CD123 expression levels in 846 acute leukemia patients based on standardized immunophenotyping

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SUPPLEMENTAL DATA

General Note

This supplement consist of two parts:

Main Supplement

Additional data supporting the main article, including:

- Cohort characteristics
- Representative examples
- Leukemic stem cell analysis
- Additional assay evaluation
- WHO classifications

Extended Supplement

Additional statistical analysis, confirming results as presented within the main article and main supplement, by using alternative statistical analysis methods, including:

- Identical figures, based on mean instead of median values (including statistics).
- Identical figures, based on 1000 intensity cut-off instead of 739 intensity cut-off (including statistics).
- Identical figures, based on individual cohorts instead of the combined cohort (including statistics).

MAIN SUPPLEMENT

1. Cohort Description / Cohort Characteristics

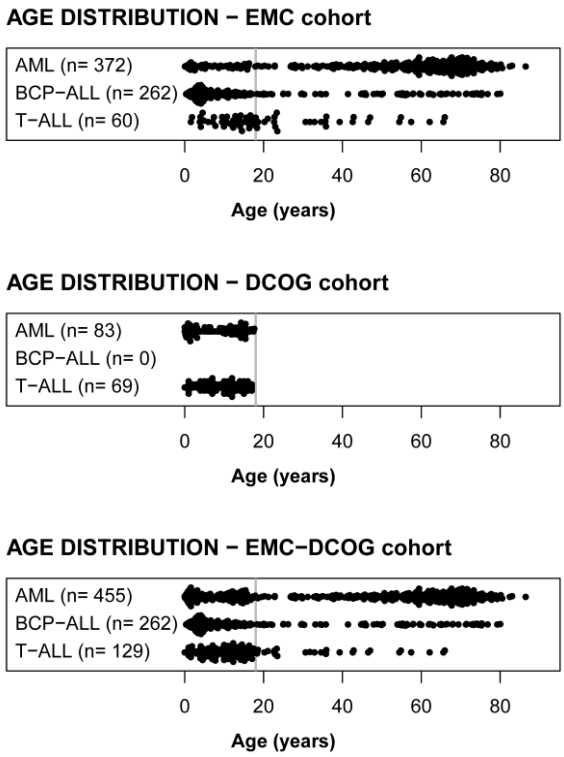
Table - Number of patients for each individual cohorts and the combined cohort.

	EMC cohort		
	Pediatric	**Adult**	**Total**
AML	56	316	372
BCP-ALL	193	69	262
T-ALL	32	28	60

	DCOG cohort		
	Pediatric	**Adult**	**Total**
AML	83	-	83
BCP-ALL	-	-	0
T-ALL	69	-	69

	EMC-DCOG cohort		
	Pediatric	**Adult**	**Total**
AML	139	316	455
BCP-ALL	193	69	262
T-ALL	101	28	129

Figure - Age distribution for each individual cohort and the combined cohort.



Evaluated EMC-DCOG cohort consists of patients from the EMC cohort and DCOG cohort.
The EMC cohort contains consecutive AML, BCP-ALL and T-ALL patients (thus without age restriction).
The DCOG cohort contains consecutive pediatric AML and T-ALL patients (thus with age restriction).

Combining the EMC cohort and DCOG cohort resulted in a skewed age distribution for AML and T-ALL patients, since the DCOG cohort only contains pediatric patients and the EMC cohort contains both pediatric and adult patients. Age distribution of BCP-ALL patients was not affected, since the DCOG cohort does not include any BCP-ALL patients. Age distributions are visualized for each cohort and the combined cohort.

All statistical analysis including age as parameter, were performed in two cohorts:

- EMC-DCOG More patients, but enriched for pediatric patiants.
- EMC cohort Fewer patients, but normal representative age distribution.

Table - Subgroups for AML and BCP-ALL patients, based on the WHO 2008 classification.

AML	
WHO 2008	**Count**
AML with t(8;21)(q22;q22)	23
AML with inv(16)(p13q22)	20
AML with t(15;17)	29
AML with t(9;11)(p21.3;q23.3)	29
AML with mutated *NPM1*	91
AML with mutated *CEBPA*	13
AML with mutated *RUNX1*	6
AML with myelodysplasia-related changes	70
AML therapy-related	36
AML without maturation	25
AML with minimal differentiation	17
AML with maturation	19
Acute myelomonocytic leukemia	15
Acute monoblastic/monocytic leukemia	21
Acute erythroleukemia	14
Acute megakaryoblastic leukemia	13
Other	14

BCP-ALL	
WHO 2008	**Count**
with t(9;22)(q34;q11.2), *BCR-ABL1*	23
with t(v;11q23); *KMT2A* rearranged	14
with t(12;21)(p13;q22) *TEL-AML1* (*ETV6-RUNX1*)	50
with t(1;19)(q23;p13.3) *TCF3-PBX1*	4
with high-hyperdiploidy (chromosome number > 50)	46
with hyperdiploidy (chromosome number > 46)	50
with normal karyotype	30
with pseudodiploid karyotype	31
Other	14

2. EuroFlow panels

Table - EuroFlow tubes used for evaluation of CD123 expression

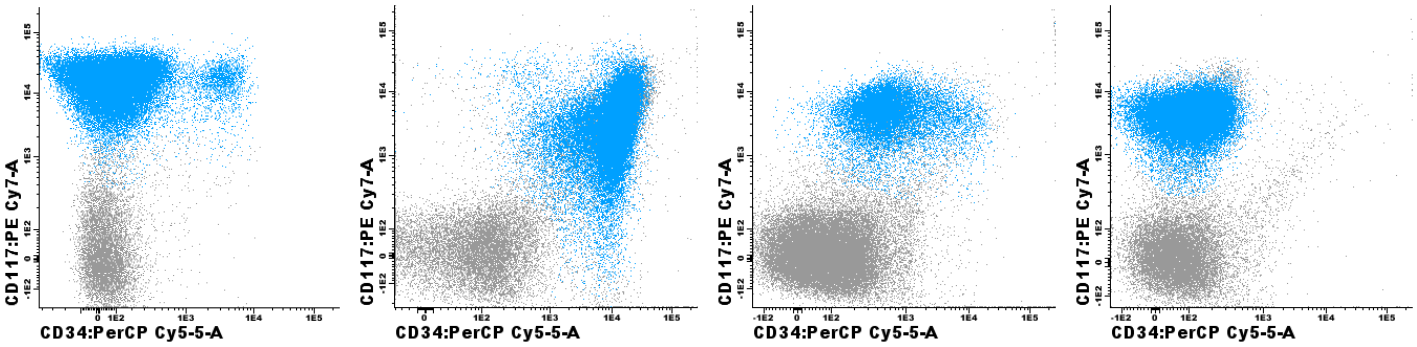
	PB	PO	FITC	PE	PerCPCy5	PeCy7	APC	APCH7
BCP-ALL Tube 4	CD21	CD45	CD15 CD65	NG2	CD34	CD19	CD123	CD81
T-ALL Tube 4	cyCD3	CD45	CD44	CD13	HLADR	CD45RA	CD123	smCD3
AML-MDS Tube 6	HLADR	CD45	CD42a CD61	CD203c	CD34	CD117	CD123	CD4

Staining and acquisition procedures were performed according to EuroFlow guidelines (1) and flow cytometry was performed on FACSCanto II flow cytometers (BD) with EuroFlow instrument settings (2). Common markers are shown in grey.

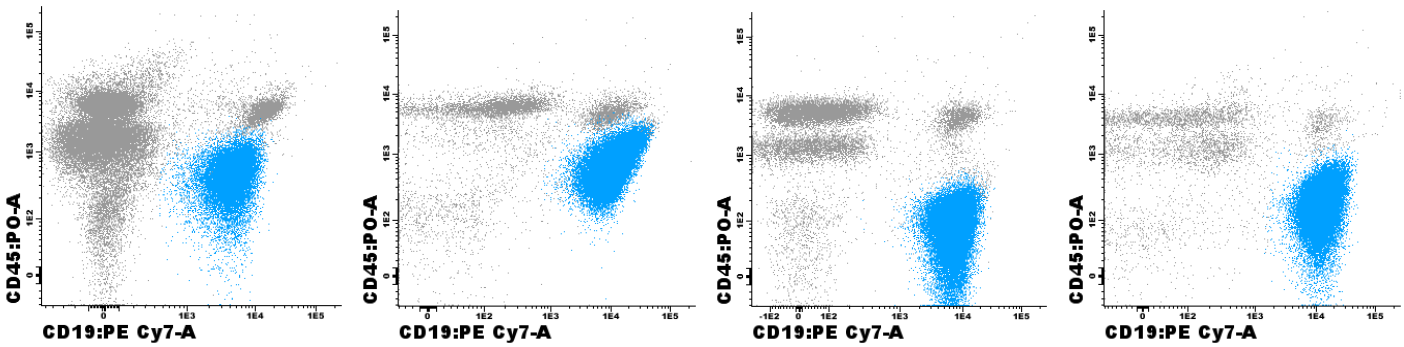
3. Malignant populations

Figure - Representative plots for various blast populations.

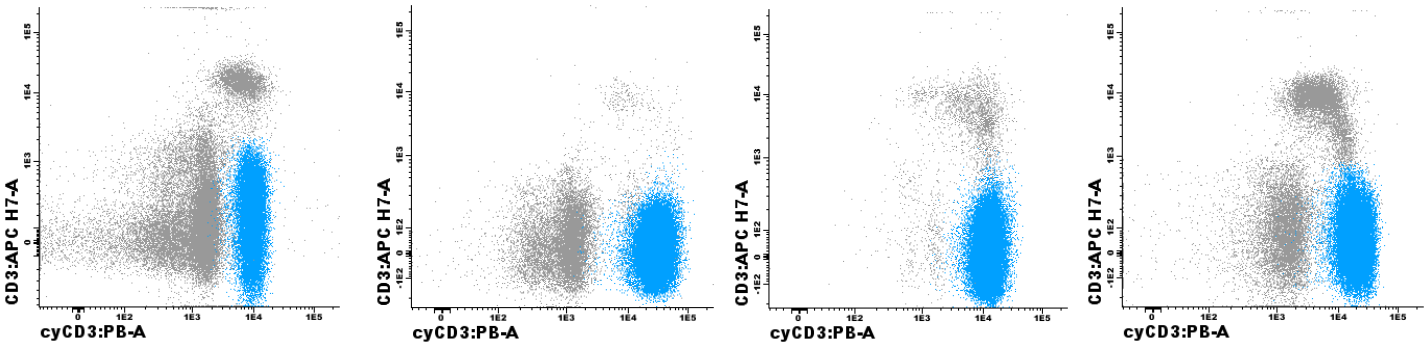
AML blasts



BCP-ALL blasts



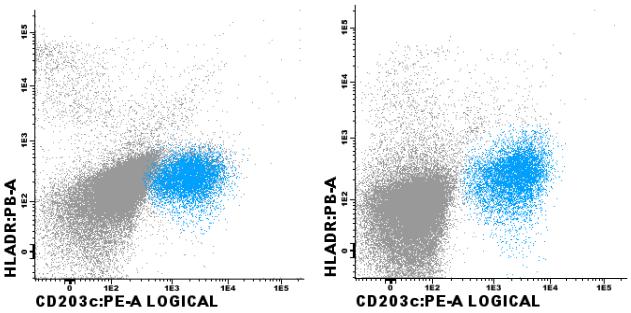
T-ALL blasts



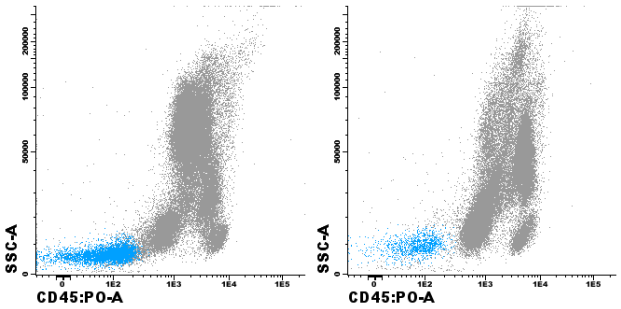
4. Normal populations

Figure - Representative plots for various normal populations.

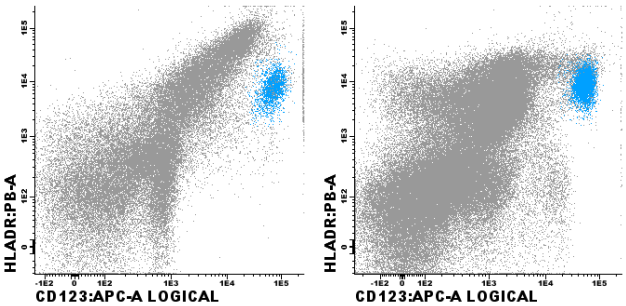
Basophils



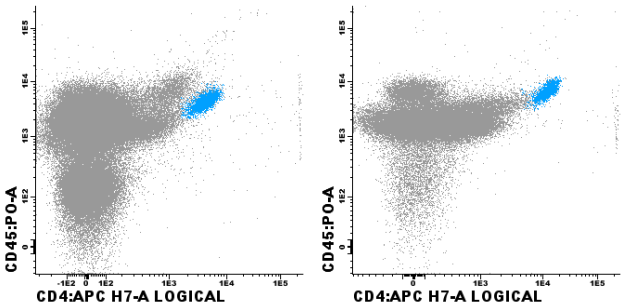
Nucleated Red Blood Cells



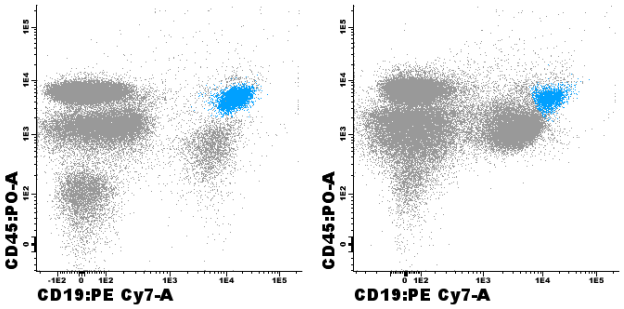
Plasmacytoid dendritic cells



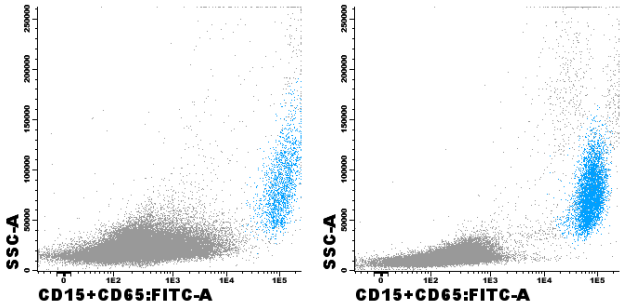
T-Lymphocyes



Mature B-Lymphocytes



Granulocytes



5. LSC analysis

Leukemic stem cell (LSC) data was available for 32 out of 56 pediatric AML cases from the EMC cohort.

- Data from our earlier LSC study (3) was used, thus only patients enrolled in this study were evaluated.
- Only CD34 positive AML cases were eligible for LSC evaluation. LSC are defined as the CD34 positive CD38 negative fraction of the tumor population. Generally CD34 negative AML cases did not have enough LSC to evaluate CD123 expression in a reliable way.

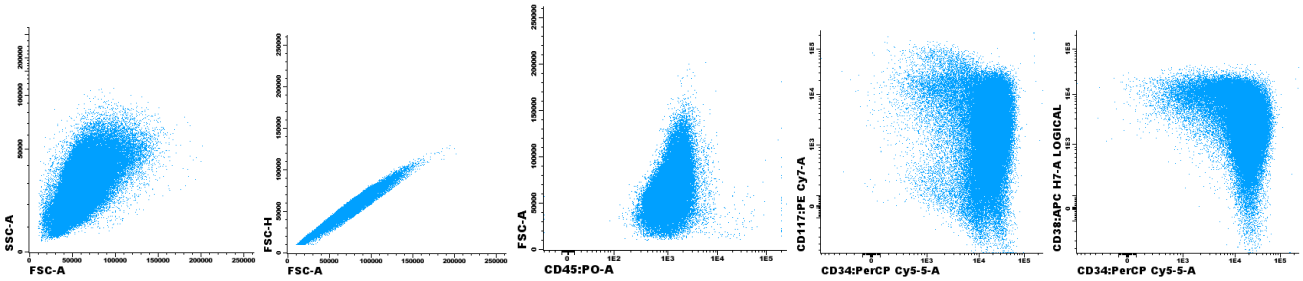
Detailed information regarding the LSC study and performed LSC assay was previously published (3).

In summary, immunophenotyping was performed using the following EuroFlow tube:

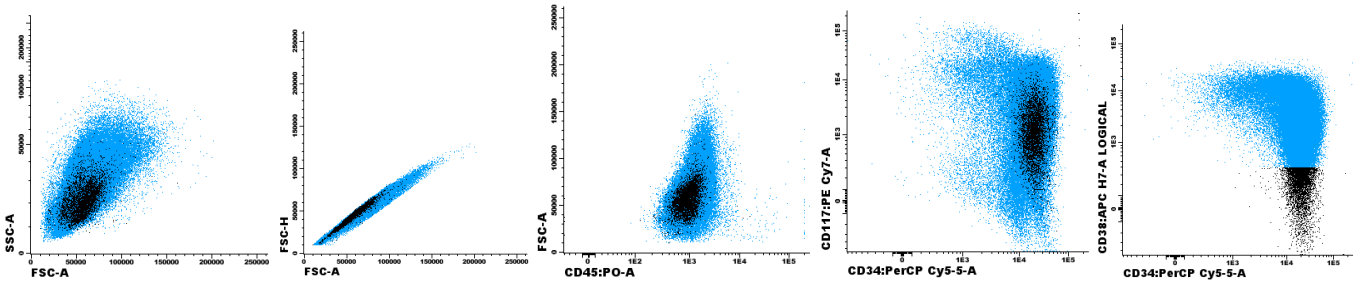
PB	PO	FITC	PE	PerCPCy5	PeCy7	APC	APCH7
HLADR	CD45	CD123	CD56	CD34	CD117	CD7	CD38

Manual analysis was performed using the following gating strategy:

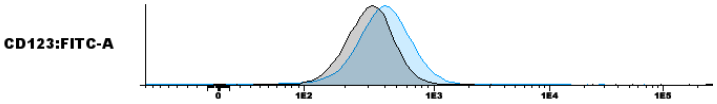
1. Identification of tumor population.



2. Identification of CD34 positive CD38 negative tumor fraction (LSC).



3. Comparison of CD123 expression on LSC versus non-LSC



6. Assay Stability

Figure - NRBC and PDC grouped by acquisition year

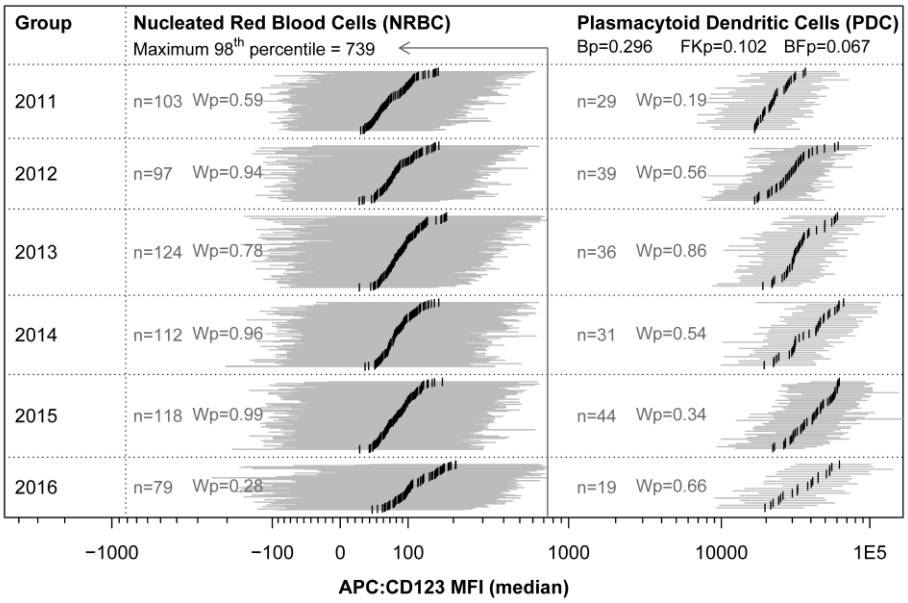
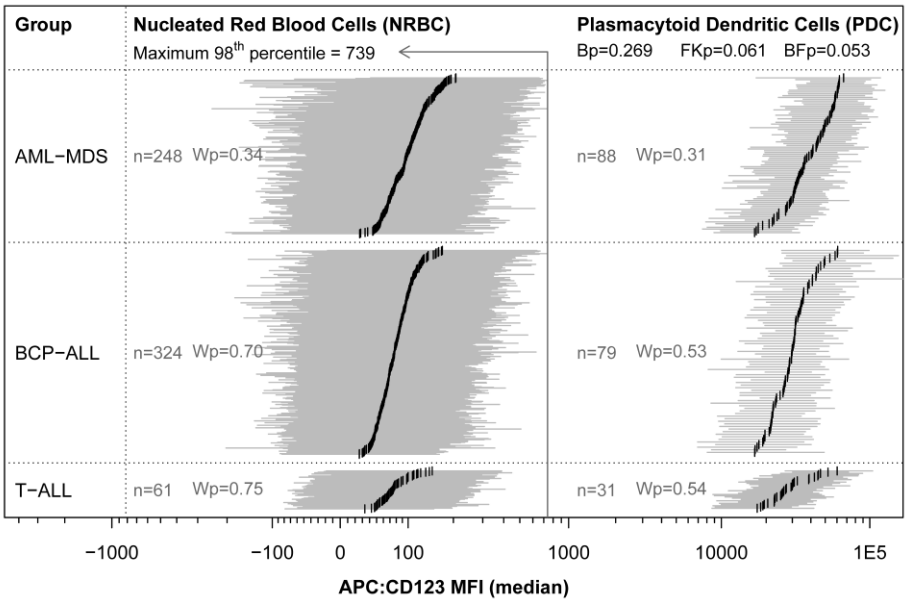


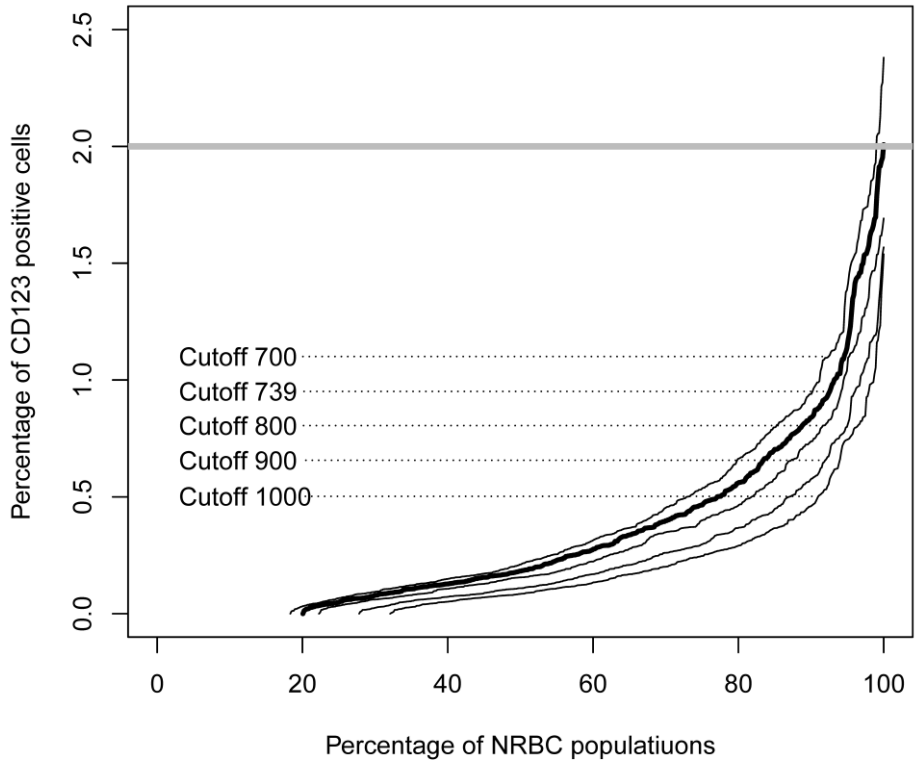
Figure - NRBC and PDC grouped by acquisition panel



Ledged for indicated probability values
Bp = Bartlett test
FKp = Fligner-Killeen test
BFp = Brown-Forsythe test
Wp = Mann-Whitney-U test (after Bonferroni correction)

7. Evaluation CD123 positivity cut-offs

Figure - Influence of various positivity cut-offs on the number of CD123 positive NRBC



CD123 positivity cut-off was defined as the highest 98th percentile among 633 normal NRBC populations. Using this objective positivity cut-off (located at 739 intensity), all 633 normal NRBC populations had less than two percent CD123 false-positive cells (and 95 percent had less than 1 percent CD123 false-positive cells). In addition, various other arbitrary positivity cut-offs (700, 800,900 and 1000) were evaluated as well, which resulted in highly comparable numbers of CD123 false-positive NRBC.

Note: CD123 false-positive assuming NRBC are always CD123 negative (by definition).

8. Precursors

CD123 expression on CD34-positive BCP, CD34-negative BCP and CD34-positive myeloid precursors was evaluated in a limited number of bone marrow samples that were considered normal during routine diagnostic evaluation and acquired between January 2011 and December 2016.

Following populations were identified in the sixth tube of the EuroFlow AML/MDS panel (**supplement 2**):

- CD34+ B cell precursors (orange)
- CD34− B cell precursors (blue)
- CD34+ Myeloid precursors (pink)

Figure – Two representative samples

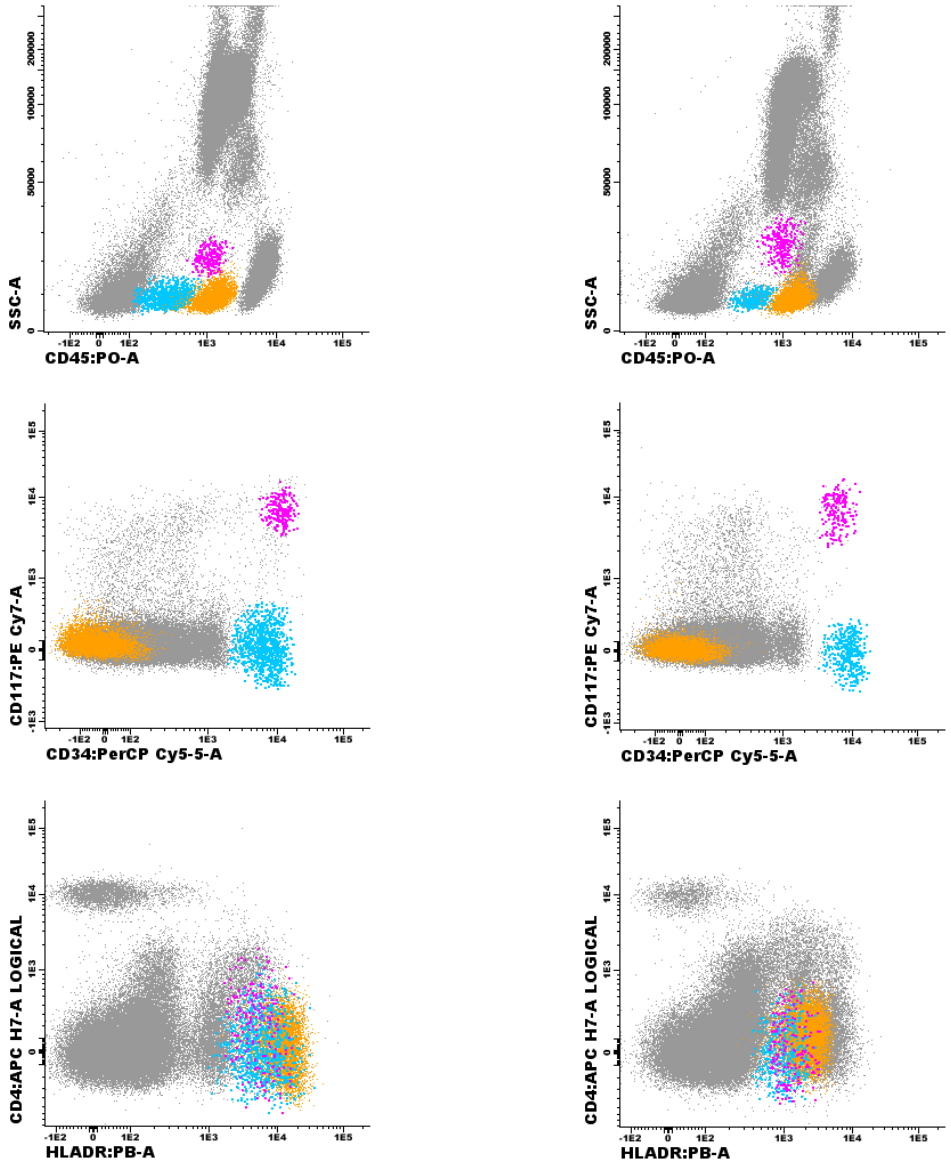
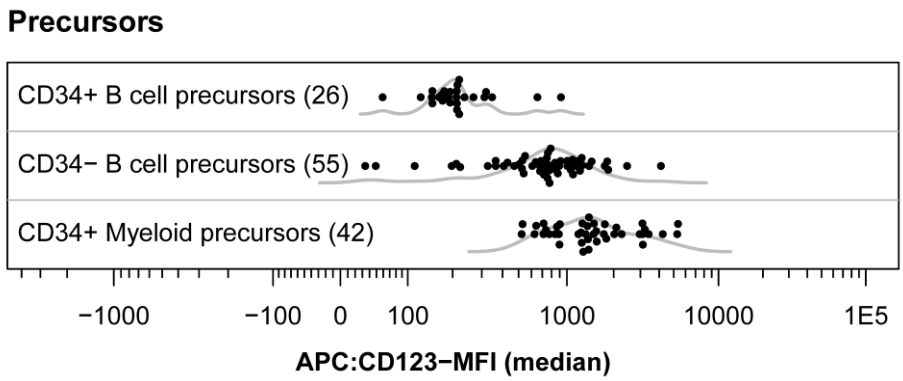


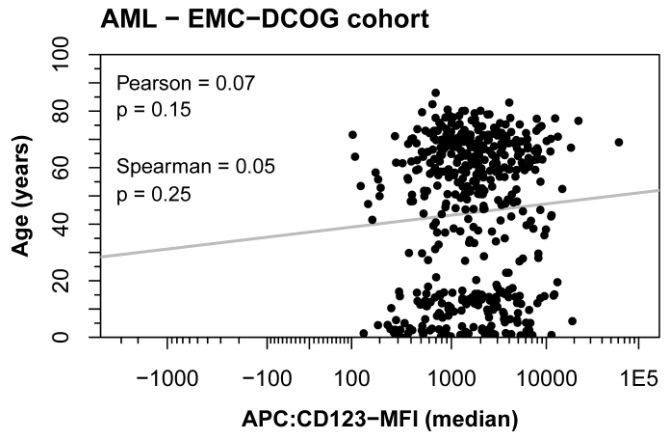
Figure – CD123-MFI for normal B cell precursors and myeloid precursors

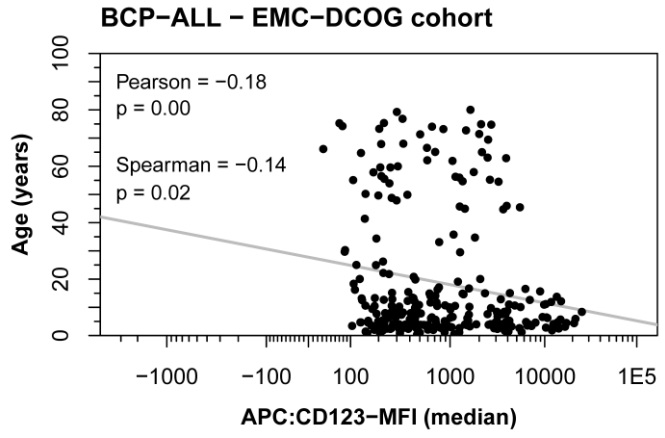


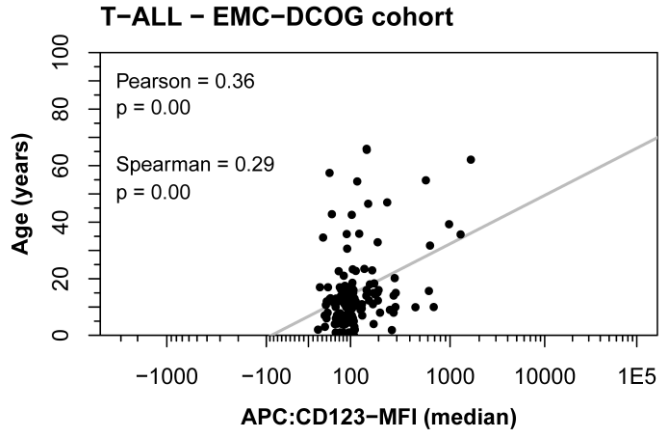
In conclusion CD123-MFI was low in CD34-positive BCP, higher in CD34-negative BCP ($p<0.001$) and highest in CD34-positive myeloid precursors ($p<0.001$).

9. Continuous analysis of CD123-MFI versus age

Figure - Correlation between CD123-MFI and age (in AML, BCP-ALL and T-ALL)







Continuous analysis revealed that age negatively correlated to CD123-MFI in BCP-ALL, positively correlated to CD123-MFI in T-ALL and did not correlate to CD123-MFI in AML.

10. CD123-MFI versus WHO classification for AML and BCP-ALL

Figure - CD123-MFI for AML patients, grouped by WHO classification (number of patients indicated between brackets)

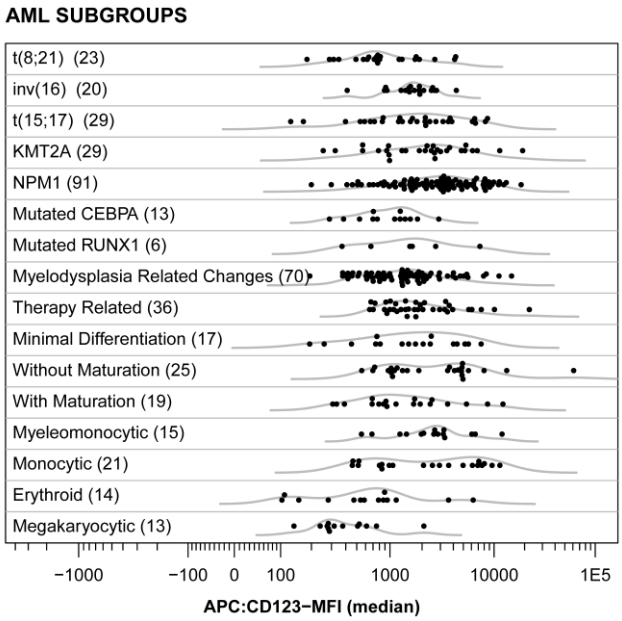


Figure - CD123-MFI for AML patients, grouped by FLT3-ITD status (number of patients indicated between brackets)

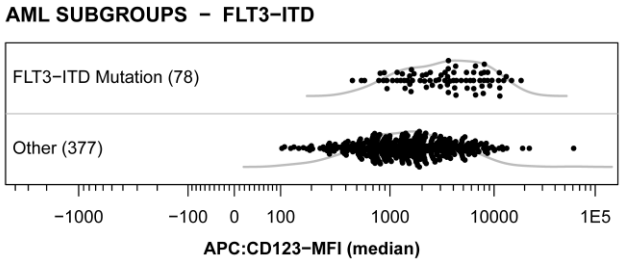
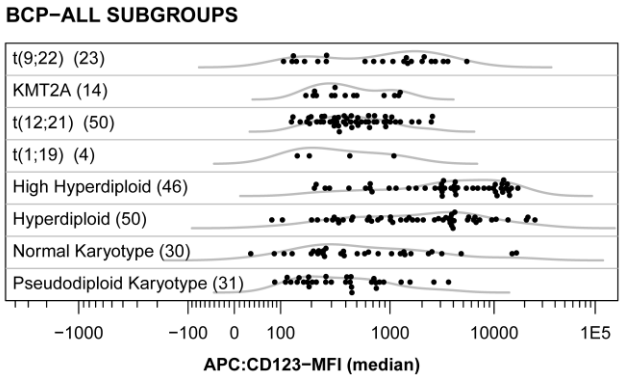
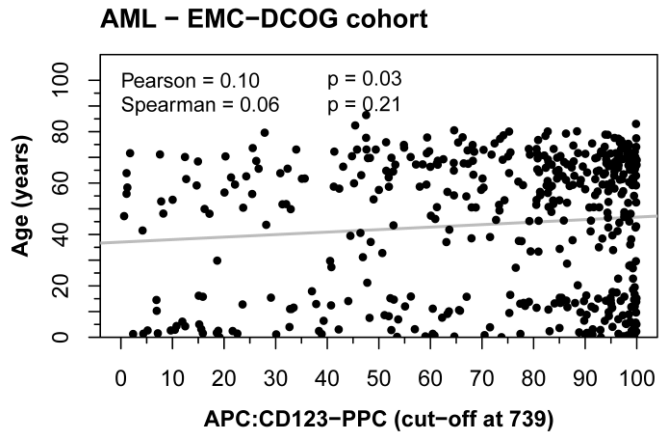


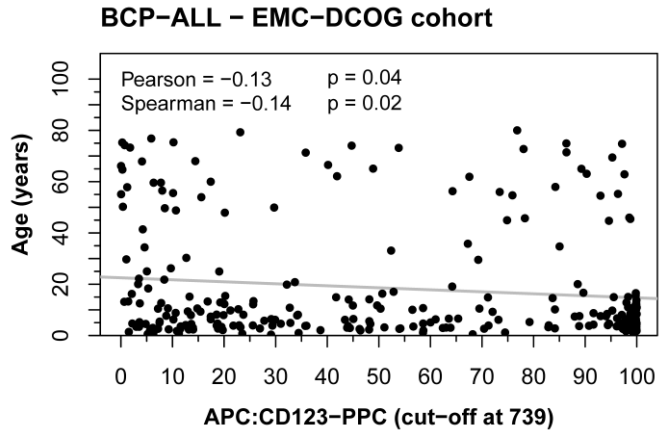
Figure - CD123-MFI for BCP-ALL patients, grouped by WHO classification (number of patients indicated between brackets)

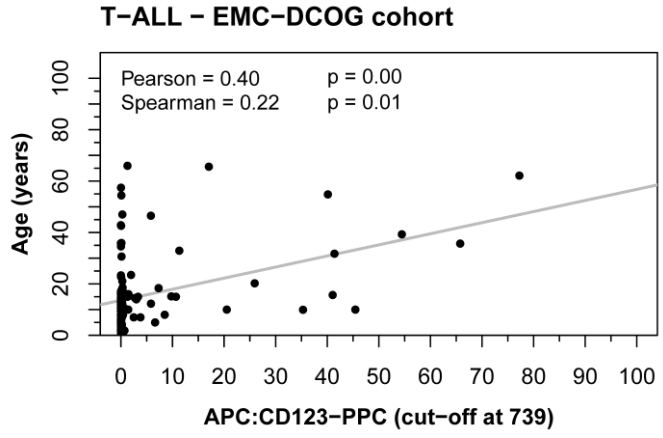


11. Continuous analysis of CD123-PPC versus age

Figure - Correlation between CD123-PPC (based on the 739 cut-off) and age (in AML, BCP-ALL and T-ALL)







Continuous analysis revealed that age negatively correlated to CD123-PPC in BCP-ALL, positively correlated to CD123-PPC in T-ALL and did not correlate to CD123-PPC in AML.

12. CD123-PPC versus WHO classification in AML and BCP-ALL

Figure - CD123-PPC (based on the 739 cut-off) for AML patients, grouped by WHO classification

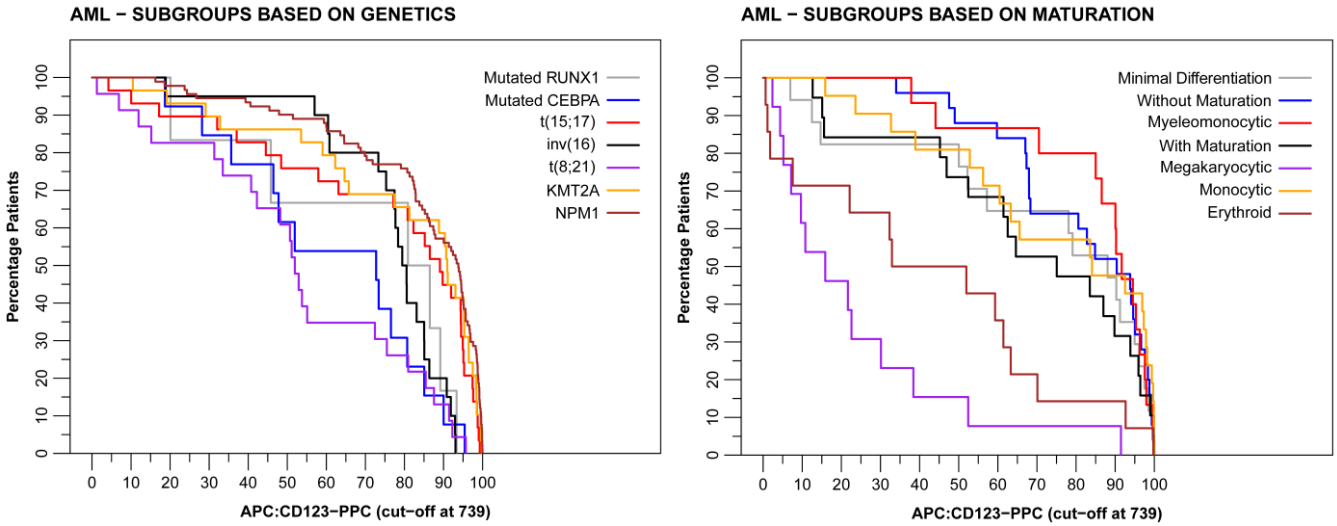
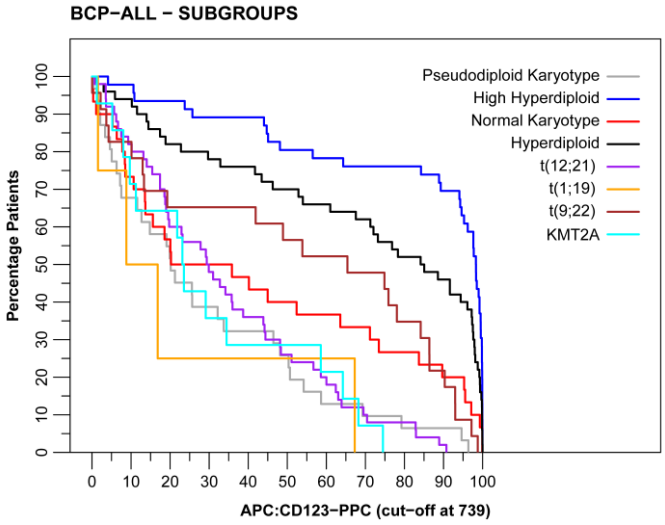


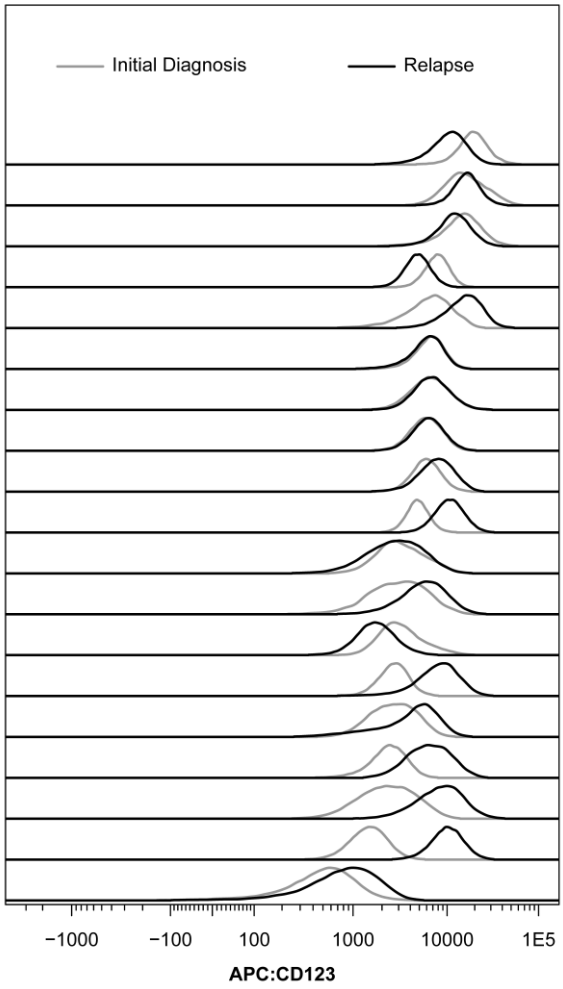
Figure - CD123-PPC (based on the 739 cut-off) for BCP-ALL patients, grouped by WHO classification



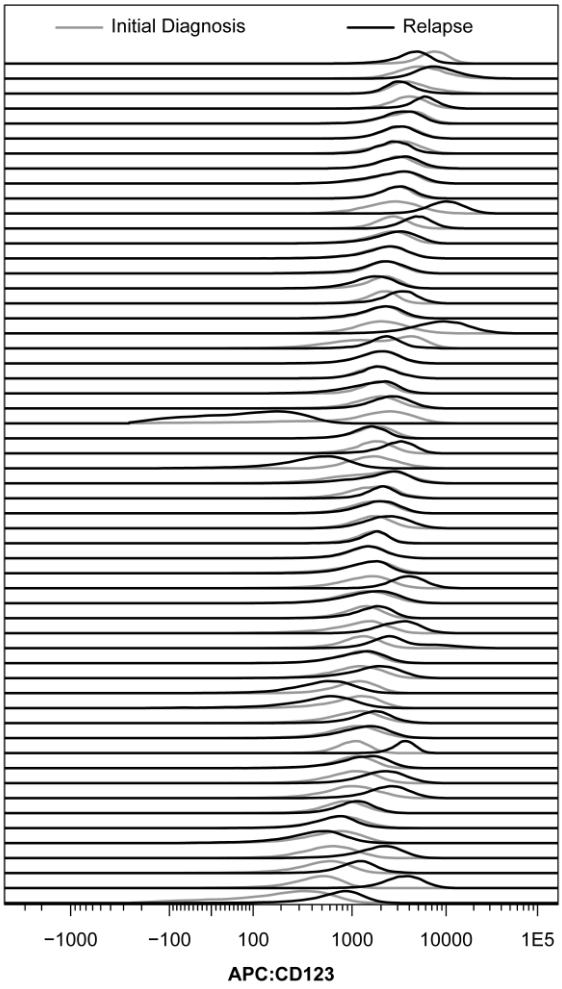
13. CD123 distribution of DC versus RX

Figure - CD123 expression at initial diagnosis (DX) and relapse (RX) in AML and BCP-ALL

BCP-ALL (n=19)



AML (n=57)



14. Time-To-Relapse

Figure – Delta CD123 expression (after logicile transformation) for initial diagnosis versus relapse, where positive delta indicates higher CD123 expression at relapse (compared to initial diagnosis).

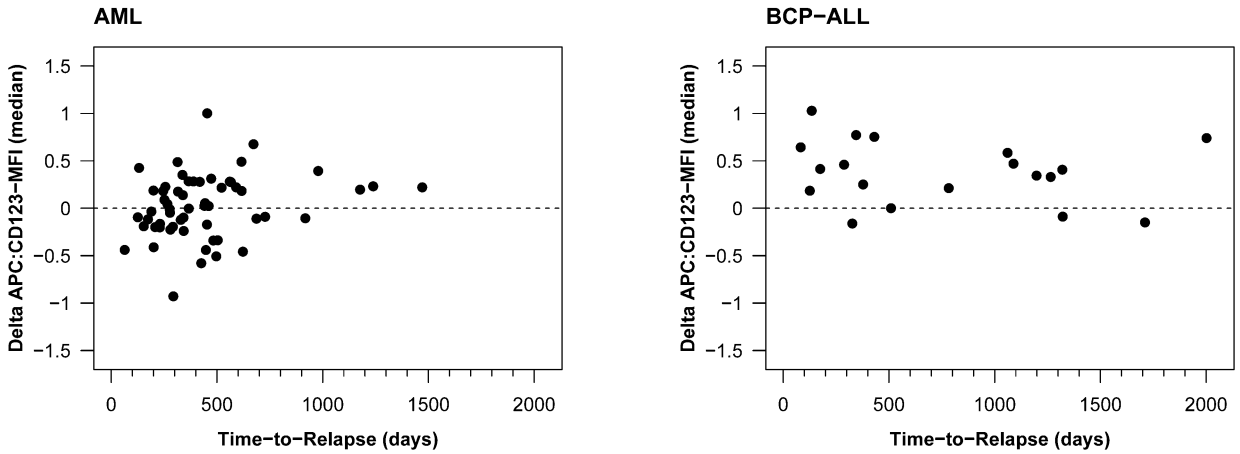


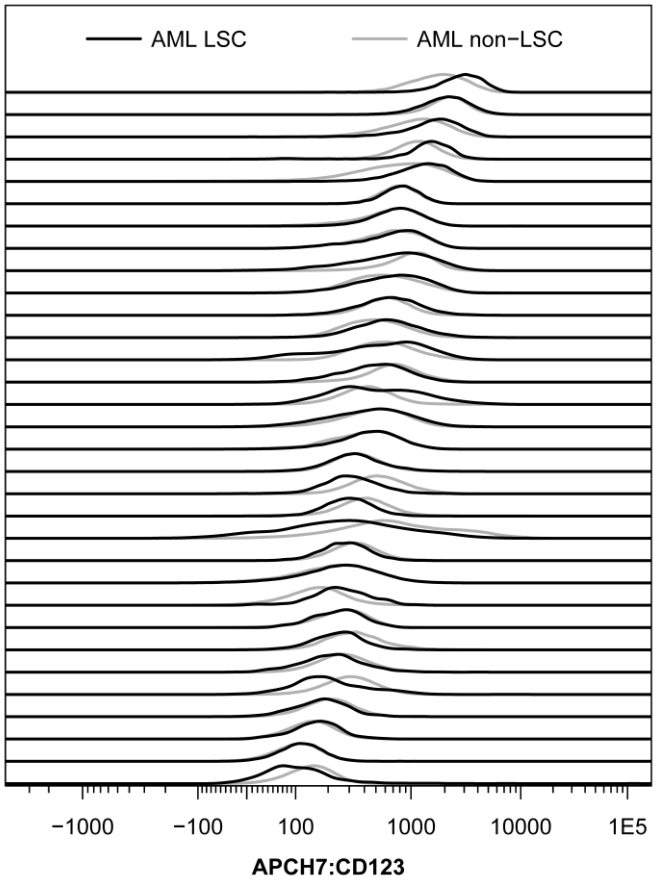
Table – Patient characteristics

AML	Loss	Gain
Myeleomonocytic	1	2
inv(16)	2	1
Minimal Differentiation	-	4
With Maturation	2	1
KMT2A	4	4
Without Maturation	2	1
NPM1	8	5
t(8;21)	-	1
Other	2	2
Monocytic	2	1
Myelodysplasia Related Changes	4	4
t(15;17)	-	1
Mutated RUNX1	-	1
Erythroid	-	1
Therapy Related	1	-

BCP-ALL	Loss	Gain
Normal Karyotype	-	2
t(12;21)	1	2
High Hyperdiploid	-	1
Hyperdiploid	-	2
Other	1	4
t(9;22)	-	1
Pseudodiploid Karyotype	1	2
KMT2A	-	2

15. CD123 distribution of LSC versus non-LSC in AML

Figure - CD123 expression of LSC and non-LSC in AML



Sufficient numbers of cells were measured to generate robust kernel density estimations.
Expression levels were highly comparable for LSC (shown in black) and non-LSC (shown in grey).

Reference

1. van Dongen JJ, Lhermitte L, Bottcher S, Almeida J, van der Velden VH, Flores-Montero J, Rawstron A, Asnafi V, Lecrevisse Q, Lucio P and others. EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. Leukemia 2012;26:1908-75.
2. Kalina T, Flores-Montero J, van der Velden VH, Martin-Ayuso M, Bottcher S, Ritgen M, Almeida J, Lhermitte L, Asnafi V, Mendonca A and others. EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. Leukemia 2012;26:1986-2010.
3. Hanekamp D, Denys B, Kaspers GJL, Te Marvelde JG, Schuurhuis GJ, De Haas V, De Moerloose B, de Bont ES, Zwaan CM, de Jong A and others. Leukaemic stem cell load at diagnosis predicts the development of relapse in young acute myeloid leukaemia patients. J Immunol Methods. 2018 Mar 9. pii: S0022-1759(17)30126-6. doi: 10.1016/j.jim.2018.03.005. [Epub ahead of print].