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

Supplemental Materials

Multiparametric Flow Cytometry (MFC) analysis

At diagnosis, we performed immunophenotyping on erythrocyte-lysed whole BM samples according to standardized operating procedures previously described[1]. BM samples were delivered from AIEOP Centers at room temperature and processed within 48 hours from collection. We incubated 500,000 nucleated cells per analysis at room temperature for 15 minutes 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. We adopted the 8-color MoAbs combinations listed in Table S5a[1, 2].

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. We routinely optimized the instrument set-up, analyzing Cytometer Setup and Tracking Beads, and normal peripheral blood (PB) T lymphocytes, as previously indicated[1, 2].

As concern MFC-MRD, we processed and analyzed the samples with the same procedure and instruments adopted at diagnosis, using 2,000,000 nucleated cells per analysis and acquiring at least 500,000 events[3]. MoAbs combinations included those markers previously shown as the most relevant for MRD detection in BCP-ALL[3-6]. Between June 2014 and May 2016, one 8-colors combination was adopted, including the following MoAbs[3, 7] (Table S5b). 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 the dry 10-colors preformulated DuraClone 10 Color Custom Mix (Beckman Coulter, Inc., Brea, CA, USA) [3, 7](Table S5c). Those samples were acquired 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. We routinely optimized the instrument set-up, analyzing Flow Check Fluorospheres (Beckman Coulter), and normal peripheral blood (PB) T lymphocytes, as previously indicated[1, 2].

Altogether, we adopted the 8-colors combination in 141 of 210 (67.1%) samples [27 of 43 (62.8%) samples on Day 8, 45 of 73 (61.6%) samples on Day 15, 30 of 40 (75.0%) samples on Day 33, 39 of 54 (72.2%) samples on Day 78]. Whereas we used the 10-colors DuraClone in 69 of 210 (33.3%) samples [16 of 43 (37.2%) samples on Day 8, 28 of 73 (38.3%) samples on Day 15, 10 of 40 (25%) samples on Day 33, 15 of 54 (27.8%) samples on Day 78].

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)[3](Table S5).

Standard operating procedures for the detection of myelomonocytic switch

To standardize the approach to switch detection, we analyzed all the 210 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, on a CD34/CD58 plot, we gated CD34 negative to strong positive and CD58 strong positive events. Finally, we analyzed the selected events on CD371/CD19, when available (or alternatively CD19/SSC), and CD45/SSC dot plots, to detect the presence of switch and define its expression as the single or two-populations pattern.

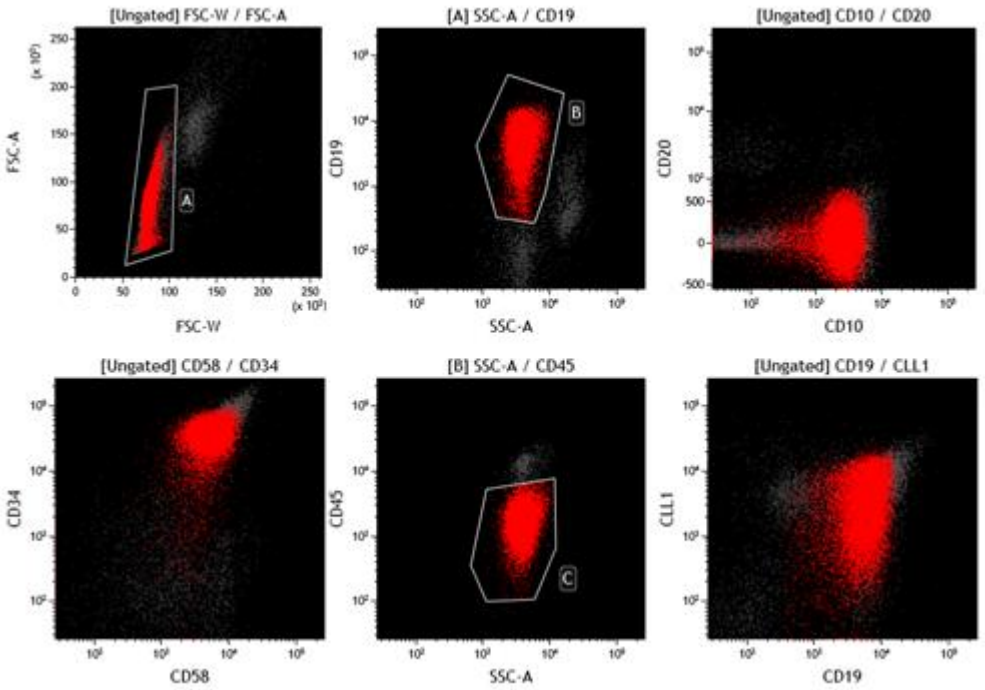
Table S1: BCP-ALL final risk group stratification according to the AIEOP-BFM ALL 2009 protocol

High risk group
Hypodiploidy (DNA index < 0.8)
t(4;11)(q21;q23);*KMT2A-AFF1*
Day 8 (morphology): ≥ 1,000 blasts/µL in PB
Day 15 (MFC-MRD): ≥ 10% blasts in BM
Day 33 (morphology): non-complete remission (≥ 5% blasts in BM)
PCR-MRD at TP1(Day 33) and TP2 (Day 78):
PCR-MRD ≥ $5x10^{-4}$ at TP2
PCR-MRD Slow Early Response (SER): MRD ≥$5x10^{-4}$ at TP1 and MRD pos <$5x10^{-4}$ at TP2
Standard risk group
No high-risk criteria and PCR-MRD negative at TP1 and TP2
Intermediate risk group
Any condition other than high and standard risk group

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

Figure S1: Persistency of CD371 expression on blasts cells during Induction: in samples still positive for blast cells during Induction, CD371 expression was always kept, confirming CD371 to be a useful marker for MRD detection in CD371+ BCP-ALL.

a) CD371 positive BCP-ALL: immunophenotype at diagnosis



b) CD371 positive BCP-ALL: MFC-MRD on Day 15 of induction therapy

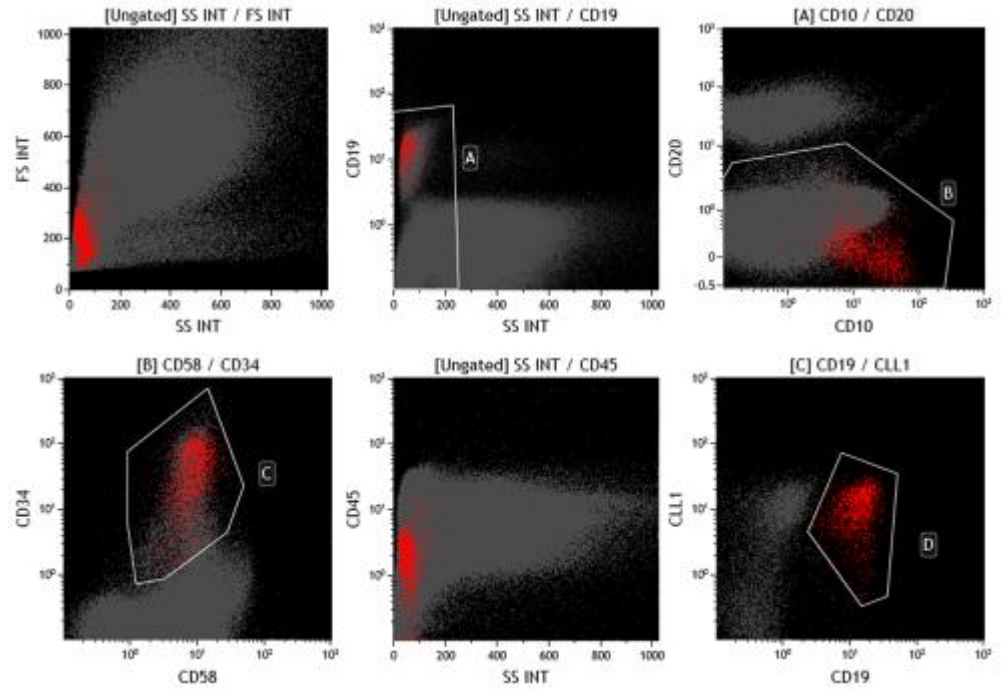


Figure S2: Expression of monocytic markers in the two-population switch pattern on Day 15: when we test two-population switch pattern samples with an extensive panel including myelomonocytic antigens, only the second population on blasts cells (SW-blasts) showed the expression of CD14, CD11b, CD33. Whereas the first population (unchanged lymphoblasts), keeping the same immunophenotype of diagnosis, was consistently negative for those antigens.

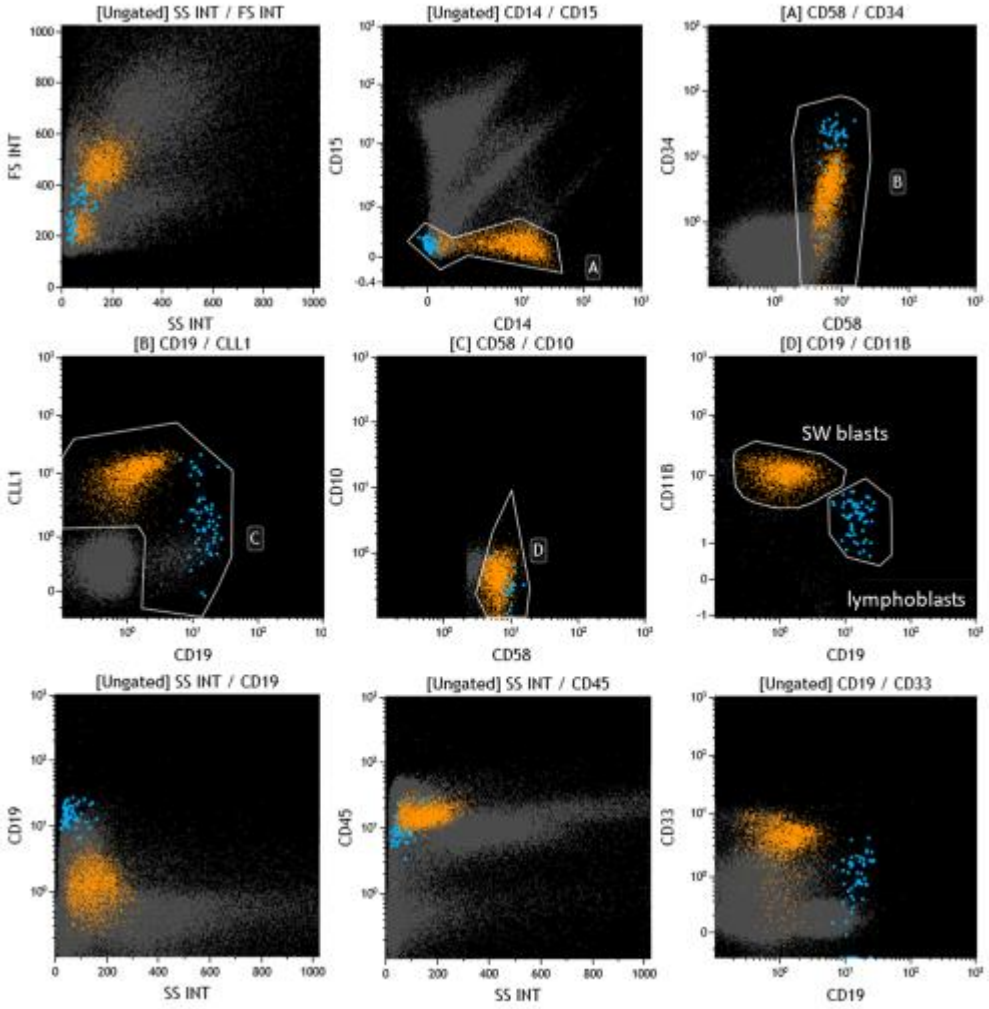


Figure S3: IG/TR receptor rearrangements study in the two-population switch pattern samples: when we sorted the second population of blasts cells (SW-blasts) and investigated IG/TR expression, it shared identical IG/TR receptor rearrangements (VH3JHC and VKIIIKDE) with the single population of blast cells detected at diagnosis.

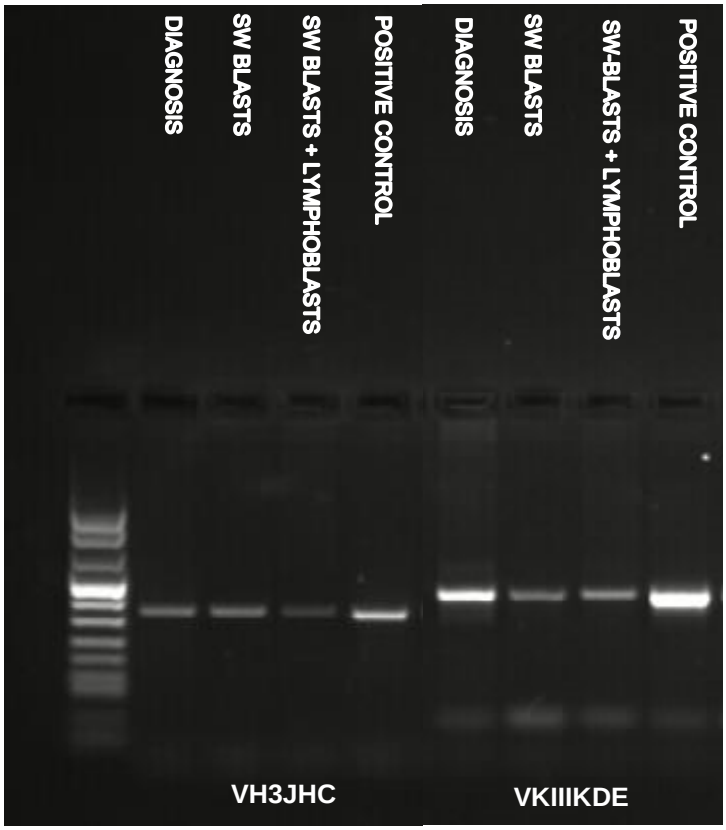


Table S2: The role of antigen CD371 (a) and CD2 (b) in predicting BCP-ALL myelomonocytic switch on Day 15 of Induction Therapy.

a

	SWITCH+	SWITCH-	TOTAL
CD371+	51	22	73
CD371-	4	753	757
TOTAL	55	775	830

Sensitivity = 0.93 (95% CI ± 0.067)

Specificity = 0.97 (95% CI ± 0.011)

Positive Predictive Value = 0.70

Negative Predictive Value = 0.99

Accuracy = 0.97

b

	SWITCH+	SWITCH-	TOTAL
CD2+	31	10	41
CD2-	24	765	789
TOTAL	55	775	830

Sensitivity = 0.56 (95% CI ± 0.13)

Specificity = 0.99 (95% CI ± 0.01)

Positive Predictive Value = 0.76

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)

Table S3 Details on high-risk features in CD371 positive **(a)** and CD371 negative- **(b)** BCP-ALL in the AIEOP cohort

a: CD371 positive BCP-ALL at diagnosis (27 patients)

HR criteria (hierarchical)	N	Additional HR criteria
HR MRD	4	HR MFC-MRD Day 15 (N=2)
No CR D33	0	
t(4;11) positivity	1	SER MRD (N=1)
PPR	3	SER MRD (N=1)
Hypodiploidy	0	
SER MRD	16	HR MFC-MRD D15 (N=7)
HR MFC-MRD D15	3	

b: CD371 negative BCP-ALL at diagnosis (149 patients)

HR criteria (hierarchical)	N	Additional HR criteria
HR MRD	21	PPR - HR MFC-MRD D15 (N=1)
		No CR D33 - HR MFC-MRD D15 (N=3)
		No CR D33 (N=1)
		No CR D33 – PPR – HR MFC-MRD d15 (N=2)
		No CR D33 – t(4;11) positivity – PPR (N=1)
		HR MFC-MRD D15 (N=3)
No CR D33	3	PPR – SER MRD – HR MFC-MRD D15 (N=1)
		SER MRD – HR MFC-MRD D15 (N=1)
		SER MRD (N=1)
t(4;11) positivity	4	PPR (N=1)
PPR	46	SER MRD – HR MFC-MRD D15 (N=6)
		SER MRD (N=2)
		HR MFC-MRD D15 (N=12)
Hypodiploidy	7	HR MFC-MRD D15 (N=1)
SER MRD	37	HR MFC-MRD D15 (N=16)
HR MFC-MRD D15	31	

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

Table S4: DUX4 rearrangements analysis in the Austrian and Czech cohort. Almost all the DUX4 rearrangements were associated with CD371 positivity a diagnosis. We confirmed CD371 to be a surragate marker of DUX4 fusions in BCP-ALLs with high sensitivity (76.5%) and specificity (99.5%).

Patients (420)	**CD371 positive (n=35)**	**CD371 negative (n=385)**
DUX4 positive	27	1
DUX4 negative	4*	380**
DUX4 not tested	1	4

4*CD371 positive (all with weak expression) DUX4 negative: 2 KMT2A::AFF1; 1 hyperdiploid; 1 ZNF384
380**CD371 negative: DUX4 negative or not tested by exclusive genetic subtype (ETV6::RUNX1, TCF3::PBX1, KMT2A:AAFF1, high hyperdiploid, hypodiploid, iAMP21)
Abbreviations: DUX4: DUX4 rearrangements

Figure S4: Outcome analysis: 5-year cumulative incidence of relapse of CD371^{pos} BCP-ALLs vs. CD371^{neg} BCP-ALLs

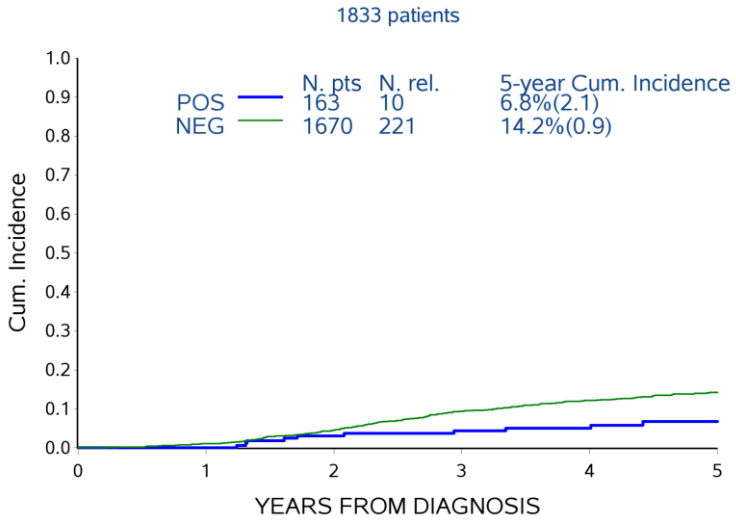


Table S5: Antibody panel used for BCP-ALL immunophenotyping at diagnosis and MRD monitoring in AIEOP cohort.

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	cyCD79a	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 (Beckman Coulter, Inc., Brea, CA, USA)

FITC	**PE**	**ECD**	**PC5.5**	**PC7**	**APC**	**APC-ALEXA700**	**APC-Cy7/ ALEXA750**	**V450**	**V500**
CD58	CD9	CD123	CD371	CD19	CD34	CD10	CD38	CD20	CD45
SYTO16	CD22	-	7AAD	CD19	CD3	CD45	CD34	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

References

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