Phenotypic profiling of CD34+ cells by advanced flow cytometry improves diagnosis of juvenile myelomonocytic leukemia

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Supplementary Material and Methods
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Flow cytometry

Whole BM or PB samples were stained with both the 8-color EuroFlow ALOT and the AML/MDS/MPN antibody panels. In particular, ALOT allowed assignment of the B-lymphoid (CD34+/CD19+/cyCD79a+) *vs* early lymphoid progenitors (CD34+/CD7+/CD3-) *vs* neutrophil (CD34+/CD7-/CyMPO+) lineage of CD34+ cells and also the lineage dissection of CD34-negative cells. Moreover, we identified aberrant HPC precursor cell subsets based on the presence of immunophenotypes which are not found in normal BM. These specifically included the presence of any CD34+ HPC population (>0.01%) showing CyMPO+/CD7+, CyMPO+/CyCD3+, CyMPO+/CyCD79a+, CyMPO+/CD19+, CD7+/CyCD79a+, CD7+/CD19+ and combinations of these immunophenotypes, together with overexpression (>median +3SD of normal BM levels) of CD34+ and/or abnormally high FSC and SSC values.

The AML/MDS/MPN antibody panel consists of a set of back bone markers in tubes 1 to 3 (CD34, CD117, HLA-DR and CD45) and additional lineage-specific markers for the identification of progenitors committed to the three major BM myeloid lineages (neutrophil, monocytic and erythroid cells). For each sample, >500,000 nucleated cells were measured on a FACSCanto II flow cytometer (BD Biosciences). For data analysis, the Infinicyt™ software (Cytognos SL) was used.

Supplementary Tables

Table S1. Eight-color flow cytometry antibody panel used for the characterization of CD34+HPC

Fluorochrome position for antibody markers								
Antibody combination	**PB**	**PO**	**FITC**	**PE**	**PerCP -Cy5.5**	**PC7**	**APC**	**APC-H7**
ALOT	CyCD3	CD45	CyMPO	CyCD79a	CD34	CD19	CD7	SmCD3
AML/MDS Tube 1	HLA-DR	CD45	CD16	CD13	CD34	CD117	CD11b	CD10
AML/MDS Tube 2	HLA-DR	CD45	CD35	CD64	CD34	CD117	IREM-2	CD14
AML/MDS Tube 3	HLA-DR	CD45	CD36	CD105	CD34	CD117	CD33	CD71

Abbreviations: PB, pacific blue; PO, pacific orange; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP-Cy5.5; peridinin–chlorophyll–proteincyanin 5.5; Cy7, cyanin 7; APC, allophycocyanin; ALOT, acute leukemia orientation tube; H7, hilite7; Cy, cytoplasmic; Sm, surface membrane.

Table S2. Clinical and laboratory findings of 3 additional JMML patients

Patient code	AGE (Years)	Gender	Splenomegaly	WBC x10^9/L	Monocytes x10^9/L	% Blasts (by morphology)	Genetic subgroup[§]	Caryotype
JMML 32	M	3	P	8.8	6.0	7.0	*PTPN11*	47, XY, +8
JMML 33	F	6	P	14.4	1.6	12.0	*PTPN11*	46, XX
JMML 34	M	1	A	7.0	1.2	1.0	*PTPN11*	46, XY

F, Female; M, Male; P, present; A, Absent; n.k., Not Known; § *PTPN11*or *K-RAS* or *N-RAS* or *RAS* are intended as somatic mutations (germline *status* was excluded based on buccal swab testing) , *CBL* is intended as germline mutation ± loss of heterozygosity (LOH), NF-1 is intended as clinical diagnosis of Neurofibromatosis type 1.

Table S3. Percentage of total CD34+ HPC and their immunophenoptypic subsets: predictive score values in control subjects and JMML patients

Control code	Source of sample	% of total CD34+ HPC[§]	% B cell precursors	% CD7+ precursors	% Neutrophil precursors	% Monocytic precursors	% Erhytroid precursors	% Aberrant precursors	Score model 1 *	Score model 2 **
CTR 1	BM	0.94	42.11	5.01	35.45	4.31	5.13	0.00	3.100	-5.578
CTR 2	BM	1.00	60.14	1.44	25.11	4.71	3.16	0.00	0.891	-4.255
CTR 3	BM	2.12	63.57	2.17	23.72	1.25	3.04	0.05	1.415	-3.703
CTR 4	BM	2.21	71.16	1.78	21.09	1.49	1.73	0.36	1.621	-1.836
CTR 5	BM	1.97	60.07	1.28	25.25	0.68	3.17	0.00	0.792	-4.350
CTR 6	BM	2.34	62.64	1.86	23.97	0.36	1.85	0.00	1.151	-1.985
CTR 7	BM	0.72	52.37	2.87	27.99	11.77	3.65	0.00	1.776	-4.314
CTR 8	BM	0.67	27.00	3.12	54.09	7.19	0.54	0.00	1.930	0.703
CTR 9	BM	0.59	3.65	5.91	77.66	6.54	1.85	0.20	3.945	0.030
CTR 10	BM	1.77	67.05	0.67	24.78	0.38	3.10	0.00	0.415	-4.543
CTR 11	BM	5.35	67.77	4.04	16.03	6.77	0.34	0.36	2.995	1.455
CTR 12	BM	1.36	47.29	2.99	41.41	3.00	1.83	0.73	2.904	-1.391
CTR 13	BM	1.51	67.77	2.38	21.57	2.69	1.85	0.22	1.790	-1.726
CTR 14	BM	1.15	52.69	1.41	30.24	4.97	2.27	0.00	0.872	-2.869
CTR 15	BM	5.31	70.62	1.64	n.d.	n.d.	n.d.	0.45	1.665	n.d.
CTR 16	BM	2.50	76.51	1.30	18.06	1.60	n.d.	0.05	0.876	n.d.
CTR 17	BM	0.96	65.72	2.10	22.04	1.00	4.48	0.10	1.444	-6.003
CTR 18	BM	4.07	84.35	0.84	5.58	1.25	1.92	0.04	0.577	-2.602
CTR 19	BM	2.18	57.83	6.01	21.18	1.00	5.69	0.20	4.007	-5.961
CTR 20	BM	2.83	61.82	2.60	15.53	3.14	1.84	0.06	1.695	-1.601
CTR 21	BM	2.59	64.68	3.39	20.23	3.21	1.01	0.05	2.170	0.098
CTR 22	BM	2.90	57.30	6.23	20.46	3.81	5.74	0.33	4.331	-5.930
CTR 23	BM	2.07	66.34	4.00	15.42	3.28	0.37	0.00	2.475	1.408
CTR 24	BM	0.57	34.03	4.22	n.d.	n.d.	n.d.	0.00	2.611	n.d.
CTR 25	BM	1.03	11.27	7.05	n.d.	n.d.	n.d.	0.06	4.448	n.d.
CTR 26	BM	0.84	25.87	5.55	n.d.	n.d.	n.d.	1.90	6.178	n.d.
CTR 27	BM	1.33	47.21	3.02	n.d.	n.d.	n.d.	0.82	3.024	n.d.
CTR 28	BM	1.22	15.67	4.59	59.5	10.22	5.21	0.00	2.840	-5.897
CTR 29	BM	1.76	41.51	1.17	33.04	8.92	0.87	0.11	0.868	-0.787

Patient code	Source of sample	% of total CD34+ HPC[§]	% B cell precursors	% CD7+ precursors	% Neutrophil precursors	% Monocytic precursors	% Erhytroid precursors	% Aberrant precursors	Score model 1 *	Score model 2 **
JMML 1	PB	1.78	0.81	26.63	39.91	22.15	0.03	0.96	17.862	13.200
JMML 2	PB	2.01	0.51	63.00	15.00	6.00	0.23	11.52	55.617	30.977
JMML 3	PB	2.22	0.03	78.84	n.d.	n.d.	n.d.	6.11	57.602	n.d.
JMML 4	BM	10.05	13.90	7.89	46.69	24.42	0.05	1.55	7.120	3.846
	PB	6.39	0.65	8.10	46.00	18.00	0.05	0.10	5.156	3.951
JMML 5	PB	5.55	0.01	81.87	0.30	1.00	0.41	16.98	75.178	40.08
JMML 6	BM	2.78	64.46	3.72	15.02	10.95	0.20	2.86	6.433	1.536
JMML 7	BM	2.44	23.17	8.56	46.00	18.00	0.20	0.65	6.235	3.943
	PB	0.33	0.32	6.86	n.d.	n.d.	n.d.	0.16	4.475	n.d.
JMML 8	BM	3.42	2.42	85.82	n.d.	n.d.	n.d.	11.41	69.576	n.d.
	PB	2.57	5.71	88.45	n.d.	n.d.	n.d.	3.88	60.326	n.d.
JMML 9	PB	0.08	34.78	9.78	39.22	16.29	0.00	0.00	6.051	4.865
JMML 10	PB	0.85	0.57	22.34	45.21	20.00	0.36	1.08	15.381	10.546
JMML 11	PB	1.23	6.77	5.26	n.d.	n.d.	0.20	2.84	7.357	2.302
JMML 12	PB	0.32	1.20	6.01	40.00	49.00	0.91	0.43	4.339	1.558
JMML 13	BM	4.67	0.27	85.88	3.97	12.73	0.33	11.74	70.090	42.201
	PB	3.51	0.02	81.85	1.53	10.38	0.02	16.51	74.486	40.684
JMML 14	BM	8.32	0.46	88.45	n.d.	n.d.	n.d.	6.09	63.518	n.d.
	PB	8.89	0.11	89.98	n.d.	n.d.	n.d.	7.44	66.415	n.d.
JMML 15	BM	3.43	31.83	24.16	37.00	29.00	0.13	8.53	27.268	11.814
	PB	1.86	4.35	37.00	23.24	35.96	0.22	2.18	26.040	18.059
JMML 16	BM	6.50	66.49	3.59	15.00	6.12	0.15	6.61	11.769	1.550
JMML 17	PB	2.53	0.52	28.66	53.46	9.57	0.14	2.84	21.833	14.036
JMML 18	BM	2.91	2.63	48.52	32.50	3.29	0.71	5.91	38.555	23.019
	PB	2.20	0.64	54.68	38.52	4.78	0.04	3.23	38.495	27.137
JMML 19	BM	2.23	55.36	1.48	26.15	1.64	1.81	0.45	1.566	-2.111
JMML 20	BM	4.23	2.85	19.38	41.02	17.14	0.39	6.97	22.058	9.027
JMML 21	BM	3.33	9.83	58.00	11.00	2.86	0.14	15.92	58.879	28.631
JMML 22	BM	2.48	2.52	3.43	57.80	6.03	0.60	0.68	3.104	0.762
JMML 23	BM	0.52	2.29	23.11	n.d.	n.d.	1.43	3.48	19.324	9.246
JMML 24	BM	2.95	2.61	39.23	29.64	3.44	0.19	15.34	46.429	19.216
JMML 25	BM	2.48	n.d.	n.d.	28.08	18.5	0.71	n.d.	n.d.	n.d.

JMML 26	BM	17.75	n.d.	n.d.	n.d.	6.16	n.d.	n.d.	n.d.	n.d.
JMML 27	BM	5.49	4.39	69.92	n.d.	4.72	0.07	6.48	52.618	34.671
JMML 28	BM	3.04	0.89	28.48	39.9	15.50	0.88	8.10	29.320	12.783
JMML 29	BM	1.09	0.82	14.95	65.0	17.50	0.26	2.68	13.120	7.028
JMML 30	BM	1.55	4.42	61.61	0.43	0.53	0.49	23.67	72.307	29.876
JMML 31	BM	1.11	2.5	73.17	1.61	3.00	0.19	8.30	57.257	36.099
	PB	0.47	2.21	81.88	0.90	0.94	0.10	2.05	53.618	40.57

* Score calculation formula used for model 1: 0.619* %CD7+ CD34+ precursors + 1.444* % CD34+ aberrant precursors; ** Score calculation formula used for model 2: 0.497* %CD7+ CD34+ precursors - 1.573* % CD34+ erythroid precursors; [§]Percentages of CD34+ HPC are referred to total nucleated cells; Percentages of each immunophenotypic subset are referred to 100% of CD34+ HPC. Note that in each row the sum of the percentages referred to the individual immunophenotypic subsets may not reach the value of 100 as the values of minor subsets such as mast cells and dendritic cells are not reported here.
The % of CD34+ HPC, CD34+ B cell precursors, CD7+ CD34+ precursors and CD34+ aberrant cell precursors were all calculated from the A LOT tube. The % of CD34+ neutrophil precursors was calculated from the AML/MDS Tube 1, the % of CD34+monocytic precursors from AML/MDS Tube 2 and the % of CD34+ erythroid precursors from AML/MDS Tube 3.
Abbrevations: n.d., not determined; CTR, control subject; JMML, patients.

Table S4. Score values obtained with predictive score model 1 and model 2.

Group	**Score values for model 1**				**Score values for model 2**			
	Mean (SD)	Median (range)	1st quartile	3rd quartile	Mean (SD)	Median (range)	1st quartile	3rd quartile
CONTROL	2.235 (1.38)	1.790 (0.414-6.178)	1.151	2.995	-2.680 (2.45)	-2.600 (-6.003-1.455)	-4.543	-0.787
JMML	34.237 (25.60)	22.268 (1.565-75.178)	7.356	57.602	16.710 (14.33)	12.783 (-2.111-42.201)	3.846	29.876

The algorithm used to calculate score values with model 1was: 0.619* %CD7+ CD34+ precursors + 1.444* % CD34+ aberrant precursors; the algorithm used to calculate the score in model 2 was: 0.497* %CD7+ CD34+ precursors - 1.573* % CD34+ erythroid precursors.

Table S5. Percentage of total CD34+ HPC and their immunophenoptypic subsets: predictive score values in a validation cohort of control subjects and JMML patients.

Patient code	Source of sample	% total CD34+ HPC[§]	% B cell precursors	% CD7+ precursors	% Neutrophil precursors	% Monocytic precursors	% Erhytroid precursors	% Aberrant precursors	Score model 1 *	Score model 2 **
CTR 30	BM	0.63	55.61	3.10	n.d.	n.d.	4.35	0.09	2.048	-5.301
CTR 31	BM	2.41	70.33	2.0	n.d.	n.d.	1.48	0.07	1.338	-1.333
CTR 32	BM	1.95	73.61	5.41	n.d.	n.d.	1.41	0.28	3.751	0.473
CTR 33	BM	1.36	73.80	1.28	n.d.	n.d.	1.46	0.10	0.936	1.660
JMML 32	BM	1.94	3.96	24.88	44.50	18.02	0.49	13.65	35.386	11.605
	PB	1.48	1.49	27.64	54.76	8.98	0.04	0.24	17.447	13.686
JMML 33	BM	9.84	0.25	45.11	18.50	8.37	0.01	2.67	32.033	22.424
	PB	11.58	0.23	45.78	32.33	8.10	0.06	6.92	38.318	22.678
JMML 34	BM	1.30	1.53	78.28	n.d.	n.d.	n.d.	15.32	71.137	n.d.

Table S6. Percentage of total CD34+ HPC and their immunophenoptypic subsets in non-confirmed JMML patients.

Patient code	Source of sample	% of total CD34+ HPC[§]	% B cell precursors	% CD7+ precursors	% Neutrophil precursors	% Monocytic precursors	% Erhytroid precursors	% Aberrant precursors	Score model 1 *	Score model 2 **
non-confirmed JMML 1	BM	1.99	69.80	2.21	17.18	1.27	1.51	0.35	1.867	-1.281
non-confirmed JMML 2	BM	3.49	68.11	3.32	12.77	0.46	0.26	1.61	4.380	1.242
non-confirmed JMML 3	BM	0.95	26.56	2.18	59.0	6.93	3.86	0	1.349	-4.988
non-confirmed JMML 4	BM	2.27	62.59	4.44	21.86	7.55	0.12	1.88	5.463	2.051
non-confirmed JMML 5	PB	0.09	4.29	4.74	76.88	12.20	0.65	0	2.932	1.335
non-confirmed JMML 6	PB	1.25	15.41	1.36	66.0	8.03	0.75	0.11	1.000	-0.503
non-confirmed JMML 7	PB	1.59	94.75	3.32	n.d.	n.d.	n.d.	0.12	2.227	n.d.
non-confirmed JMML 8	PB	0.36	3.81	5.31	57.60	13.31	0.17	0	3.285	2.374
non-confirmed JMML 9	BM	4.26	66.37	4.08	14.58	2.53	0.25	0.84	3.738	1.636
	PB	1.44	20.62	5.01	57.38	5.96	0.46	0.640	4.024	1.769

* Score calculation formula used for model 1: 0.619* %CD7+ CD34+ precursors + 1.444* %CD34+ aberrant precursors; ** Score calculation formula used for model 2: 0.497* %CD7+ CD34+ precursors - 1.573* %CD34+ erythroid precursors; [§]Percentages of CD34+ HPC are referred to total nucleated cells; Percentages of each immunophenotypic subset are referred to 100% of CD34+ HPC. Note that in each row the sum of the percentages referred to the individual immunophenotypic subsets may not reach the value of 100 as the values of minor subsets such as mast cells and dendritic cells are not reported here.

The % of CD34+ HPC, CD34+ B cell precursors, CD7+ CD34+ precursors and CD34+ aberrant cell precursors were all calculated from the ALOT tube. The % of CD34+ neutrophil precursors was calculated from the AML/MDS Tube 1, the % of CD34+ monocytic precursors from AML/MDS Tube 2 and the % of CD34+ erythroid precursors from AML/MDS Tube 3.

Abbrevations: n.d., not determined; non-confirmed JMML, patients with non-confirmed diagnosis of JMML.

Supplementary Figures

Figure S1. Intersected gating strategy used to identify total CD34+ HPC and their distinct immunophenotypic subsets. Starting from the selection of total CD34+ cells identified as $CD45^{low}SSC^{intermediate\ (int)}$ (purple dots, **Panel A)** we illustrate how CD34+ neutrophil precursors, defined as CD13+CD117+HLA-DR+ cells negative for the more mature neutrophil associated markers (CD10, CD16 and CD11b), were identified in AML/MDS tube 1 (light blue dots, **Panel B**). **Panel C** illustrates an example of the gating performed in AML/MDS tube 2 for the identification of CD34+ monocytic precursors (green dots) defined as CD64+CD117+HLA-DR+, negative for the more mature markers of monocytic lineage (CD14 and IREM). **Panel D** illustrates an example of the gating strategy performed in AML/MDS tube 3 for the identification of early CD34+ erythroid precursor cells based on their unique $CD36^{int}$CD117+HLA-DR+$CD105^{heterogeneous(het)}$CD33- immunophenotype (brown dots). **Panel E** illustrates an example of the gating strategy performed in Acute Leukemia Orientation Tube (ALOT) for the identification of myeloid precursors (CyMPO+CD7-; yellow dots), B cell precursors (CD34+cyCD79a+; light green dots), and CD7+sCD3- early lymphoid precursors (dark blue dots); in **panel F**, a representative case of JMML and CTR depicted aberrant/unusual immunophenotypes of CD34+ HPC identified with ALOT tube (e.g., CD7+cyMPO+ and/or CD7+cyCD79a+; red dots).

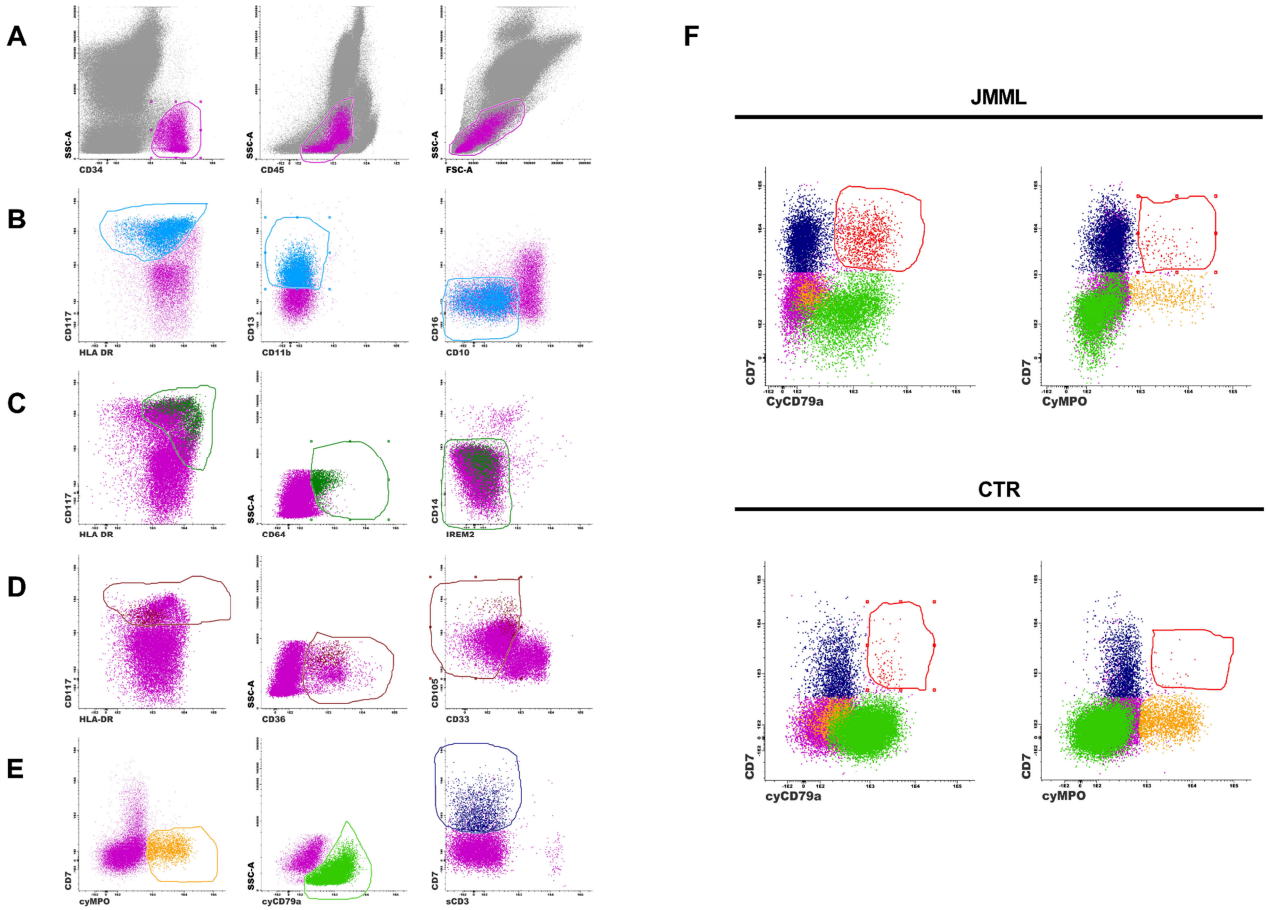

Figure S2. Distribution of different immunophenotyic subsets in CD34+ HPC of JMML samples. Immunophenotypic profiles of each CD34+ HPC subset collected from either BM or PB source were not statistically significative as reported in each box plot graph ($p \geq 0.05$).

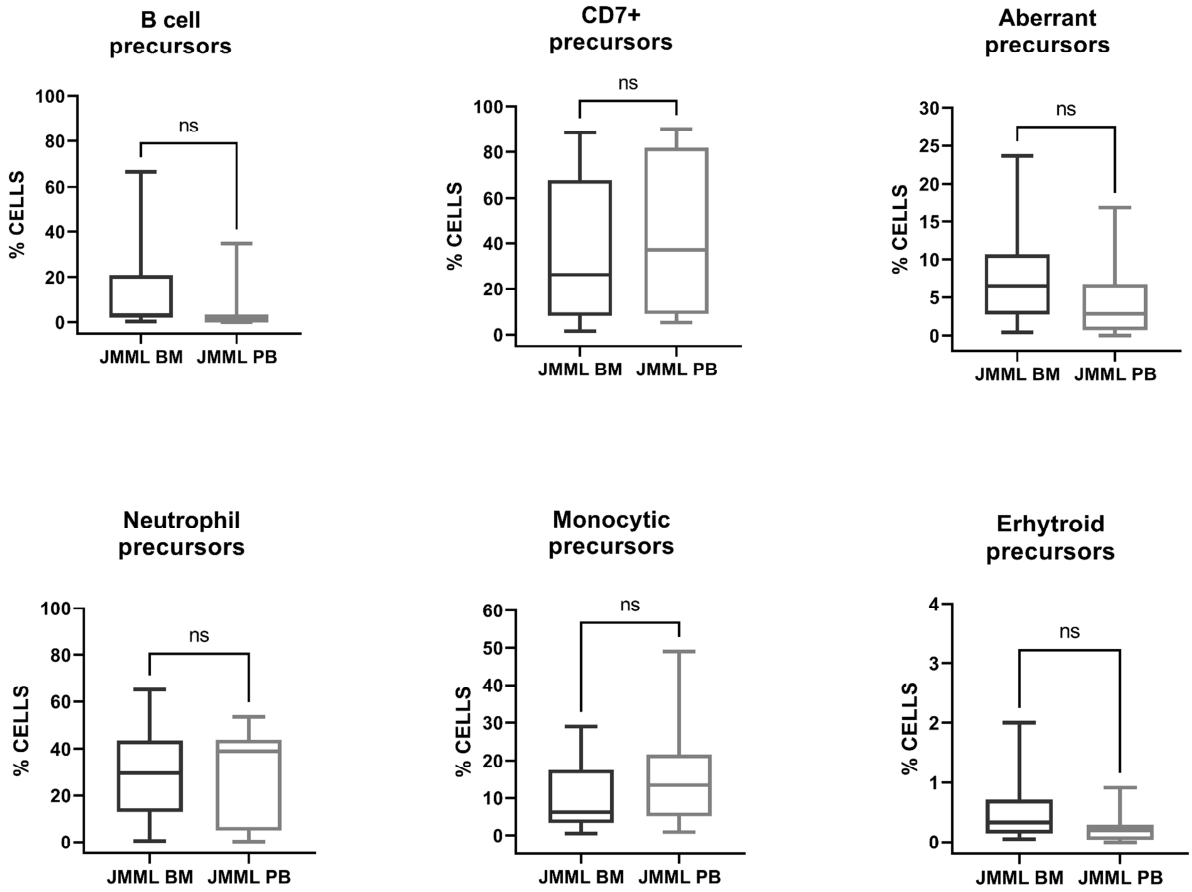

Figure S3. Phenotypic profile of CD34+ HPC in non-malignant control subjects according to the age-matched range of JMML patients. Each scatter plot graph represents the frequencies of CD34+ HPC subsets found in the control samples aged less than 4 years and those with an age greater than or equal to 4 years. No statistical differences were found among them. (p>0.05).

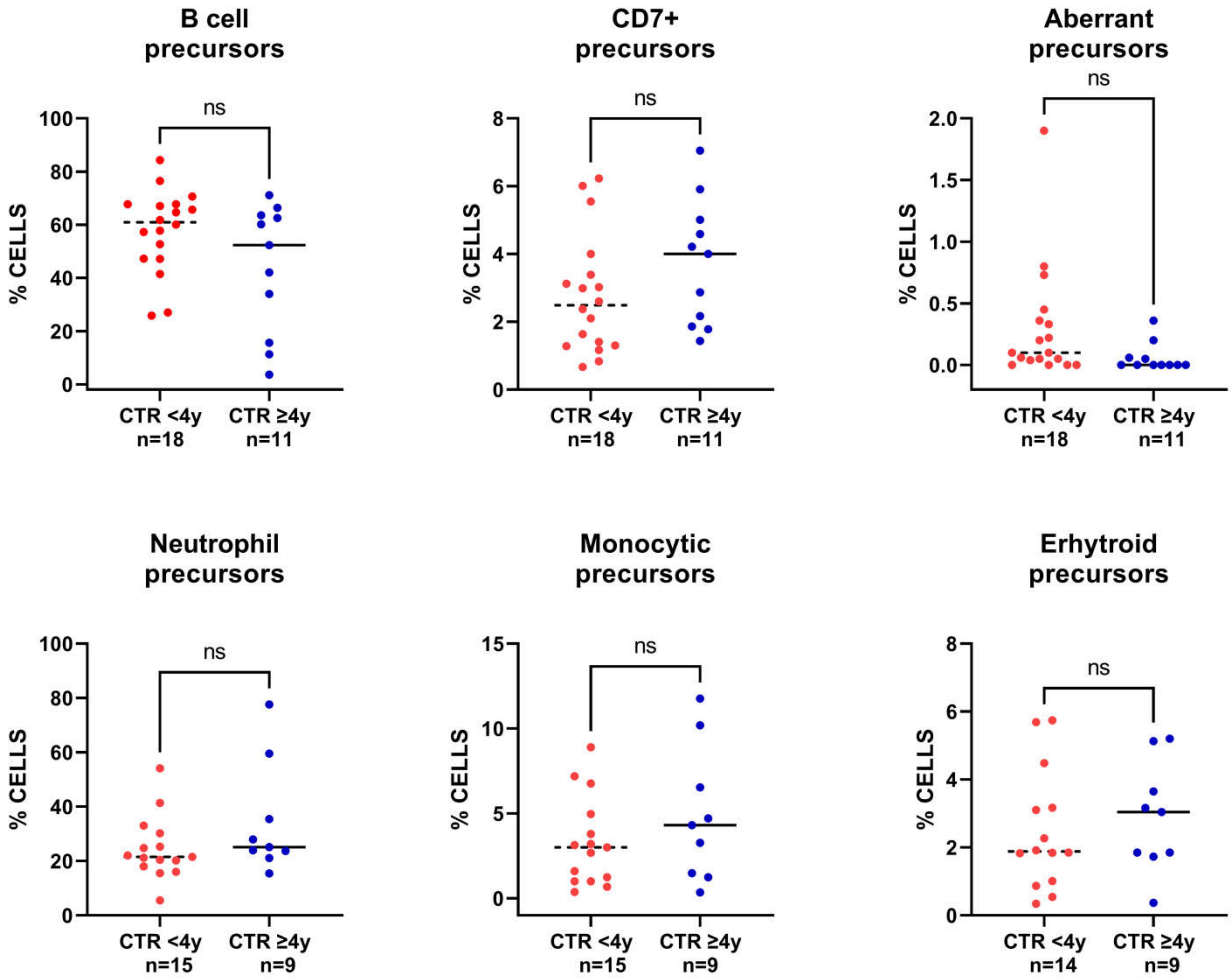

Figure S4. Comparison of phenotypic profile of CD34+ HPC in control series. The frequencies of CD34+ HPC subsets found in healthy hematopoietic stem cell donors (n=5) and children without hematological malignancies (n=27) were almost similar in both groups.

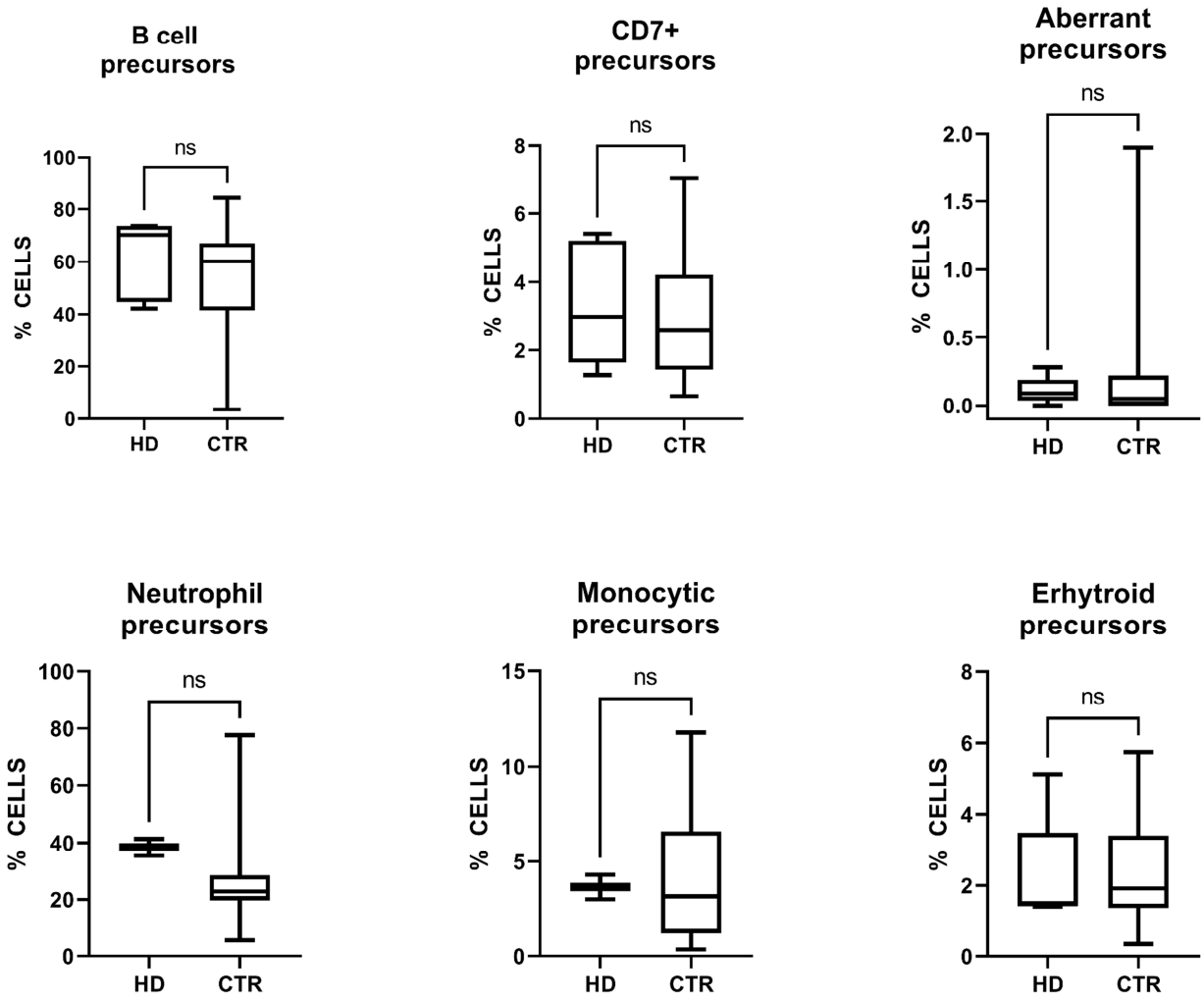

Figure S5. Maturing CD34-negative hematopoietic cell populations. The distribution of immunophenotypically defined CD34- more mature populations of BM cells from JMML patients and control subjects are represented in each box plot graph. Compared to controls, JMML patients showed significantly increased percentages of monocytic cells ($p<0.0001$), NK cells ($p<0.001$) and eosinophils ($p<0.01$) at the expense of reduced numbers of maturing neutrophils and B-cells ($p<0.01$ and $p<0.05$ respectively), with no significant differences in erythroid and T cells.

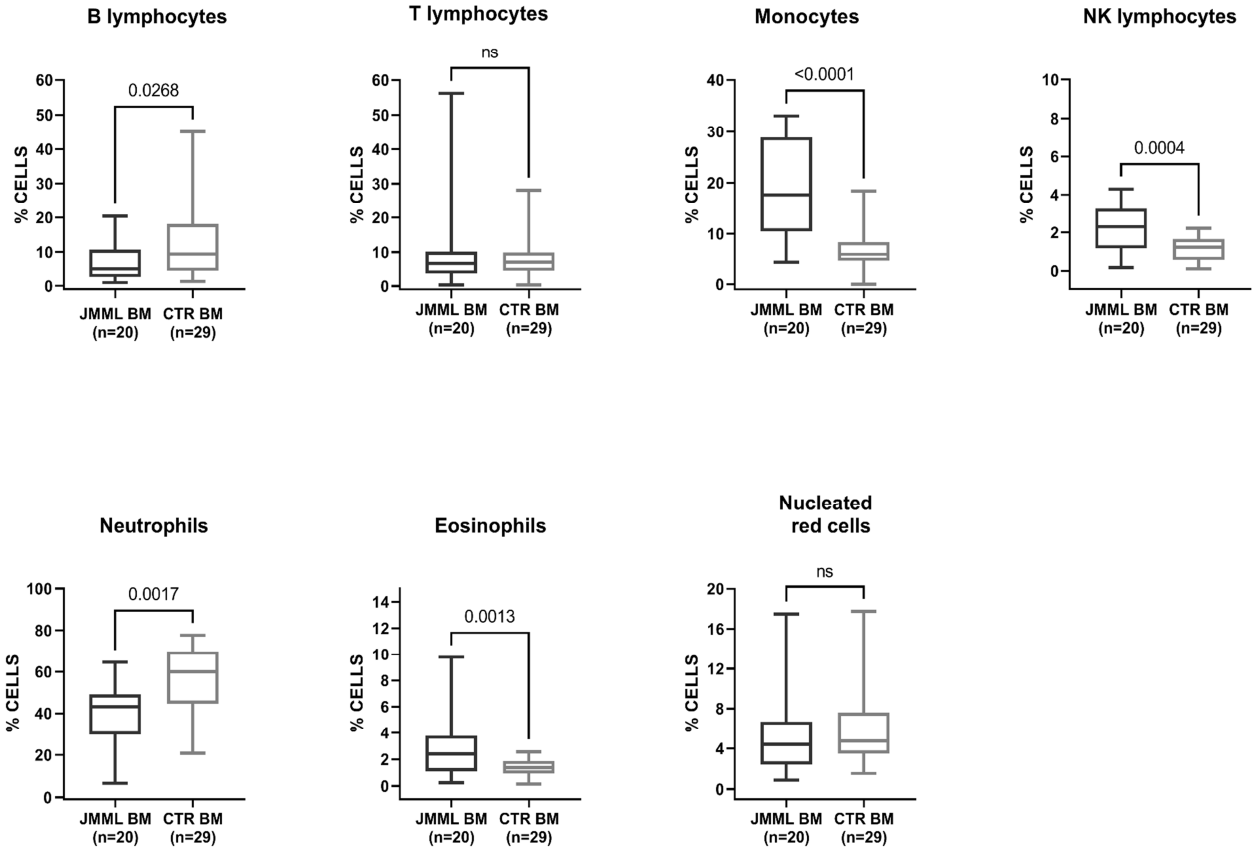

Figure S6. Discriminatory potential of the phenotypic parameters in CD34+ HPC by ROC curve analysis. The cross validated area under the curve (AUC) was used to rank the discriminatory potential between JMML and controls of each individual cell population, the most discriminatory (AUC above the threshold of 0.9) being CD7+ CD34+ precursors (AUC = 0.944), CD34+ aberrant precursors (AUC = 0.943), CD34+ erythroid precursors (AUC = 0.936) and CD34+ B-cell precursors (AUC = 0.930).

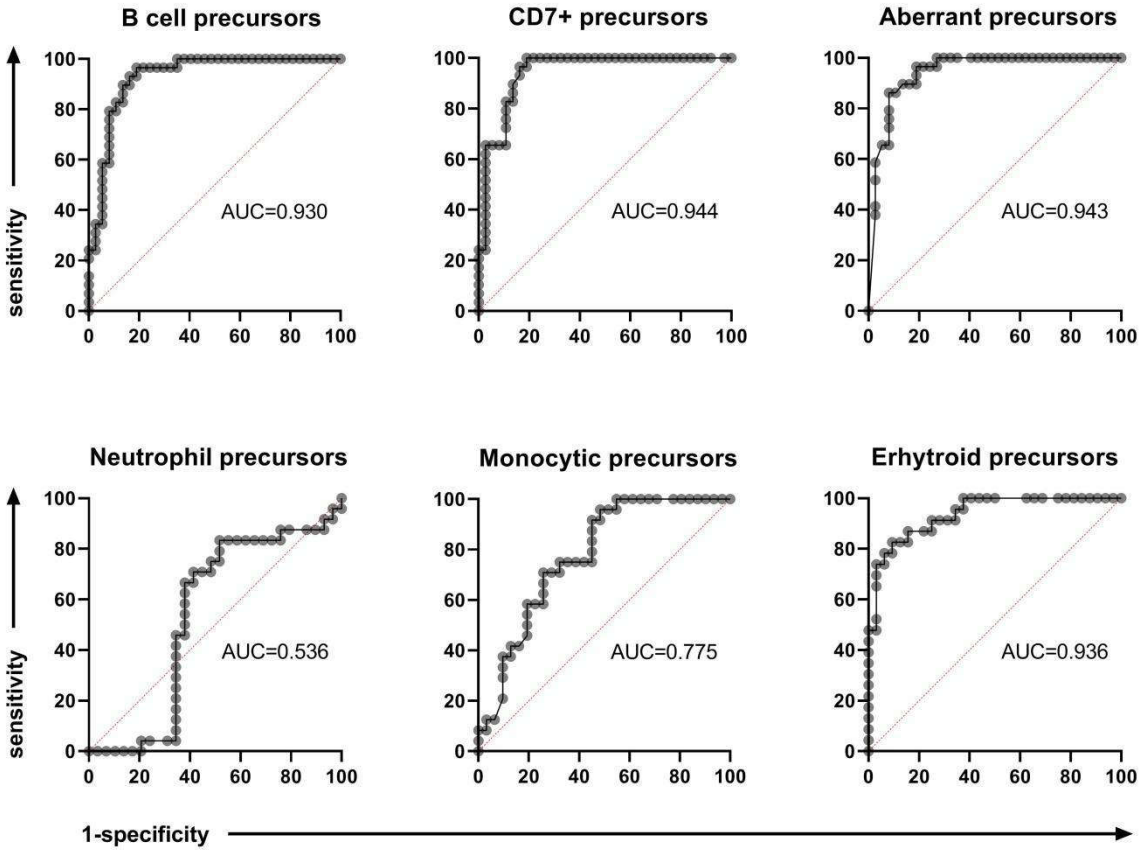

Figure S7. Percentages of CD34+ HPC in JMML, non-confirmed JMML and control samples. When both BM and PB samples were available, both sources were represented with separated Box plot graphics.

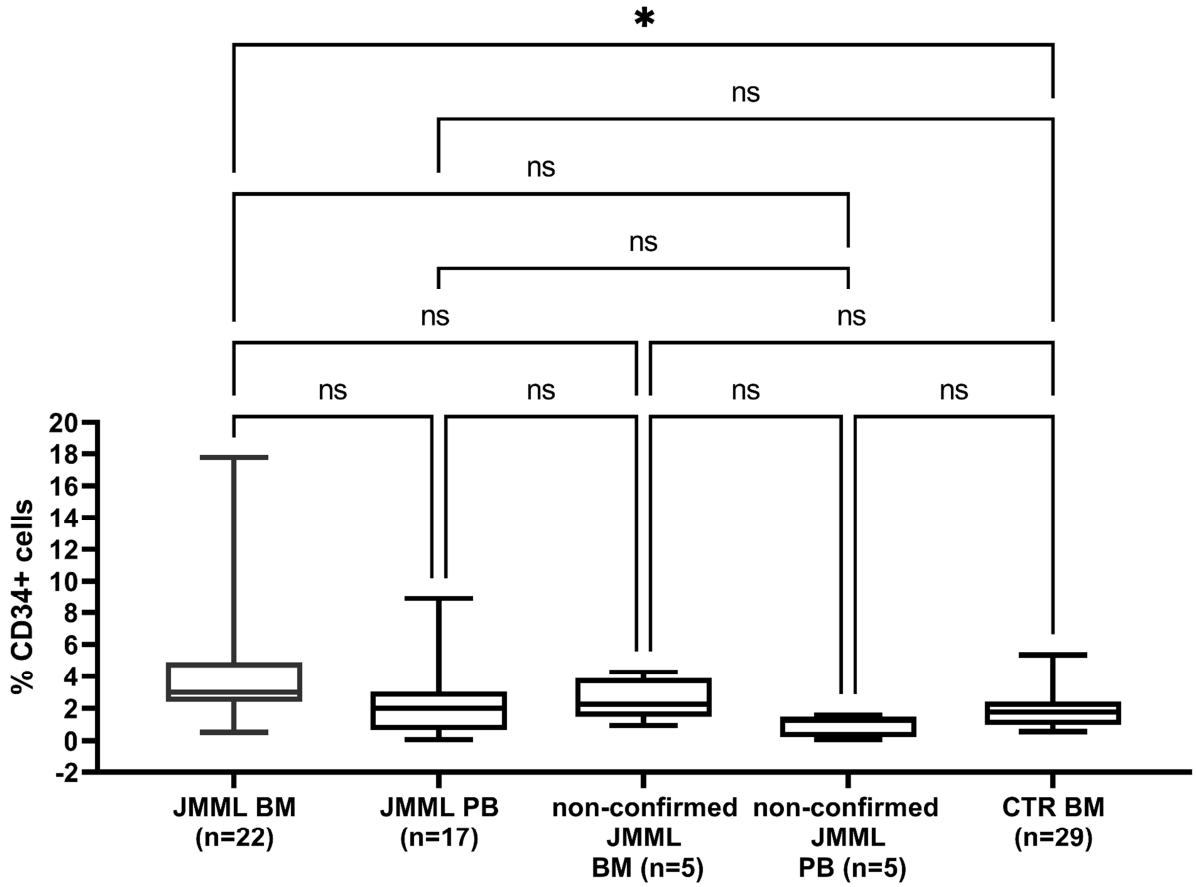

Figure S8. Distribution of genetic JMML subgroups along score values obtained with either score model 1 or score model 2. Panels A and **B** show the distribution of score values for JMML patients classified according to their underlying genetic mutation profiles. Control subjects are also reported in the graphic for comparison purposes. The CBL mutated patients are the only subgroup with a score non-significantly different from that of the control group. **Panels C** and **D** show the CD34+ phenotypic signature based on either score model 1or score model 2 applied to JMMLs in comparison with the control group. Each JMML-associated mutation is labelled with a specific color (red=CBL, blue=NF-1, green=RAS, black=PTPN11, purple=not determined). In **panel C** by applying score model 1, six out of 37 analyzed JMML samples (16.2%) fall below the maximum value of CTR samples (6.18), specifically: 3 CBL samples (JMML#9, #12 and #19), 2 K-RAS samples (JMML#7 and #22), and 1 PTPN11 sample (JMML#4). In **panel D** by applying score model 2, two out of 31 analyzed samples (6.4%) fall below the maximum value of CTR samples (1.45), specifically: 1 CBL sample (JMML#19) and 1 K-RAS sample (JMML#22).