CI ± 0.004)

Positive Predictive Value = 0.86

Negative Predictive Value = 0.97

Accuracy = 0.96

Abbreviations: SWITCH+: presence of switching behavior; SWITCH-: absence of switching behavior; CD371/CD2+: any positivity of CD371/CD2 antigen at diagnosis; CD371/CD2-: negativity of CD371/CD2 antigen at diagnosis. CI: confidence interval)

4.4 MCF-MDR on Day 15: impact of lineage switch on the assignment of risk group

Of the 50 CD371+ patients showing a lineage switch on Day 15, 47 (94.0%) displayed a two-populations pattern of blast cells. To understand the role of each population in the final assessment of MFC-MRD and risk assignment, we analyzed the two populations individually. In the first population, characterized by an immunophenotype overlapping to the diagnosis (orange population in Figure 3b), blast cells ranged from 0.03% up to 21.29% of all nucleated cells (mean 2.56%, median 0.42%, sample standard deviation 4.20). In the second population, showing a reduction of CD19 and CD34 intensity, an intensification of CD45, and an increase of SSC (blue population in Figure 3b), blast cells ranged from 0.01% to 22.28% of all nucleated cells (mean 2.57%, median 0.41%, sample standard deviation 4.70). In the final assessment of MFC-MRD on Day 15, the exclusion of the second population from the blast count would have underestimated the risk group in 12 of 47 (25.5%) patients (Table 7). We always included both the populations in the analysis of MFC-MRD on Day 15 in our Center.

Table 7: Difference in risk group assignment by MFC-MRD on Day 15 between the exclusion and inclusion of the second population of blast cells in presence of a two-population pattern lineage switch

MFC-MRD	First population alone		First + second population	
Risk group	n	%	N	%
<0.1%	6	12.8	0	0
0.1 to <10%	39	78.0	39	83.0
≥ 10%	2	4.0	8	17.0

Abbreviations: MFC: multiparametric flow cytometry; n: number of patients; % percentage; MRD: minimal residual disease. First population: in lineage switch, population of blast cells keeping the same immunophenotype of diagnosis; Second population: in lineage switch, population of blast cells showing myelomonocytic antigens

4.5 Response to the Induction Therapy and final risk group

CD371+ BCP-ALL showed an inferior response to Induction chemotherapy according to AIEOP BFM ALL 2009 protocol than CD371- BCP-ALL, with a significantly higher rate of patients enrolled in the final high-risk therapeutic arm [27 of 75 (36.0%) CD371+ patients vs. 345 of 747 (19.7%) CD371- patients, $p < 0.001$], even if not associated with high-risk features at diagnosis [hypodiploidy, t(4;11)(q21;q23);KMT2A-AFF1] (Table 8). At a separate analysis of each re-evaluation time point during Induction phase, we did not observe any difference in the response to prednisone therapy, evaluated as the absolute blast count on a PB sample on Day 8 [prednisone poor response (blast cells > 1,000/µL): 3 of 75 (4.0%) CD371+ samples vs. 52 of 747 (7.0%) CD371- available samples, $p = 0.47$]. As well, no difference was found in failure to achieve morphological complete remission on BM on Day 33 [non-complete remission: 0 of 72 (0%) CD371+ available samples vs. 10 of 739 (1.4%) CD371- available samples, $p = 1.0$]. On the other hand, CD371+ BCP-ALL showed a lower rate of patients assigned to the standard-risk group according to MFC-MRD on Day 15 [16 of 75 (21.3%) CD371+ samples vs. 284 of 735 (38.6%) CD371- available samples, $p= 0.01$]. Regardless, the most important finding in the response to the Induction Therapy was the significantly high proportion of CD371+ patients with a PCR-MRD at Time Point 1 (Day 33) and Time Point 2 (Day 78) belonging to the high-risk group [22 of 73 (30.1%) CD371+ available samples vs 67 of 714 (9.4%) CD371+ available samples, $p < 0.001$]. To notice, most of them responded to the definition of Slow Early Response (SER) [18 of 73 (24.7%) CD371+ available samples vs. 48 of 714 (6.7%) CD371- available samples]. Additional details on high-risk features in our cohort are summarized in Table 9.

Table 8: Response to Induction Therapy and risk group stratification according to AIEOP BFM ALL 2009 protocol criteria.

	CD371+		CD371-		TOTAL		*P*
	n	**%**	**n**	**%**	**n**	**%**	
Prednisone response							0.47
Good (PGR)	72	96.0	695	93.0	767	93.3	
Poor (PPR)	3	4.0	52	7.0	55	6.7	
MCF-MRD Day 15							**0.01**
<0.1%	16	21.6	284	38.6	300	37.1	
≥0.1-<10%	46	62.2	375	51.0	421	52.0	
≥10%	12	16.2	76	10.4	88	10.9	
Response to Induction IA							1.0
CR Day 33	72	100.0	729	98.6	801	98.8	
Non-CR Day 33	0	0.0	10	1.4	10	1.2	
PCR-MRD at TP1							**< 0.001**
Negative	11	15.1	262	36.6	273	34.6	
POS < 5×10^{-4}	28	38.3	349	48.8	377	47.8	
POS ≥ 5×10^{-4}	34	46.6	104	14.6	138	17.5	
PCR-MRD at TP2							**0.007**
Negative	43	57.5	543	76.0	585	74.3	
POS < 5×10^{-4}	27	37.0	152	21.3	179	22.7	
POS ≥ 5×10^{-4}	4	5.5	19	2.7	23	2.9	
PCR-MRD FINAL							**< 0.001**
Standard	11	15.1	253	35.4	264	33.6	
Medium	40	54.8	394	55.2	434	55.1	
MRD-SER	18	24.6	48	6.7	66	8.4	
High	4	5.5	19	2.7	23	2.9	
FINAL RISK GROUP							**< 0.001**
Standard	12	16.0	256	34.2	268	32.6	
Medium	36	48.0	345	46.1	381	46.3	
High	27	36.0	147	19.7	174	21.1	

Abbreviations: CD371+: any positivity (strong, weak, partial positive) of CD371 antigen at diagnosis; CD371-: negativity of CD371 antigen at diagnosis; MFC: multiparametric flow cytometry; MRD: minimal residual disease; PCR: polymerase chain reaction; POS: positive; SER: slow early response; TP: time point. **Data not available:** prednisone response: 1; MFC-MRD: 14; response to Induction IA: 12; PCR-MRD at TP1: 35; PCR MRD at TP2: 36

Table 9: Details on high-risk features in CD371 positive **(a)** and CD371 negative **(b)** BCP-ALL

a: CD371 positive BCP-ALL at diagnosis

HR criteria (hierarchical)	N	%	Additional HR criteria
t(4;11) positivity	1	1.3	SER MRD (N=1)
Hypodiploidy	0	0.0	/
Prednisone poor response	3	4.0	SER MRD (N=1)
HR MFC-MRD Day 15	3	4.0	/
Non-complete remission Day 33	0	0	/
SER MRD	16	21.3	HR MFC-MRD Day 15 (N=7)
HR MRD	4	5.3	HR MCF-MRD Day 15 (N=2)
TOTAL	27	36.0	/

b: CD371 negative pB-ALL at diagnosis

HR criteria (hierarchical)	N	Additional HR criteria
t(4;11) positivity	4	PPR (N=1)
Hypodiploidy	7	HR MCF-MRD Day 15 (N=1)
Prednisone poor response	46	SER MRD-HR MCF-MRD Day 15 (N=6) HR MCF-MRD Day 15 (N=2) HR MCF-MRD Day 15 (N=12)
HR MFC-MRD Day 15	31	/
No complete remission Day 33	3	PPR-SER MRD-HR MCF-MRD Day 15 (N=1) SER MRD-HR MCF-MRD Day 15 (N=1) SER MRD (N=1)
SER MRD	37	HR MCF-MRD Day 15 (N=16)
HR MRD	19	No CR-t(4;11)-PPR (N=2) No CR-PPR-HR MCF-FLOW Day 15 (N=1) No CR-HR MCF-FLOW Day 15 (N=3) No CR (N=1) No CR-PPR-HR MCF-FLOW Day 15 (N=2) No CR-t(4;11)-PPR(N=1)
TOTAL	147	/

Abbreviations: HR: high risk; N: number of patients; SER: slow early response; MRD: minimal residual disease; MFC: multiparametric flow cytometry; PPR: prednisone poor response; CR: complete remission

4.6 CD371 positive BCP-ALL: comparison between MFC-MRD and PCR-MRD on Day 15

We compared MCF-MRD values with PCR-MRD findings at the same time point. PCR-MRD data were available in 38 of 72 (52.8%) CD371+ patients. We found a concordance of FCM-MRD and PCR-MRD in 31 of 37 (83.8%) samples when we considered all the CD371+ available data (Table 10a). The concordance was higher (23 of 26 samples, concordance: 88.5%) when we compared MFC-MRD and PCR-MRD on Day 15 in those samples with a lineage switch (26 of 38 samples, 68.4%) (Table 10b). For MFC-MRD detection, we always considered the total amount of blasts, calculated as the sum of the two blast cells populations, when present.

Table 10: Comparison between MFC-MRD and PCR-MRD on Day 15 in CD371+ BCP-ALL. The analysis was performed on the total number of CD371+ available samples **(a)** and then was limited to those with a lineage switch on Day 15 **(b)**

		PCR-MRD Day 15		
		≥ 0.01%	**< 0.01%**	**Total**
MFC-MRD Day 15	**≥ 0.01%**	27	7	34
	< 0.01%	0	4	4
	Total	27	11	38

		PCR-MRD Day 15		
		≥ 0.01%	**< 0.01%**	**Total**
MFC-MRD Day 15	**≥ 0.01%**	23	3	26
	< 0.01%	0	0	0
	Total	23	3	26

5. Discussion

Our study allowed to define a new subset of BCP ALL, characterized by the aberrant expression of CD371 at diagnosis and a potential switch to myelomonocytic lineage limited to the first phase of Induction Therapy of AIEOP BFM ALL 2009 protocol. It is the first time that the aberrant expression of CD371 antigen in BCP blast cells is fully described. The CD371 antigen is typical of myelomonocytic lineage, being expressed on normal monocytes, granulocytes, basophils, most of the AML blasts, and leukemic stem cells[90-96]. No association with normal hematopoietic stem cells, T and B lymphocytes, and ALL blasts has ever been described until now[89,92,95,99]. In our cohort, 9.1% of pediatric BCP-ALL patients expressed CD371 antigen at diagnosis. Such a frequency can justify the use of CD371 MoAb in the panel for the immunophenotyping of B-lineage ALL at diagnosis, and its introduction as a marker of MRD in positive samples.

In contrast with the aberrant expression of some myeloid markers like CD33 or CD13 in ALL blast cells, CD371 positivity defined a specific subtype of BCP-ALL, with peculiar clinical and biological features in our cohort. This subtype of BCP-ALL was not associated with any of the traditional high-risk features of BCP-ALL at diagnosis, like hypodiploidy and t(4;11)(q21;q23);KMT2A-AFF1 rearrangement, nor hyperleukocytosis. To notice, CD371+ BCP ALL was more frequent in children older than 10 years of age. Even if age is recognized as an adverse prognostic factor worldwide, it was not considered strong enough to be chosen as a high-risk criterium in AIEOP BFM ALL 2009 protocol. We did not analyze the potential association of CD371+ BCP ALL with t(9;22)(q34.1;11.2);BCR-ABL1 rearrangement, as per the therapeutic protocol.

The immunophenotype at diagnosis showed some peculiarities. A high rate of CD371+ BCL ALL (13.3%) belonged to the immature group B-I ALL (CD19 positive, CD10 negative) of EGIL classification. At the same time, no patient was included in the B-III ALL group. No data are available on B-IV ALL, as they were excluded from the present study according to AIEOP BFM ALL 2009 protocol. CD371+ BCP ALL showed a brighter intensity of CD34, CD45, and CD58 antigens at diagnosis. Those three antigens are usually expressed on more than one cellular lineage. CD34 is a pan-lymphoid and pan-myeloid antigen, associated with immaturity[34]. CD45 is expressed in almost all hematopoietic cells, excepting for erythrocytes and plasma

cells. CD58 is widely distributed on cells of both hematopoietic and non-hematopoietic origin (peripheral blood lymphocytes monocytes, granulocytes, B lymphoblastoid cell lines, platelets, vascular endothelium, smooth muscle, fibroblasts, and approximately 40% of bone marrow cells). Regardless, we did not find any significant association with markers for dominant lineage assignment other than B-lineage, as iCD3, iMPO, CD7, CD13, CD33, CD65, CD64, CD117[42]. Altogether, the association with a more immature immunophenotype and the expression of antigens usually shared among different cell lineages may suggest potentially high plasticity of CD371+ BCP-ALL, and a propensity to lineage switch.

In our study, we observed a myelomonocytic switch in 68% of CD371+ BCP-ALL. It was transient and limited to the first phase of Induction Therapy according to the AIEOP BFM protocol. In our cohort of CD371+ BCP-ALL, we did not detect any switch at diagnosis. We observed a myelomonocytic switch on Day 8 (61.9% of the available samples) and Day 15 (69.4%), whereas we never detected it on Day 33 and Day 78. As previously described by Slamova and colleagues, steroid administration seemed to be responsible for that, even if the biologic mechanism of this phenomenon is not known yet. To notice, in our Center we observed one case of CD371+ BCP-ALL with an analog myelomonocytic lineage already present at diagnosis. It had received a steroid treatment before the diagnosis, and for that reason was excluded from the final analysis as per the therapeutic protocol. Such a finding confirms the role of steroids in modulating CD371 BCP-blasts plasticity.

Myelomonocytic lineage switch was characterized by a down-regulation of CD19 and CD34 antigens, together with an up-regulation of CD45, an increase of SSC, and strong expression of CD58. In 2002, Slamova et colleagues described a similar phenomenon in a group of BCP-ALL with an aberrant expression of the CD2 antigen in the absence of MLL rearrangements at diagnosis. To notice, the authors demonstrated the relationship between the leukemic cells and the monocytoid population, detecting the same patient-specific Ig/TCR gene rearrangements in both the populations[77]. Based on those findings and considering the potential impact of a myelomonocytic switch on MCF-MRD detection, we tried to build up a systematic approach to lineage switch in our cohort of BCP-ALL. The purpose was to optimize switch detection and make it reproducible. Indeed, the assessment of myelomonocytic lineage switch during MRD monitoring was not always easy, due to its heterogeneity among samples from different CD371+ BCP-ALL or even from the

same BCP-ALL at different time-points. We summarized lineage switch in two distinct patterns: a one-population pattern, and a two-population pattern, respectively. Of those, the first one might be analyzed more easily. When a single population was detected, it shared antigens of BCP and myelomonocytic lineage, with just a change in lymphoid antigen intensity. This phenomenon may be assimilated to an antigen remodeling due to therapy, as previously described[109]. The most challenging analysis was the detection of the two-population pattern and the interpretation of its role in MRD quantification on Day 15. Two questions came out: should the monocytoid population (CD19 dim, CD45 bright) be considered as part of blast cells and included in MFC-MRD quantification on day 15? Secondly, should chemotherapy be changed to a myeloid schema in the presence of a preponderant monocytoid population on Day 15?

In our Center we decided to include the monocytoid (CD19 dim, CD45 bright) in MFC-MRD final count on Day 15. That because its immunophenotype suggested a direct link with lymphoblasts, keeping CD19 positivity, even if with reduced intensity. Moreover, the monocytoid population could not be assigned to any other normal population. Its dimension varied widely, from the sensitivity level up to 21% in our cohort. Consequently, it might influence the final value of MFC-MRD on Day 15 and the associated risk group definition profoundly. Indeed, the comparison between an MFC-MRD value based on CD19 bright population alone vs. the sum of the two populations showed a potential underestimation of the risk group on Day 15 in 12 patients in our cohort. Besides, the comparison between MFC-MRD and PCR-MRD on Day 15 demonstrated a higher concordance among the two techniques, when both the populations were included in the analysis, confirming the accuracy of this choice.

The detection of a prevalent monocytoid population may suggest the opportunity of a therapeutic switch towards a treatment protocol for acute myeloid therapy. Regardless, this option was never adopted in our cohort, considering the initial lymphoblastic origin of the monocytoid population. This choice may find additional support in the recent manuscript by Hrusak and colleagues on treatment strategies in ambiguous lineage leukemias[16]. As the lineage switch completely disappeared on Day 33 and Day 78 of Induction Therapy, we may conclude that in the presence of a CD371+ BCP-ALL with a myelomonocit switch, it is mandatory to carry on a therapeutic protocol addressed towards lymphoblastic leukemia.

Besides, CD371+ BCP-ALL showed a slower response to the Induction Therapy in comparison to CD371- BCP-ALL. The significantly higher rate of patients enrolled in the final high-risk therapeutic arm well described this phenomenon. To notice, the high incidence of Slow Early Response (SER) in the evaluation of PCR-MRD on Day 33 and Day 78 was a peculiarity of CD371+ BCP-ALL. That finding may suggest a higher sensitivity of blast cells to the drug combination administered during the second part of Induction Therapy (cyclophosphamide, cytarabine, 6-mercaptopruine) or a reduced sensitivity to the steroid therapy. To support the second hypothesis, MCF-MRD on Day 15 showed a lower rate of standard-risk group in CD371+ BCP-ALL. Despites, no difference was observed in response to prednisone therapy at Day 8 between CD371+ and CD371- BCP-ALL.

The association with a higher rate of patients included in the final high-risk group highlights the importance of detecting precocious markers of lineage switch. In Slamova and collegues' study, myelomonocytic switch was always associated to an aberrant CD2 expression at diagnosis. Consequently, we analyzed which marker of CD2 and CD371 might better predict the lineage switch. In our study, both the antigens showed a high accuracy, whereas CD371 was complexly a more sensitive predictor of lineage switch.

Our study had several limitations. Firstly, we conducted a retrospective analysis and consequently, some data were missing. Secondly, in our study the number of CD371+ BCP-ALL was low (75 in total). To overcome this aspect, we have built a multicenter data collection, involving all the other members of AIEOP-BFM Flow Network. The study is still ongoing. Thirdly, we used different MoAb panels for MFC-MRD detection in different temporal era. Moreover, monocyte-lineage markers as CD14 and CD15 were not included in any of the MoAb panels for MRD.

Finally, the follow-up time of our patients is still too short to understand the real prognostic impact of CD371+ BCP-ALL.

6.Conclusions

In conclusion, we identified a new subtype of pediatric BCP-ALL, characterized by the aberrant expression of CD371 and a potential myelomonocytic switch during the first phase of Induction Therapy. An accurate identification of the lineage switch is mandatory to properly assess MFC-MRD on Day 15 in these patients. This is particularly important, considering that CD371+ BCP-ALL is associated with a slower response to Induction chemotherapy and a higher rate of patients included in the therapeutic high-risk group in comparison with CD371- BCP ALL.

CD371 antigen is an accurate predictive marker of lineage switch, with a high sensitivity and specificity. Even in presence of a prevalent monocytoid population during lineage switch, chemotherapy should be carried on according to a therapeutic protocol for ALL, without changing it to drugs specific for AML.

7.References

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COLLATERAL PROJECTS AND PUBLICATIONS

Traumatic and bloody lumbar puncture at diagnosis in children with acute lymphoblastic leukemia: a St. Jude Total XV and Total XVI cohort study

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Background: Traumatic and bloody lumbar punctures (TLPs and BLPs, respectively) at the diagnosis of pediatric acute lymphoblastic leukemia (ALL) impair the interpretation of central nervous system status and are associated with worse outcomes. We previously analyzed risk factors for TLP and BLP in 958 patients with ALL treated at St. Jude Children's Research Hospital (St. Jude) between 1984 and 1998. With an incidence of 19% and 5% at diagnosis, respectively, TLPs and BLPs were associated with unmodifiable and modifiable risk factors (URFs and MRFs, respectively). URFs included age less than 1 year and black race. MRFs included a platelet count (PLT) of $<100 \times 10^9$/L, a less experienced practitioner, a non-dedicated procedural setting, and no sedation. Since then, preventive measures have been adopted for the first lumbar puncture (LP) at St. Jude: patients undergoing an LP ideally have a PLT of $\geq 100 \times 10^9$/L; the procedure is performed by the most experienced practitioner available; and we use a dedicated procedure area and general anesthesia.

Objectives: To determine the incidence and risk factors of TLPs and BLPs, defined by the presence of at least 10 and 500 red blood cells per microliter of cerebrospinal fluid, respectively, at the diagnosis of pediatric ALL after the introduction of preventive measures.

Patients and Methods: We performed a retrospective cohort study on children with newly diagnosed ALL who were consecutively enrolled on the Total XV and Total XVI protocols at St. Jude from October 2000 through March 2017. Patients who received their first LP at a center other than St. Jude or did not have a PLT within 24 hours of undergoing LP were excluded.

Results: A total of 1006 patients (median age, 5.68 years at diagnosis; range, 0.12 to 18.89 years) were included in our study. Of the 1006 LPs at diagnosis, 80 (7.9%) were TLPs and 15 (1.5%) were BLPs. Of the 80 TLPs, 47 (58.7%) showed leukemia blasts (vs 30.9% [286/926] of non-TLPs; $P < 0.001$). Of the 15 BLPs, 13 (86.7%) showed leukemia blasts (vs 32.3% [320/991] of non-BLPs; $P < 0.001$). Regarding the adopted preventive measures, 561 patients received a platelet transfusion within 24 hours of LP because of a low PLT, but in 135 (13.4%) of these cases, a repeat PLT was not evaluated before the procedure (see Table). Therefore, of 871 available PLTs within 24 hours of LP, 638 (73.2%) were $\geq 100 \times 10^9$/L and 779 (89.4%) were $\geq 75 \times 10^9$/L. Experienced practitioners (defined as those having performed at least 200 procedures during the study period) performed 880 (89.2%) of the 986 LPs for which practitioner information was available. General anesthesia was used in 948 (96.7%) of 980 LPs for which anesthesia information was available. All the procedures were performed in a dedicated area. We saw no significant differences in the incidence of TLP with respect to these MRFs. TLP was associated with age less than 1 year, black race, T lineage, a white blood cell count (WBC) of $\geq 50 \times 10^9$/L both at diagnosis and within 24 hours of LP, and a higher international normalized ratio (INR) in univariate analysis (see Table). Multivariable analysis, performed on variables available for all patients and showing $P < 0.1$ in univariate analysis, confirmed the association between TLP and age less than 1 year, black race, and a WBC of $\geq 50 \times 10^9$/L at diagnosis (see Table). INR also remained significant when adjusted for age at diagnosis, race, and WBC at diagnosis. We found no significant association between BLPs and the analyzed variables.

Conclusions: Best practices were used in almost all cases in our recent cohort, and the incidences of TLP and BLP at diagnosis decreased markedly (to 7.9% and 1.5%, respectively). This decrease can be ascribed to the adoption of preventive factors (requirements for a PLT of ≥100 × 10^9/L, if possible; the most experienced practitioner; general anesthesia; and a dedicated procedure area). The previous association of TLP with the URFs age less than 1 year and black race was confirmed by this study. Our analysis suggests that LPs must be performed carefully in infants and black patients, as well as in those with WBCs of ≥50 × 10^9/L, and that it may be preferable to delay the first LP until peripheral blood blasts disappear and the INR is normal.

Table: Factors associated with incidence of traumatic lumbar puncture

Variable	Patients (N)	TLP (N)	OR	95% CI	Univariate *P*	Multivariable *P*
Age at diagnosis					**0.036**	**0.086**
1–9 years	724	50	1.00			
<1 year	12	3	**4.49**	**1.18–17.12**	**0.028**	**0.039**
≥10 years	270	27	1.50	0.92–2.45	0.107	0.299
Sex					0.963	
Male	576	46	1.00			
Female	430	34	0.99	0.62–1.57		
Race					0.054	0.110
Non-Hispanic white	758	52	1.00			
Black	150	19	**1.97**	**1.13–3.44**	**0.017**	**0.041**
Other	98	9	1.37	0.65–2.88	0.402	0.411
Lineage					**0.019**	0.250
B	838	59	1.00			
T	168	21	**1.89**	**1.11–3.19**		
WBC at diagnosis					**0.001**	**0.033**
<50 × 10^9/L	759	48	1.00			
≥50 × 10^9/L	247	32	**2.20**	**1.37–3.54**		
WBC within 24 hours of LP					**0.038**	
<50 × 10^9/L	838	60	1.00			
≥50 × 10^9/L	167	20	**1.76**	**1.03–3.01**		
Therapeutic protocol					0.074	0.052
Total XV	408	40	1.00			
Total XVI	598	40	0.66	0.42–1.04		
Final risk group					0.907	
Low	459	36	1.00			
Standard/High	547	44	1.03	0.65–1.63		
Day of LP execution					0.974	
Monday	231	18	1.00			
Tuesday	145	12	1.07	0.50–2.29	0.866	
Wednesday	170	12	0.90	0.42–1.92	0.783	
Thursday	164	13	1.02	0.48–2.14	0.961	
Friday	214	18	1.09	0.55–2.15	0.811	
Saturday	69	5	0.92	0.33–2.59	0.881	
Sunday	13	2	2.15	0.44–10.46	0.342	

Anesthesia					0.689	
Nitrous oxide and IV anesthesia	585	43	1.00			
Propofol-based IV anesthesia	363	32	1.22	0.76–1.96	0.417	
IV sedation other than propofol	32	3	1.30	0.38–4.45	0.672	
Practitioner experience (N of LPs performed during the study period)					0.295	
≥200 LPs	880	65	1.00			
51–199 LPs	47	2	0.56	0.13–2.35	0.426	
11–50 LPs	46	6	1.88	0.77–4.60	0.166	
<10 LPs	13	2	2.28	0.49–10.50	0.290	
Platelet count within 24 hours of each LP					1.000	
≥100 × 10^9/L	638	51	1.00			
76–99 × 10^9/L	141	11	0.97	0.49–1.92	0.939	
51–75 × 10^9/L	44	3	0.84	0.25–2.81	0.780	
26–50 × 10^9/L	36	3	1.05	0.31–3.53	0.942	
≤25 × 10^9/L	12	1	1.05	0.13–8.27	0.966	
No PLT after platelet transfusion	135	11	1.02	0.52–2.02	0.952	
INR	747	61	6.46	2.30–18.14	**<0.001**	**0.024**
aPTT	741	62	0.98	0.94–1.03	0.463	

Abbreviations: N, number; TLP, traumatic lumbar puncture; OR, odds ratio; CI, confidence interval; WBC, white blood cell count; LP, lumbar puncture; IV, intravenous; PLT, platelet count; INR, international normalized ratio; aPTT, activated partial thromboplastin time.

Analysis was performed with a logistic regression model. Multivariable analysis was performed using variables available for all patients and showing $P < 0.1$ at univariate analysis. The analysis of INR was adjusted for age at diagnosis, race, and WBC at diagnosis.

Multiplex CRISPR/Cas9-Based Genome Editing of Mouse Hematopoietic Stem Cells Recapitulates Acute Erythroid Leukemia and Identifies Therapeutic Targets

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603. Oncogenes and Tumor Suppressors /or

602. Disordered Gene Expression in Hematologic Malignancy, including Disordered Epigenetic Regulation

(Character limit without space: 3800)

Introduction. Acute erythroid leukemia (AEL) is a high-risk leukemia subtype of poorly understood genetic basis. In integrated comparative genomic analysis of 159 AEL and 1509 non AEL myeloid tumors, we identified 5 age-related AEL subtypes with distinct genomic features and outcome: adult AEL with bi-allelic alterations in *TP53* (31%), frequently co-occurring with alterations in *DNMT3A, BCOR, EZH2, RB1,* or *NFIX*; *NPM1*-mutated (12%); *KMT2A*-mutated/rearranged (11%), mostly co-mutated with *STAG2*; pediatric, *NUP98*-rearranged (4%) and adult, *DDX41*-mutated (3%). Thirty-seven percent of cases lacked an identifiable exclusive recurrent founding genetic alteration but were enriched in mutations in *ASXL1*, *TET2* and splicing factors.

Methods. To explore the roles and cooperativity of the identified alterations in leukemogenesis we used CRISPR-Cas9 genome editing to induce combinations of loss-of-function mutations in 9 recurrently mutated genes in AEL (*Tp53*, *Tet2*, *Dnmt3a*, *Asxl1*, *Ezh2*, *Stag2*, *Bcor*, *Ppm1d*, *Rb1* and *Nfix*). Based on patterns of mutation association, we generated 6 pools of lentiviral vectors (Table 1) with different combinations of single guide RNA (sgRNA) to induce multiplex genome editing in Cas9-eGFP-mouse lineage-negative hematopoietic stem cells (HSCs). Transduced cells were transplanted into lethally irradiated congenic mice. Tumors were characterized by morphology, immunophenotyping, and genomic analysis (sequencing of sites of editing, and exome, methylation and transcriptome sequencing). Ex vivo drug screening was performed to test sensitivity to 192 therapeutic agents, including conventional chemotherapeutic agents, and compounds targeting epigenetic regulators, protein kinases and cell cycle checkpoints.

Results. In contrast to the uniform representation of guide RNAs observed in HSCs pre-transplant, tumors exhibited enrichment of specific sgRNAs with tumors of specific phenotype.

We frequently observed bi-allelic editing of *Tp53, Bcor, Tet2*, *Dnmt3a*, *Rb1* and *Nfix* in agreement with the presence of bi-allellic loss in patients. Concomitant editing and inactivation of *Tp53*/*Bcor*/*Dnmt3a*, or *Tp53*/*Bcor*/*Rb1*/*Nfix* promoted the development of acute erythroid leukemia ($GATA1^{+}$/$RUNX1^{+}$/$GlyA^{+/-}$$Ter119^{+/-}$ and B220/CD19/PAX5/CD3/Mac1/Gr1-). Concomitant editing of *Tp53*/*Bcor*/*Tet2* promoted the development of B-lineage ALL, and editing of *Dnmt3a* and *Tet2* without *Tp53* promoted T-cell ALL. Leukemic clones from primary tumors were serially transplantable across multiple different genetic backgrounds, with the same dominant clone present in all transplanted mice. Notably, mice that did not develop leukemia showed enrichment of different combinations of sgRNAs for *Tet2, Dnmt3a* and/or *Asxl1,* genes commonly mutated in clonal hematopoiesis of indeterminate potential, confirming that these mutations are alone not sufficient to induce leukemogenesis. Additional somatic mutations were acquired during clonal expansion of leukemic cells such as alterations of *Notch1* and *Ikzf1* in T-ALL; *Setd2* at the serial passaging of T-ALL; *Ptpn11*, *Kit* (D816V), *Kras* (Q61L) and *Lmo7* in the AEL models; and *Sf3b3* in the B-ALL model (Fig 1A). Tumors with mutated *Tp53* acquired aneuploidy whereas *Tet2*-mutated cells were genomically stable (Fig. 1B). Unsupervised hierarchical clustering of gene expression profiling identified three subgroups (Fig. 1C) associated with distinct genotypes and methylation profiles (Fig. 1D). *Tet2*-mutated tumors showed increase of hypermethylated sites and co-mutated *Bcor*/*Dnmt3a* leukemic cells showed loss of methylation.

Eleven tumors representative of key AEL genotypes from the established models were selected to explore drug sensitivity. Sensitivity to individual drugs was associated with genotype with co-mutated *Tet2/Dnmt3a* T-ALL cells sensitive to bromodomain and histone methyltransferase inhibitors; co-mutated *Bcor/Tp53/Dnmt3a* or *Bcor/Rb1* AEL cells to CDK9 inhibitor (LY2857785); *Tp53* mutations alone were exclusively sensitive only to PARP inhibition (Tolazoparib). In vivo mouse studies are currently ongoing.

Conclusions: We successfully generated genetically defined models of AEL, demonstrated the role of *Tp53* and *Bcor* mutations in driving the erythroid phenotype, and showed that sensitivity to different classes of compounds is genotype-dependent. These results provide the rational for testing new targeted agents in AEL.

Table 1. Pools of sgRNAs used to induce multiplex genome editing in Cas9-EGFP-mouse lin- HSCs. For each pool of sgRNAs at least 4 primary recipient mice were transplanted (n=5 for pool1/ 2/3 and n=4 for pool5/6). Primary tumors were serially transplantable in secondary and subsequent passages.

Pool#	**sgRNAs**	**Phenotypes and Genotypes of Established Primary Tumors**	**Median Latency in Primary Recipients** (days)
Pool1	*Tp53*, *Bcor*, *Dnmt3a*, *Tet2*, *Ezh2*, *Asxl1*, and *Ppm1d*	**AEL**: *Tp53* and *Bcor* (n=1); *Tp53* (n=1); **B-ALL**: *Tp53*, *Tet2* and *Dnmt3a* (n=1)	87 (78-138)
Pool2	*Dnmt3a*, *Tet2,* and *Stag2*	-	-
Pool3	*Dnmt3a, Tet2*, *Ezh2*, and *Asxl1*	**T-ALL**: *Dnmt3a* and *Tet2* (n=1)	141
Pool4	*Tp53*, *Bcor*, and *Dnmt3a*	**AEL**: *Tp53*, *Bcor* and *Dnmt3a* (n=2)	116 (94-138)
Pool5	*Tp53*, *Bcor, Rb1* and *Nfix*	**AEL**: *Bcor*, *Nfix* (n=1); *Tp53*, *Bcor*, *Rb1* and *Nfix* (n=1); **Mixed AEL/B-ALL**: *Tp53*, *Bcor*, *Rb1*, and *Nfix* (n=1); *Tp53*,	92 (89-94)

		Bcor and *Nfix* (n=1)	
Pool6	*Tp53*, *Bcor*, and *Rb1*	**AEL**: *Bcor* and *Rb1* (n=1); **Mixed AEL/B-ALL**: *Bcor* and *Rb1* (n=1); *Tp53*, *Bcor* and *Rb1* (n=1); *Tp53* and *Bcor* (n=1)	94 (45-101)
NTG	Non-targeting guide RNA	-	-

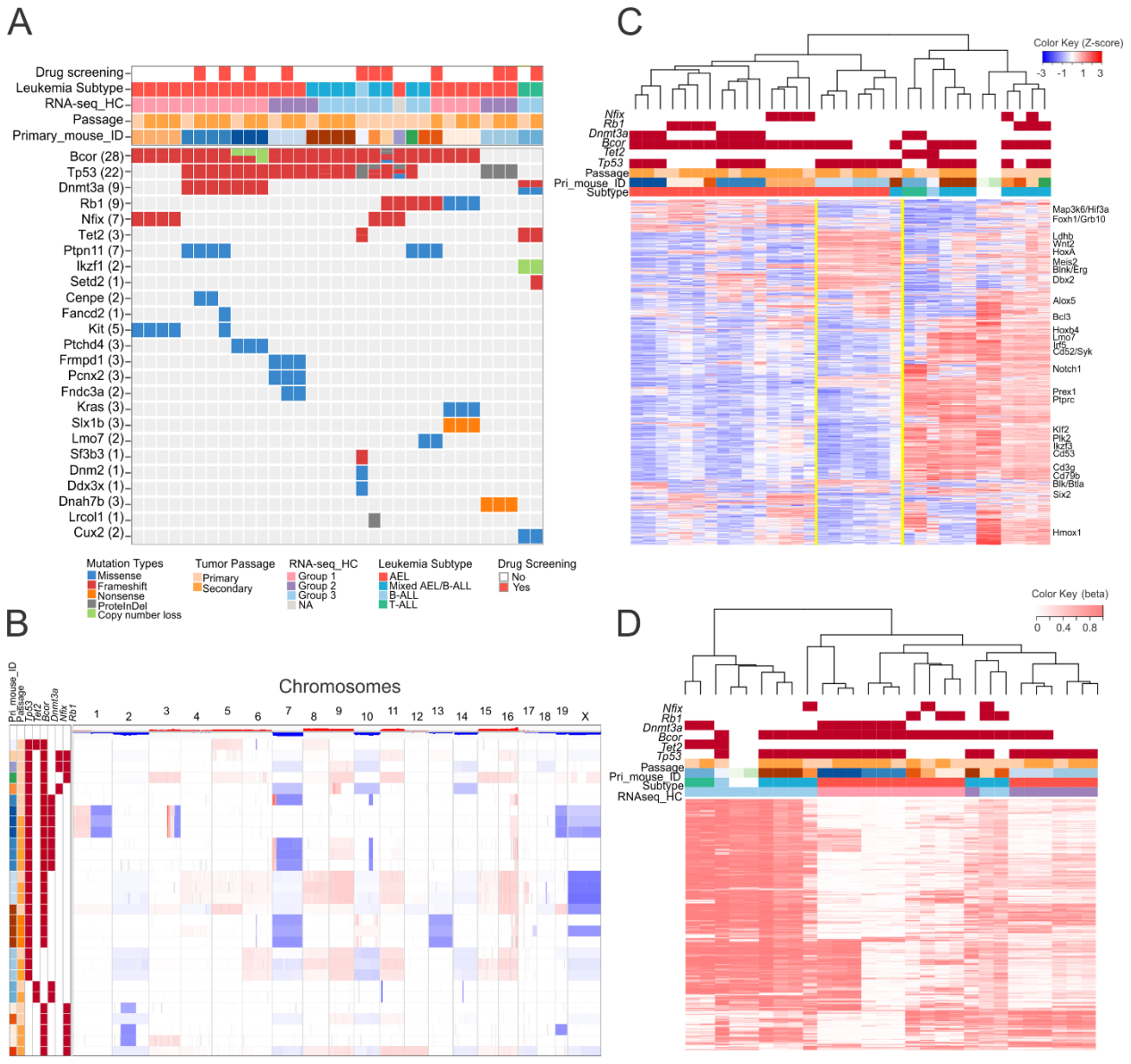

Figure 1. Multiplex CRISPR/Cas9-based genome editing of murine lin-HSCs recapitulates the genomic complexity of human AEL. A) Schematic representation of mutations induced by CRISPR/genome editing or spontaneously acquired during leukemia evolution. Each column is a different mouse. **B**) Copy number data from whole exome sequenced and visualized by Integrative Genomics Viewer. Leukemic cells harboring mutations in *Tp53* acquired gross aneuploidies mostly commonly

involving chromosomes 1, 7, 8, 9, 10, 13, 16 and 19. **C**) Unsupervised hierarchical clustering (UHC) identified three main gene expression subgroups: group 1 included samples with combination of mutations in *Tp53*/*Bcor*/*Dnmt3a*; *Bcor*/*Nfix*; *Bcor*/*Rb1*. Overexpressed genes in this group included: *Grb10*, *Hif3a*, and *Map3k6*. Group 2 included leukemic cells lacking mutations in *Dnmt3a* but harboring mutated *Tp53*/*Bcor*. Important overexpressed genes included *Wnt2*, *Hif*, *Hoxa9*, *Hoxa7*, *and Alox5*. Group 3 including samples with a lymphoid phenotype. **D**) Heatmap of beta values, with 0 for unmethylated CpG in white, and 1 for methylated CpG in red. Captured methylation sequencing data were generated using Agilent SureSelect Mouse Methyl-seq. Top 1000 most variable CpGs for each chromosome were selected based on MAD score using M-value (log2Ratio) and used for the UHC. Three main groups were depicted and they highly correlated with genotype and gene expression profile. Abbreviation: Pri_mouse_ID: primary mouse ID. Each colors represents a different leukemia model generated by a primary mouse.

Case Report

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Aspergillus flavus Disseminated Infection in Paediatric Acute Lymphoblastic Leukaemia: A Case Report

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Abstract

Here we report the case of a disseminated *Aspergillus flavus* infection in an adolescent affected by acute lymphoblastic leukaemia at the beginning of first-line chemotherapy. Association of surgery and combination antifungal therapy (high dose liposomal amphotericin B and caspofungin) allowed infection improvement. 18-FDG PET-MRI was used for response-to therapy monitoring.

Introduction

Incidence of invasive fungal infections (IFI) has progressively increased over the past few decades [1]. Invasive aspergillosis (IA) is associated with the highest morbidity and mortality in immunocompromised patients [2,3]. *Aspergillus flavus* is the second leading cause of IA affecting mostly upper airways and skin [4,5]. Invasive cutaneous asperigillosis (ICA) is a rare condition characterized by more or less itching macules, papules, plaques or haemorrhagic bullae, potentially evolving into necrotic ulcers covered by a dark eschar. Primary ICA, deriving from fungus direct inoculation into an injured site, can be distinguished from secondary ICA, usually resulting from systemic dissemination of inhaled hyphae through the blood stream [6-10]. Immunological deficiency in paediatric haemato-oncology patients is due both to the malignancy and chemotherapy regimen, emerging as the main predisposing factors to IFI [11]. Early diagnosis, effective therapy and accurate response-to-therapy monitoring are mandatory in these patients, in order to achieve infection control and reintroduce chemotherapy as soon as possible.

Here we report the case of a proven invasive *Aspergillus flavus* infection in an adolescent affected by acute lymphoblastic leukaemia (ALL), with particular attention to clinical presentation, therapy schedule and response-to-therapy monitoring.

Description

A 17 years old girl followed at Paediatric Oncology-Haematology Clinic of Padova for B-precursor ALL developed a progressive painful swelling of the left forearm. Diagnosis of B-III ALL according to EGIL classification [12] was done in August 2016; central nervous system involvement was excluded. Soon after the diagnosis, the patient was enrolled in AIEOP BFM ALL 2009 international protocol. After the end of the first chemotherapeutic phase Induction A (prednisone 60 mg/m^2/day for 28 days; vincristine 1.5 mg/m^2 weekly for 4 doses; daunorubicin 30 mg/m^2 weekly for 4 doses; PEG-asparaginase 2500 UI/m^2 for 2 doses every 2 weeks) the patient developed a painful swelling of the left forearm without alteration of overlying skin. According to personal history of a recent accidental trauma and ultrasound findings, the lesion was initially interpreted as a muscular haematoma. When fever appeared, the girl was hospitalized and wide spectrum antibiotic therapy was started (ceftazidime 40 mg/kg/day TID, amikacin 15 mg/kg QD; teicoplanin 10 mg/kg BID the first day, then 10 mg/kg QD), by considering concomitant severe neutropenia and possible haematoma superinfection. For the persistence of fever longer than 72 h, according to our local protocol, antibiotic therapy was shifted to meropenem and antifungal therapy (liposomial amphotericin B 3 mg/kg QD) was started. During hospitalization we observed a worsening of the cutaneous lesions: the first one evolved into a hyperemic painful nodule;

three analogue new lesions appeared on the left leg and left arm, one of which covered by a haemorrhagic bullae (Figure 1).

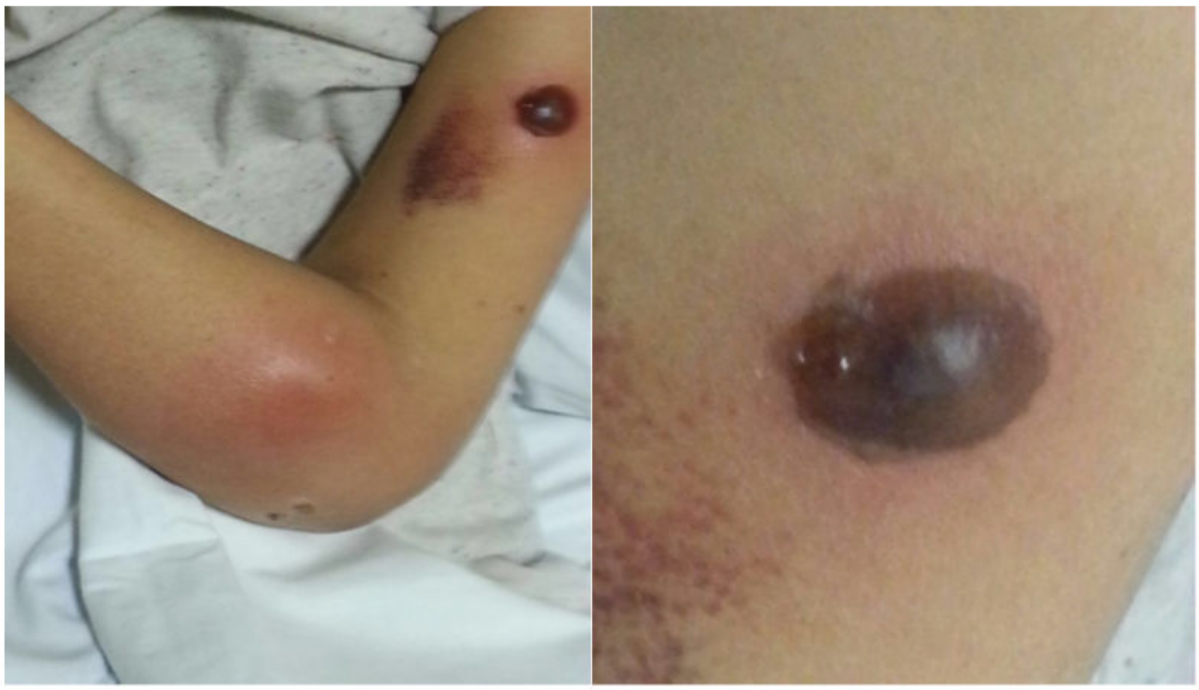

Figure 1: *Aspergillus flavus* subcutaneous lesions of the left arm

Inflammation indexes were only mildly elevated (maximum C-reactive protein value: 35 mg/L). Initial Galactomannan (GM) and 1-3,Beta-D-Glucan (BG) serum dosages were negative. Ultrasound evaluation and local Magnetic Resonance Imaging (MRI) showed subcutaneous colliquating abscesses (Figure 2). Surgical drainage was performed and abundant purulent material collected: *Aspergillus flavus* was isolated. According to previous experience at our Centre in fungal infection diagnosing and monitoring, a 18-fluorodeoxyglucose Positron Emission Tomography-MRI (18-FDG PET-MRI)) was performed. This exam allows to study anatomically and functionally the whole body, detecting disseminated lesions and minimizing radiation exposure [13]. Mild to high metabolic uptake (Standardized Uptake Value–SUV 2.4-4.8) was shown at the following sites: right leg (2 muscular lesions); left leg (2 subcutaneous lesions); right dorsal muscles (1 lesion); left arm (2 subcutaneous lesions); left underarm (1 lymphatic lesion); kidneys (2 parenchymal lesions); right upper pulmonary lobe (1 lesion). No central nervous system (CNS) involvement was detected. Eyes evaluation and echocardiography excluded respectively ocular and cardiac involvement.

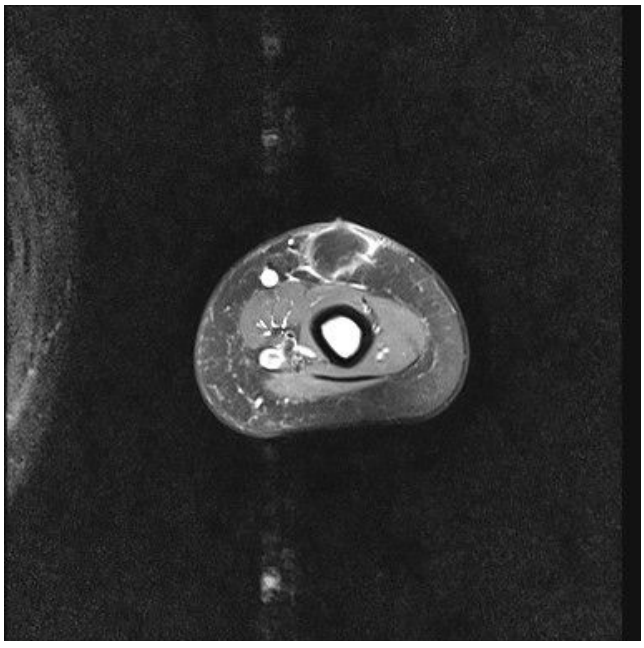

Figure 2 (A): MRI at diagnosis: left arm

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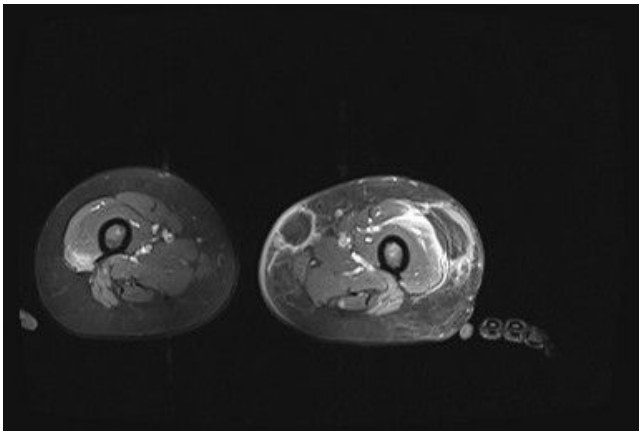

Figure 2 (B): MRI at diagnosis: left limb

Combination antifungal therapy with high dose liposomal amphotericin B (7.5 mg/kg QD) and caspofungin (70 mg QD the first day, then 50 mg QD) was started. We opted for this intensive pharmacological schedule by considering the extension of fungal infection and the necessity to control it promptly and reintroduce chemotherapy as soon as possible. Surgical curettage of subcutaneous abscesses was performed three times a week by paediatric surgeons. We observed fever resolution after 3 days of combination therapy and slow improvement of subcutaneous lesions.

GM was persistently negative, while BG increased progressively (maximum value>523 pg/ml). After 21 days of combination therapy we repeated 18-FDG PET-MRI, that showed a partial improvement of the fungal lesions. This finding allowed us to start a maintenance chemotherapy with oral methotrexate and 6-mercaptopurine waiting for a complete resolution of the infection.

Discussion

Invasive aspergillosis has been emerging as an increasingly important cause of morbidity and mortality in immunocompromised children. *Aspergillus fumigatus* is the most frequent pathogen, followed by *Aspergillus flavus* and *Aspergillus terreus* [14,15]. IA usually develops soon after prolonged and severe neutropenia and commonly affects lungs, less often paranasal sinuses, central nervous system (CNS), skin and soft tissues. Disseminated form accounts for about 30% of cases [7,16]. Clinical presentation may be aspecific (i.e. prolonged fever in severe neutropenia not responding to wide spectrum antibiotic therapy) or as expression of organ damage (i.e. cough, pleural pain, and haemoptysis in lung infection or focal neurological deficits or seizures in CNS localization).

Primary cutaneous aspergillosis has been reported more frequently in children than in adults, in association with direct skin lacerations [16,17]. Our patient developed a disseminated *Aspergillus flavus* infection with multiple subcutaneous, muscular and parenchymal lesions. IA is an unexpected finding in paediatric ALL at the beginning of the first line therapy, as we observed in our patient: no antifungal prophylaxis is usually recommended in paediatric ALL [18-20]. Even if patient's personal history was positive for a traumatic event, disseminated IA was more likely the consequence of fungal vascular migration from an initial pulmonary site than direct inoculation of *Aspergillus flavus* into the skin, also considering that no cutaneous laceration was detected or referred.

IA prognosis mainly depends on early diagnosis, appropriate treatment and restoration of host defences [21]. Timely infection control is mandatory in haemato-oncology paediatric patients, considering that a prolonged interruption of chemotherapy may affect dramatically the oncological prognosis. On the other hand IA diagnosis, therapy and monitoring are still challenging. Isolation of fungal pathogen is necessary for the definition of proven IA, but is rare and usually needs surgical approaches [22].

Galactomannan (GM), a polysaccharide cell-wall component of all *Aspergillus spp*, is the most accurate marker for IA screening in adults, but has shown a wide range of true-positive and true-negative results in paediatric studies (sensitivity 0.76; specificity 0.86) [23]. In our patient serum GM was repeatedly negative, even in presence of a proven *Aspergillus flavus* infection. 1-3, Beta-D-Glucan (BG) is a cell wall component of many fungal pathogens such as *Aspergillus* spp, *Candida* spp, *Fusarium*, *Trichosporum* or *Saccharomyces*, and *Pneumocystis jiroveci*, whereas lacks in *Cryptococcus neoformans* and *Mucorales*. Its serum testing has been proving to be useful in adult patients, but data are too limited in children to recommend [24]. In our patient basal serum BG was negative and increased progressively during therapy to the maximum detectable level (>523 pg/ml). In our opinion BG should be considered as an indicator of the presence or absence of fungal infection more than an indirect estimator of disease extension. The increasing serum levels in our patient might be due to moulds destruction rather than to IA progression.

As regards radiological investigation, CT and MRI have a high accuracy in the early diagnosis of IA, but may result inadequate in evaluating disseminated disease and monitoring of residual infection. Furthermore cumulative radiation exposure due to CT scan has to be considered, especially in paediatric population [25,26]. 18-FDG PET-MRI has been recently proposed as a combined functional-anatomical methodical to better distinguish active fungal lesions from residual scars [13]. In our patient 18- FDG PET-MRI allowed to study the whole body, detected additional lesions and monitored their evolution in terms of activity and anatomical definition.

No standardized therapy are available, both for the variability of local epidemiology and paucity of clinical trials [27]. ECIL-5 guidelines recommended monotherapy admistration in IA (voriconazole 2 x 6 mg/kg the first day, then 2 x 4 mg/kg - evidence grade AI - or liposomal amphotericin B 3 mg/kg – evidence grade BI) [18]. Anyway clinical experience seems to indicate combination therapy as an efficacious treatment of IA in heamato-oncology paediatric patients, in order to accelerate the infection control and reduce chemotherapy delay. Association of caspofungin and voriconazole or caspofungin and liposomal amphotericin B has been previously described [9,28-30]. Combination therapy with high dose liposomal amphotericin B

(5-7.5 mg/kg) and caspofungin (70 mg the first day, then 50 mg/day) was well tolerated in our patient. No renal or liver impairment was detected. Moderate hypokalemia appeared and was easily corrected with intravenous potassium chloride infusion. Surgical curettage of necrotic lesions had a leading role in the improvement of subcutaneous localizations.

In conclusion, IA is a challenging complication of chemotherapy-induced immunosuppression in paediatric haemato-oncology patients. It requires a multidisciplinary approach in order to obtain promptly the most appropriate diagnosis. Aggressive therapy is mandatory to control the infection and allows the clinician to reduce chemotherapy suspension. 18-FDG PET-MRI may be useful for a more accurate diagnosis and response-to-therapy monitoring.

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