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

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## Abstract

Diagnostic criteria for juvenile myelomonocytic leukemia (JMML) are currently well defined, however in some patients diagnosis still remains a challenge. Flow cytometry is a well established tool for diagnosis and follow-up of hematological malignancies, nevertheless it is not routinely used for JMML diagnosis. Herewith, we characterized the CD34+ hematopoietic precursor cells collected from 31 children with JMML using a combination of standardized EuroFlow antibody panels to assess the ability to discriminate JMML cells from normal/reactive bone marrow cell as controls (n=29) or from cells of children with other hematological diseases mimicking JMML (n=9). CD34+ precursors in JMML showed markedly reduced B-cell and erythroid-committed precursors compared to controls, whereas monocytic and CD7+ lymphoid precursors were significantly expanded. Moreover, aberrant immunophenotypes were consistently present in CD34+ precursors in JMML, while they were virtually absent in controls. Multivariate logistic regression analysis showed that combined assessment of the number of CD34+CD7+ lymphoid precursors and CD34+ aberrant precursors or erythroid precursors had a great potential in discriminating JMMLs *versus* controls. Importantly our scoring model allowed highly efficient discrimination of truly JMML

*versus* patients with JMML-like diseases. In conclusion, we show for the first time that CD34⁺ precursors from JMML patients display a unique immunophenotypic profile which might contribute to a fast and accurate diagnosis of JMML worldwide by applying an easy to standardize single eight-color antibody combination.

# Introduction

Juvenile myelomonocytic leukemia (JMML) is a rare, unique myeloproliferative/myelodysplastic neoplasia of early childhood driven by canonical Ras-pathway mutations in *PTPN11, N-RAS, K-RAS, NF1*, or *CBL* and characterized by *in vitro* hypersensitivity of hematopoietic progenitor to granulocyte-macrophage colony-stimulating factor (GM-CSF).[1,2] JMML has an incidence of 1.2 cases per million children per year and, if untreated, patients can progress toward an uncontrolled disease.[3] Recently the World Health Organization (WHO) classification placed JMML in the group of myelodysplastic/myeloproliferative disorders.[4,5] Clinical signs of JMML are well defined.[6] Peripheral blood (PB) smears typically shows leukocytosis with monocytosis and low blast cell counts (median 2% myeloblasts),[7] and presence of immature hematopoietic precursor cells.[8] Bone marrow (BM) examination reveals hypercellularity with myelomonocytic cell proliferation, reduction of megakaryocytes and moderate increase of blasts (<20% myeloblasts). Thus, the combination of young age, hepato-splenomegaly, appearance of myeloid and erythroid precursors in the PB, and/or elevated levels of fetal hemoglobin should alert the clinicians to suspect JMML and initiate specific tests. These generally include the molecular analysis of driver mutations in the *PTPN11, K-RAS, N-RAS*, and *CBL* genes, and the search for features of neurofibromatosis type 1 (NF1) including family history,[9] in addition to the contemporary exclusion of the BCR-ABL transcript. Monosomy 7 is the most frequent cytogenetic aberration found in ~25% of JMML patients.[8,10,11]

A conventional hallmark of clonogenic JMML cells is their *in vitro* hypersensitivity to GM-CSF, although this test is laborious and poorly standardized. Flow cytometry-based assay of STAT5 hyperphosphorylation after stimulation with GM-CSF was proposed to aid in distinguishing JMML from other disease conditions.[12,13] In addition to the above diagnostic parameters the DNA methylation analysis has been also validated as prognostic biomarker potentially aiming at tailoring treatment strategies for JMML.[14-16]

Despite progress made in the diagnosis of JMML, it can still be challenging due to the existence of several diseases presenting with clinical features mimicking JMML, such as human herpesvirus infections, leukocyte-adhesion molecule deficiency, infantile malignant osteopetrosis, hemophagocytic lymphohistiocytosis, and Wiskott-Aldrich syndrome.[17-19] Diagnostic criteria for JMML have been recently revised and summarized in several comprehensive reviews.[20-23] However, in contrast to other hematological malignancies, immunophenotyping is not part of the diagnostic work-up. Despite this, Oliveira *et al.* have recently shown a significant decrease of T lymphocytes and the presence of several phenotypic abnormalities within CD34⁺ cells of JMML patients including the aberrant expression of CD7 in the majority of CD34⁺CD117⁺CD13⁺ cells, associated with a decrease or complete lack of CD19⁺CD10⁺ B-cell precursors,[24] similarly to what has been described in adults with myelodysplastic syndrome (MDS) and/or myeloproliferative neoplasm (MPN).[25,26]

Since 2012, the EuroFlow consortium has developed fully standardized approaches for immunophenotyping of hematological malignancies, including validated antibody panels, standardized sample preparation protocols, gating strategies and innovative (smart) data analysis and software tools embedded with artificial intelligence, to prospectively classify individual patients against a predefined reference database of normal/reactive and disease associated groups of subjects.[27] However, these approaches have never been applied for the diagnosis of JMML.

Herewith we applied the EuroFlow eight-color acute leukemia orientation tube (ALOT), and AML/MDS/MPN antibody panel for the phenotypic characterization of major BM myeloid lineages (neutrophil, monocytic, and erythroid cells), and other minor BM cell subsets.[28]

In this study we used such antibody panels for in depth characterization of the immunophenotypic profile of CD34⁺ hematopoietic precursor cells (HPC) of children with confirmed JMML and comparison with either normal/non-malignant cells or cells from children with JMML-like diseases. Our ultimate goal was to identify an immunophenotypic profile that could help in fast and objective differential diagnosis of JMML *versus* patients with normal/reactive BM or patients with JMML-like diseases.

# Methods

## Pediatric samples

Samples for flow cytometric immunophenotyping were collected in eight EuroFlow centers (Brazil, Czech Republic, France, Italy, the Netherlands, Poland, Spain and Switzerland) from 31 children newly diagnosed as JMML according to the WHO 2016 criteria.[4] From each child either BM (n=22) or PB (n=9) was collected. In eight of 31 JMML patients paired BM/PB samples were available, obtaining a total of 39 samples (22 BM and 17 PB) for the analyses.

The immunophenotype of JMML BM/PB cells was compared with that of BM cells from 29 children without hematological malignancies (referred in the following as control group) or with that of BM/PB cells from nine children presenting with a suspected diagnosis of JMML which was not subsequently

confirmed (referred in the following as non-confirmed JMML patients).
For validation purposes, an additional cohort consisting of four control subjects and three JMML patients was collected. Informed consent was obtained from each patient and/or his/her legal guardian.

### Flow cytometry immunophenotypic studies

Flow cytometry immunophenotyping was performed as described in *Online Supplementary Appendix* with both the eight-color EuroFlow ALOT and the AML/MDS/MPN antibody panels (*Online Supplementary Table S1*), following the EuroFlow standard operating procedures for sample preparation, instrument set-up and calibration.[27,29]
The gating strategies used to identify each cell population are described in *Online Supplementary Figure S1A-F*.

### Statistical analysis

Descriptive statistics for each CD34+ HPC subset was first performed separately for JMML cases, controls and non-confirmed JMML using median, interquartile range (IQR) (p25, p75) displayed in box plot graphics. For the comparison between groups of subjects, the non-parametric Wilcoxon rank sum test was used and the degree of discrimination was investigated using receiver operating characteristic (ROC) curves. The cross-validated area under the curve (AUC) was used to rank the discriminatory potential of each phenotypic parameter. A logistic regression model, with a binary response variable indicating the diagnosis of JMML (i.e., presence *vs.* absence), was then estimated including combinations of phenotypic parameters among those that showed the greatest discrimination potential in the ROC curve analyses. The ROC analysis through the AUC enabled us to assess the diagnostic potential in separating JMML cases from controls by the probability that, for a random pair of JMML cases and controls, the model risk score was greater in the JMML case than in the control case. The number of phenotypic parameters included in the model was guided from considerations of significance on couples, triplets etc. The number of parameters in the final models was the maximum with significant contributions that will be lost by adding further parameters. The choice of the final model was then reassessed by its potential in terms of ROC analysis. This model was used to obtain a risk score for confirming the diagnosis of JMML, where the greater the score was obtained the higher the predicted probability of JMML was observed.

## Results

### Patients and controls features

We investigated a total of 31 patients with a median age at diagnosis of 1 year (IQR, 1 month to 4 years) fulfilling clinical and hematological characteristics of JMML, as summarized in Table 1. Molecular screening performed in 30 of 31 patients (in 1 case data was not available) showed mutations in the RAS signaling pathway and/or clinical findings consistent with NF1 with a distribution in line with previous studies.[30] Specifically, 13 patients (45%) carried somatic *PTPN11* mutations, eight (26%) showed *RAS* somatic mutations (4 *N-RAS* and 4 *K-RAS*). In three cases (10%) a clinical diagnosis of NF1 was made, and six patients (21%) carried *CBL* germline mutations. In all studied patients the absence of Philadelphia chromosome (BCR/ABL rearrangement) was assessed.
The control group consisted of 29 pediatric BM samples including two healthy hematopoietic stem cell donors and 27 BM samples obtained from children non-suspected for JMML undergoing diagnostic BM aspiration for suspicious of idiopathic thrombocytopenic purpura, cytopenia after viral infection, arthralgias and transient neutropenia. Morphological examination of the BM aspirates confirmed the absence of BM involvement at the time of immunologic investigation in all cases. The median age of the children was 4 years (IQR, 1 month to 12 years).
Nine patients with clinical features mimicking JMML (median age 6 months; IQR, 0-2 years) for whom diagnosis of JMML was finally ruled out (non-confirmed JMML) were also analyzed (Table 2). Finally, we used additional samples as a validation cohort consisting of four control subjects (3 BM samples from healthy hematopoietic stem cell donors, with age of 3, 6 and 8 years, and 1 BM from a 6-year-old child without hematological disease, who underwent clinical observation for arthralgias) and three JMML patients (1 BM sample and 2 paired BM/PB samples) whose characteristics are reported in *Online Supplementary Table S2*.

### Analysis of CD34+ hematopoietic precursor cells

Overall, the median frequency of CD34+ HPC, referred to total nucleated cells, was significantly higher in BM of JMML patients (n=22) than in control BM samples (n=29) being 3.0% (IQR, 2.4-4.9%) *versus* 1.8% (IQR, 1.0-2.4%) ($P$=0.0038; Figure 1). The distribution of CD34+ HPC in PB and BM of JMML patients was 2.0% (IQR, 0.7-3.0%) *versus* 3.0% (IQR, 2.4-4.9%) ($P$=0.1155), respectively (*Online Supplementary Figure S2*). For this reason, we decided to pool all the JMML samples regardless of the collection source (BM or PB) obtaining a series of 39 samples (22 BM + 17 PB) that have been compared to CD34+ HPC from control BM samples.
Both B-cell and erythroid precursors subsets of CD34+ HPC, were strongly reduced in JMML *versus* control: 2.4% (IQR, 0.6-5.7%) *versus* 60.1% (IQR, 41.8-66.7%), ($P$<0.0001) and 0.2% (IQR, 0.1-0.6%) *versus* 1.9% (IQR, 1.7-3.6%) ($P$<0.0001). By contrast, the median percentage of monocytic precursors and CD7+ precursors were both significantly expanded within the CD34+ HPC compartment in JMML *versus* control group: 10.4% (IQR, 3.4-18.0%) *versus* 3.2% (IQR, 1.2-6.1%) ($P$=0.0004) and 28.7% (IQR, 8.3-76.0%) *versus* 2.9% (IQR, 1.5-4.4%) ($P$<0.0001), respectively. In turn, the proportion of cells showing early commitment to the neutrophil lineage was similar in JMML *versus* control samples: 32.5% (IQR, 13.0-43.1%) *versus* 23.9% (IQR, 20.3-32.3%) ($P$=0.9). Interestingly, immunophenotyp-

**Table 1.** Clinical and laboratory findings of 31 juvenile myelomonocytic leukemia patients at diagnosis.

| Patient code | Age in years | Sex | Splenomegaly | WBC x10⁹/L | Monocytes x10⁹/L | % Blasts (by morphology) | Genetic subgroup§ | Karyotype |
|---|---|---|---|---|---|---|---|---|
| JMML 1 | 2 | F | P | 50.0 | 5.5 | 5.0 | *PTPN11* | NK |
| JMML 2 | 3 | F | P | 52.4 | 3.7 | 1.0 | *K-RAS* | 46,XX |
| JMML 3 | <1 | F | P | 26.4 | 5.2 | 2.7 | *PTPN11* | 47,XX +21 |
| JMML 4 | 2 | M | P | 47.7 | 7.2 | 4.0 | *PTPN11* | 45,XY,-7 |
| JMML 5 | 1 | F | P | 23.6 | 14.5 | 5.0 | *PTPN11* | 46,XX |
| JMML 6 | <1 | F | P | 8.2 | 1.5 | 4.0 | *CBL* | NK |
| JMML 7 | 1 | M | A | 12.2 | 2.6 | 0.0 | *K-RAS* | 45,XY,-7 |
| JMML 8 | 3 | F | P | 20.5 | 1.2 | 15.0 | *PTPN11* | 46,XX |
| JMML 9 | <1 | M | P | 12.7 | 1.7 | 0.0 | *CBL* | 46,XY |
| JMML 10 | 1 | M | NK | 22.8 | 1.6 | NK | *PTPN11* | NK |
| JMML 11 | 1 | M | P | 20.0 | 3.0 | 0.0 | NF1 | NK |
| JMML 12 | 1 | F | NK | 65.0 | >1.0 | NK | *CBL* | NK |
| JMML 13 | 3 | M | P | 52.0 | 8.5 | NK | NF1 | NK |
| JMML 14 | 1 | M | P | 24.7 | 2.4 | 10.0 | *N-RAS* | 46,XY |
| JMML 15 | <1 | M | P | 19.5 | 3.8 | 1.0 | *PTPN11* | 46, XY |
| JMML 16 | <1 | M | P | 149.0 | 26.8 | 3.0 | *N-RAS* | 46, XY |
| JMML 17 | <1 | M | NK | 28.0 | 7.8 | 3.0 | *PTPN11* | 46,XY |
| JMML 18 | <1 | M | P | 65.0 | 13.7 | 8.0 | *PTPN11* | 46,XY |
| JMML 19 | 2 | M | P | 46.2 | 8.8 | 2.0 | *CBL* | NK |
| JMML 20 | 1 | F | P | 13.8 | 4.7 | 5.0 | *K-RAS* | 45,XX,-7 |
| JMML 21 | 3 | M | P | 6.7 | 2.0 | 5.5 | *PTPN11* | 45,XY,-7 |
| JMML 22 | 1 | F | P | 50.0 | 20.0 | 4.8 | *K-RAS* | 46,XX |
| JMML 23 | <1 | M | P | 14.0 | 4.9 | 2.2 | NK | 45,XY,-7 |
| JMML 24 | <1 | M | P | 10.1 | 1.3 | 8.0 | *PTPN11* | 46, XY |
| JMML 25 | <1 | F | NK | 28.4 | 7.1 | 1.4 | *CBL* | 46,XX |
| JMML 26 | 2 | F | P | 10.8 | 1.2 | 10.0 | *PTPN11* | 47,XX,+8 |
| JMML 27 | 3 | F | P | 23.5 | 9.8 | 4.8 | *PTPN11* | 45,XX,-7 |
| JMML 28 | <1 | F | P | 56.8 | 21.6 | NK. | *N-RAS* | NK |
| JMML 29 | 4 | M | P | 24.0 | 5.5 | 0.0 | *CBL* | NK |
| JMML 30 | <1 | M | P | 26.9 | 8.6 | 3.2 | *N-RAS* | 46, XY |
| JMML 31 | 2 | F | P | 34.2 | 1.9 | 0.0 | NF1 | 46, XX |

§*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), NF1 is intended as clinical diagnosis of neurofibromatosis type 1. JMML: juvenile myelomonocytic leukemia; WBC: white blood cells; F: female; M: male; P: present; A: absent; NK: not known.

ically aberrant CD34⁺ HPC subsets such as CD7⁺/cyMPO⁺/cyCD3⁻ and cyCD79a⁺/CD7⁺/cyCD3⁻ were detected in most of JMML samples (3.9%; IQR, 1.3-8.4%), while they were virtually absent in the control group (0.05%; IQR, 0.0-0.3%) ($P$<0.0001) (Figure 2A-F).

In order to rule out any age-specific effects on the comparison between JMML and non-malignant control subjects we analyzed this latter series by dividing the patients into those with an age of less than 4 years (within the age range of JMML patients) and those with an age greater than or equal to 4 years. As shown in *Online Supplementary Figure S3* no significant differences in the phenotypic profile of CD34⁺ HPC were observed.

In addition, to verify the homogeneity of our control series, we compared the phenotypic profile of the healthy hematopoietic stem cell donors (n=5) with that of the 27 children with non-malignant hematopoietic abnormalities, and we did not find any remarkable differences (*Online Supplementary Figure S4*).

Besides of CD34⁺ HPC we also dissected seven major compartments of more mature (i.e., CD34⁻) hematopoietic cells in BM, including: B and T lymphocytes, natural killer cells, monocytes, neutrophils, eosinophils, and erythroid cells. Statistical differences are reported in *Online Supplementary Figure S5*.

### Assessment of the discriminatory potential of the phenotypic profile and distribution of hematopoietic cells in juvenile myelomonocytic leukemia patients *versus* controls

Once identified those immunophenotypic parameters with significantly different expression in JMML *versus* controls, we studied their potential in discriminating between JMML and

**Table 2.** Clinical and laboratory findings of nine non-confirmed juvenile myelomonocytic leukemia patients.

| Patient code | Age in years | Sex | Splenomegaly | WBC x10⁹/L | Monocytes x10⁹/L | Final diagnosis |
|---|---|---|---|---|---|---|
| Non-confirmed JMML 1 | <1 | M | A | 30.1 | 1.5 | LAD II |
| Non-confirmed JMML 2 | <1 | M | NK | 20.4 | 1.5 | CMV infection |
| Non-confirmed JMML 3 | 2 | M | P | 7.3 | 0.8 | EBV infection |
| Non-confirmed JMML 4 | <1 | M | P | 26.8 | 2.6 | Transient myeloproliferative reaction with karyotype: 46, XY |
| Non-confirmed JMML 5 | 2 | M | P | 30.5 | 2.3 | Chronic eosinophilic leukemia [inv(9), t(8;9), *PMC1-JAK2* fusion gene] |
| Non-confirmed JMML 6 | 1 | F | P | NK (leukocytosis) | NK (monocytosis) | AML M7 |
| Non-confirmed JMML 7 | <1 | M | NK | 30.0 | NK (monocytosis) | Aphthous fever, JMML and Noonan syndrome were excluded* |
| Non-confirmed JMML 8 | 1 | F | NK | 7.9 | 0.98 | AML NOS with megakaryoblastic maturation |
| Non-confirmed JMML 9 | <1 | M | P | 20.6 | 1.1 | CMV congenital infection and Noonan syndrome (pathogenic mutation c.846 C-G in *PTPN11* in heterozygotic status) |

*This patient underwent observation because of Noonan syndrome facies not subsequently confirmed. JMML: juvenile myelomonocytic leukemia; WBC: white blood cells; M: male; F: female; NK: not known; A: absent; P: present; CMV: Cytomegalovirus; EBV: Epstein-Barr virus; LAD II: leukocyte adhesion deficiency type II; AML: acute myeloid leukemia; NOS: not otherwise specified.

control subjects. To this aim we used a ROC curve analysis approach based on the relative distribution of immature CD34⁺ HPC compartments. Then, the cross-validated AUC was used to rank the discriminatory potential of each single CD34⁺ phenotypic parameter along the range of possible values. Based on the rank of accuracy identified for each cell population the most discriminatory phenotypic parameters, measured as percentage of cells on total CD34⁺ HPC, were: CD7⁺ precursors (AUC=0.944), aberrant precursors (AUC =0.943), erythroid precursors (AUC=0.936), and B-lymphoid precursors (AUC=0.930) (*Online Supplementary Figure S6*).

### Logistic regression model for the immunophenotypic diagnosis of juvenile myelomonocytic leukemia

In order to better discriminate JMML cases from controls we developed a logistic regression model using the diagnosis of JMML (yes or no) as a dependent variable, and those immunophenotypic parameters detected within the CD34⁺ HPC having (individually) the highest discriminatory potential (i.e., AUC), as regressors.

To this aim, we combined the highest discriminatory parameter (i.e., the proportion of CD7⁺ precursors within CD34⁺ HPC: AUC=0.944) with either the percentage of CD34⁺ aberrant precursors (AUC=0.943), or the number of CD34⁺ erythroid precursors (AUC= 0.936), to obtain two different combinations of informative immunophenotypic parameters. This model was then used to obtain a risk score for being diagnosed as JMML according to the following algorithms: score model 1= (0.619 * %CD7 precursors) + (1.444 * %aberrant precursors); and score model 2 = (0.497 * %CD7 precursors) - (1.573 * %erythroid precursors). The obtained scores (*Online Supplementary Table S3*) are a scale transformation of the predicted probability of being a JMML, thus the greater the score, the greater is

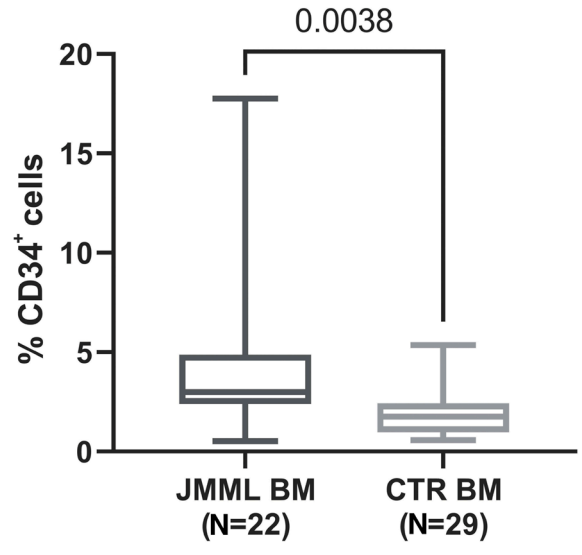


**Figure 1. Percentage of CD34⁺ hematopoietic precursor cells in juvenile myelomonocytic leukemia and control samples.** Box plot graphics show a significantly increased percentage of CD34⁺ hematopoietic precursor cells in juvenile myelomonocytic leukemia (JMML) bone marrow (BM) as compared to control (CTR) BM samples (*P*=0.0038).

the predicted probability. This probability will depend on the prevalence of JMML cases in the target population (*Online Supplementary Table S4*). The discriminatory potential (score value) of each score model was then assessed by the AUC of the linear predictor, both score models displaying high discriminatory potential between JMML and control subjects: AUC=0.973, *P*<0.0001, and AUC=0.982, *P*<0.0001, respectively (Figure 3A, B). By applying each risk score to the additional validation series of controls and JMML samples (*Online Supplementary Table S5*) we confirmed highly significant differences in the discriminatory potential with both models (*P*=0.0079 and *P*=0.0008; Figure 3C).

### Distribution of CD34⁺ hematopoietic precursor cells subsets in juvenile myelomonocytic leukemia *versus* non-confirmed juvenile myelomonocytic leukemia patients

The median percentage of CD34⁺ HPC in pooled BM/PB samples of non-confirmed JMML samples was 1.5% (IQR, 0.8-2.6%) with non-significant difference with that of JMML (*P*=0.121) even when comparing BM and PB separately (*Online Supplementary Figure S7*). In order to confirm the diagnostic value of the developed immunophenotypic scores, we subsequently compared the distribution of different subsets of CD34⁺ HPC in JMML patients with those of non-confirmed JMML patients. Overall, JMML samples showed a significantly higher proportion (median and range) of both CD7⁺ precursors with 28.7% (IQR, 8.3-76.0%) *versus* 3.7% (IQR, 2.2-4.8%) (*P*=0.0006) and aberrant precursors with 3.9% (IQR, 1.3-8.4%) *versus* 0.2% (IQR, 0.0-1.0%) (*P*=0.0047); by contrast B-cell precursors were decreased with 2.4% (IQR, 0.5-6.2%) *versus* 44.6% (IQR, 12.6-68.5%) (*P*<0.0001) (Figure 4A-F). We then applied both score model 1 and 2 to non-confirmed JMML samples to assess their risk score (*Online Supplementary Table S6*), and we obtained a highly efficient discrimination between JMML and non-confirmed JMML as shown in Figure 5A, B. The calculated discriminatory values (AUC of JMML *vs.* non-confirmed JMML) were 0.954 and 0.903 for score model 1 and 2, respectively (*data not shown*).

### Prevalence of the juvenile myelomonocytic leukemia L-associated immunophenotypic signature according to the underlying genetic mutation

We, therefore, wanted to evaluate the JMML-associated immunophenotypic profile more in-depth by analyzing each CD34⁺ phenotypic parameter within each JMML genetic subgroup in comparison with control subjects. As reported in Figure

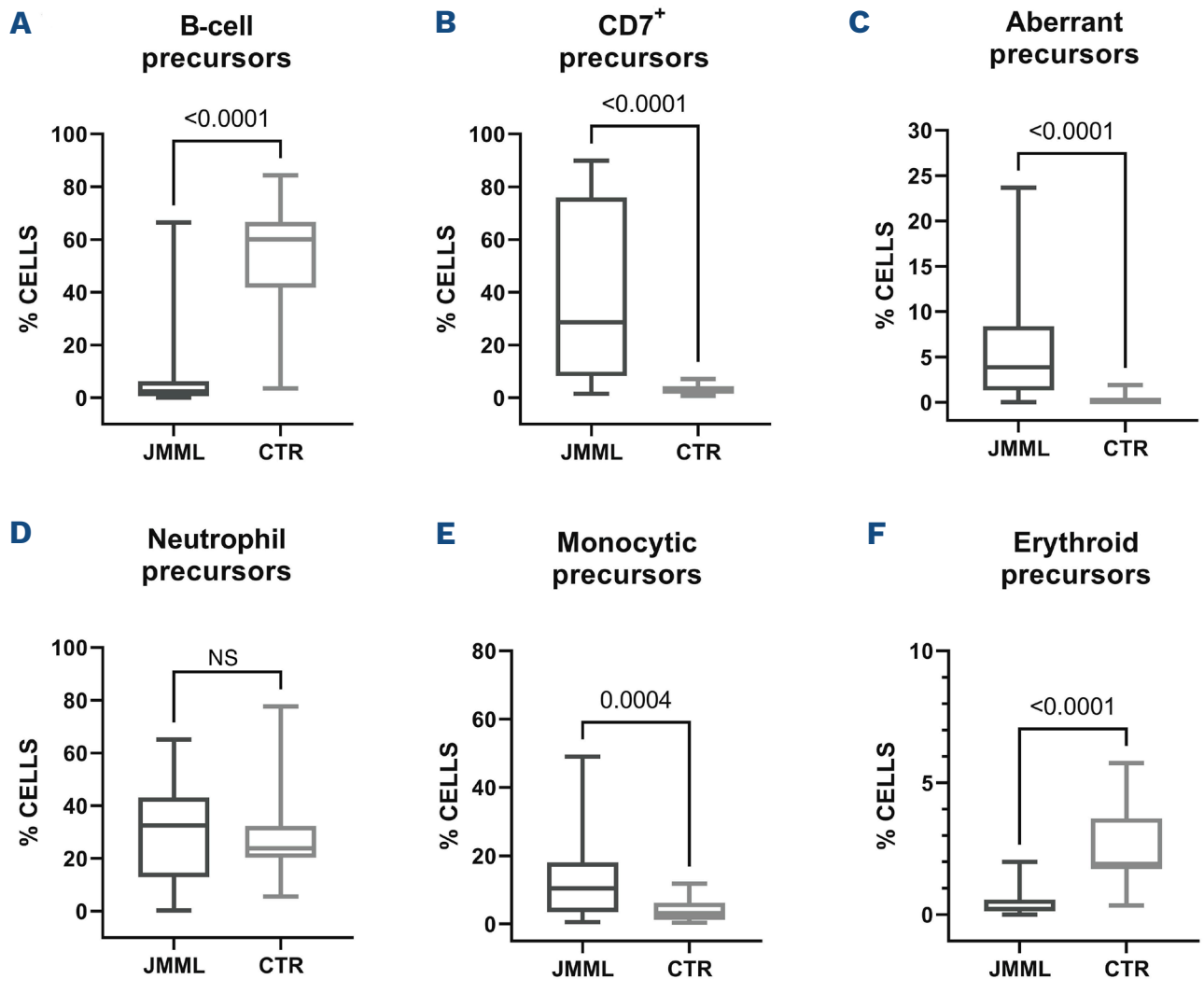


**Figure 2. Distribution of different immunophenotypic subsets in CD34⁺ hematopoietic precursor cells in juvenile myelomonocytic leukemia and control samples.** Box plot graphics show severely reduced B-cell and erythroid precursors (*P*<0.0001) (A, F) and significantly increased CD7⁺ lymphoid, CD34⁺ aberrant precursors, (both *P*<0.0001) (B, C), as well as increased monocytic precursors (*P*=0.0004) in juvenile myelomonocytic leukemia (JMML) bone marrow/peripheral blood as compared to control (CTR) bone marrow precursor cells (E). Non-significant (NS) differences were found in the distribution of neutrophil precursors (*P*≥0.05) (D). Percentages of each immunophenotypic subset are referred to 100% of CD34⁺ cells.

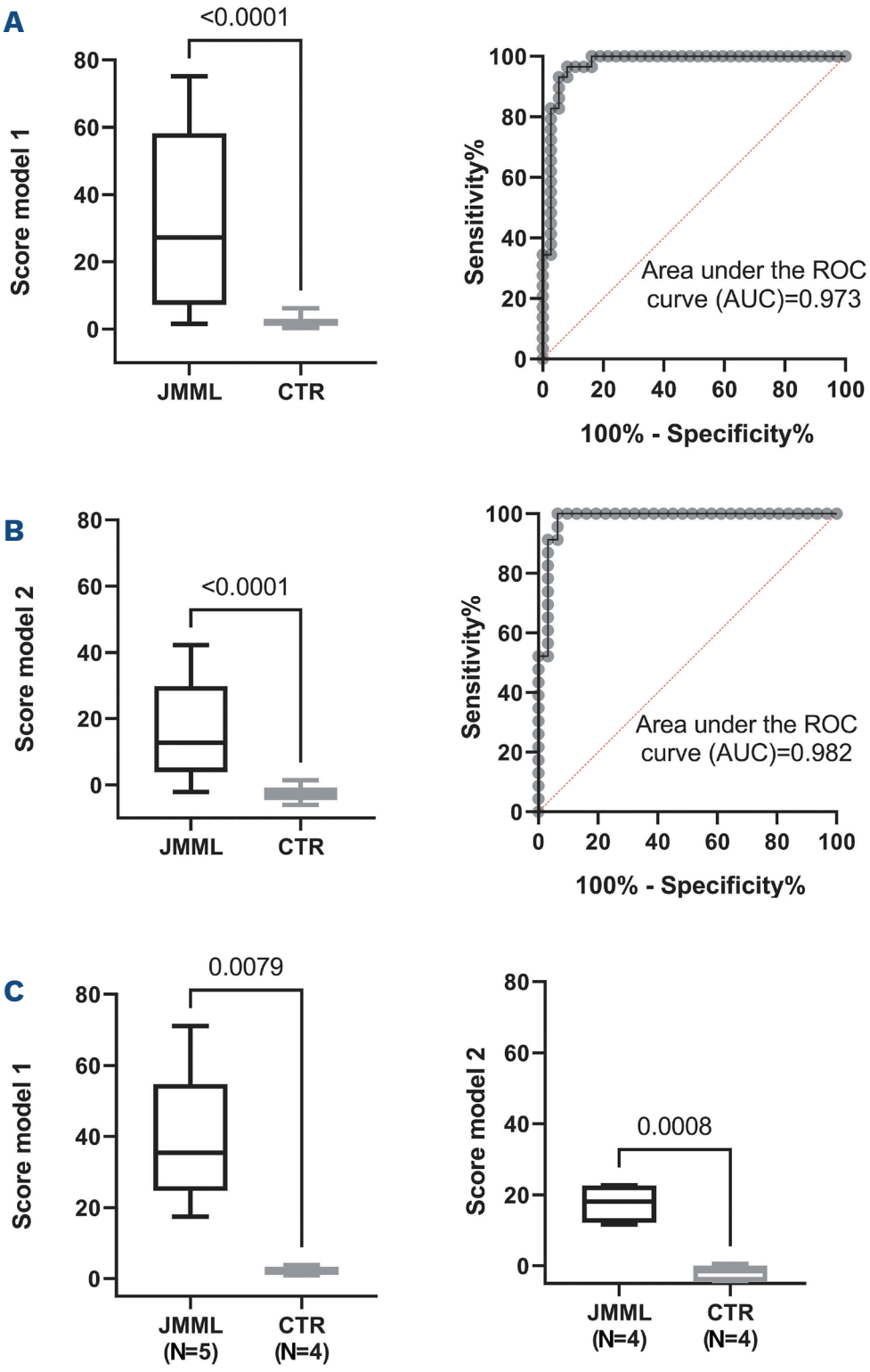


**Figure 3. Discriminatory potential of the CD34⁺ immunophenotypic profile in juvenile myelomonocytic leukemia patients *versus* controls.** The risk score for being diagnosed as juvenile myelomonocytic leukemia (JMML) is shown in the left graphs (A, B) for score model 1 (A) and score model 2 (B), respectively. The distribution of risk scores in JMML and control samples are compared in each graph showing highly significant differences. Area under the ROC curve (AUC) to assess the discriminatory potential of each score model is reported in the right graphs of both panels. (C) Represents the additional validation series of controls and JMML samples that confirms significant discriminatory potential with both models ($P$=0.0079 and $P$=0.0008).

6, CD34⁺ HPC such as B-cell precursors, CD7⁺, and aberrant precursors, maintained a significantly different expression as compared to controls even when analyzed within the *PTPN11*, NF1 and *RAS* genetic subgroups. In contrast such parameters in patients carrying germline *CBL* gene mutations were non significantly different than controls.

Despite this, JMML patients with the *CBL* mutation could still be discriminated from controls due to a significant increase in the percentage of monocytic precursors, equal to 16.9% (IQR, 8.6-26.1%) compared to 3.2% (IQR, 1.2-6.1%) ($P$<0.001), and a decrease in the percentage of CD34⁺ erythroid precursor cells being 0.5% (IQR, 0.1-1.1%) *versus* 1.9% (IQR, 1.7-3.6%) ($P$<0.01). When we applied the score model 1 or the score model 2 specifically to *CBL*-mutated JMML patients we observed a non-significant discriminatory power *versus* control with either model (*Online Supplementary Figure S8A, B*).

Finally, we displayed the distribution of each single sample along the score values according to its genetic alteration (*Online Supplementary Figure S8C, D*). Indeed, by applying the score model 1, among the six samples resulted with lowest score values (below the discriminatory value of 6.178), three were *CBL*, two were *RAS* and one was *PTPN11*. Whereas by applying the score model 2, we found one *CBL* and one *RAS* below the value of 1.455.

## Discussion

Although JMML is rare, the criteria proposed for diagnosing this malignant disease have been progressively refined over time.[11] Nevertheless, diagnosis of JMML can still be challenging especially when specific laboratory tests (e.g., molecular assays and next-generation sequencing-based genetic diagnostics) are not available. Moreover, a con-

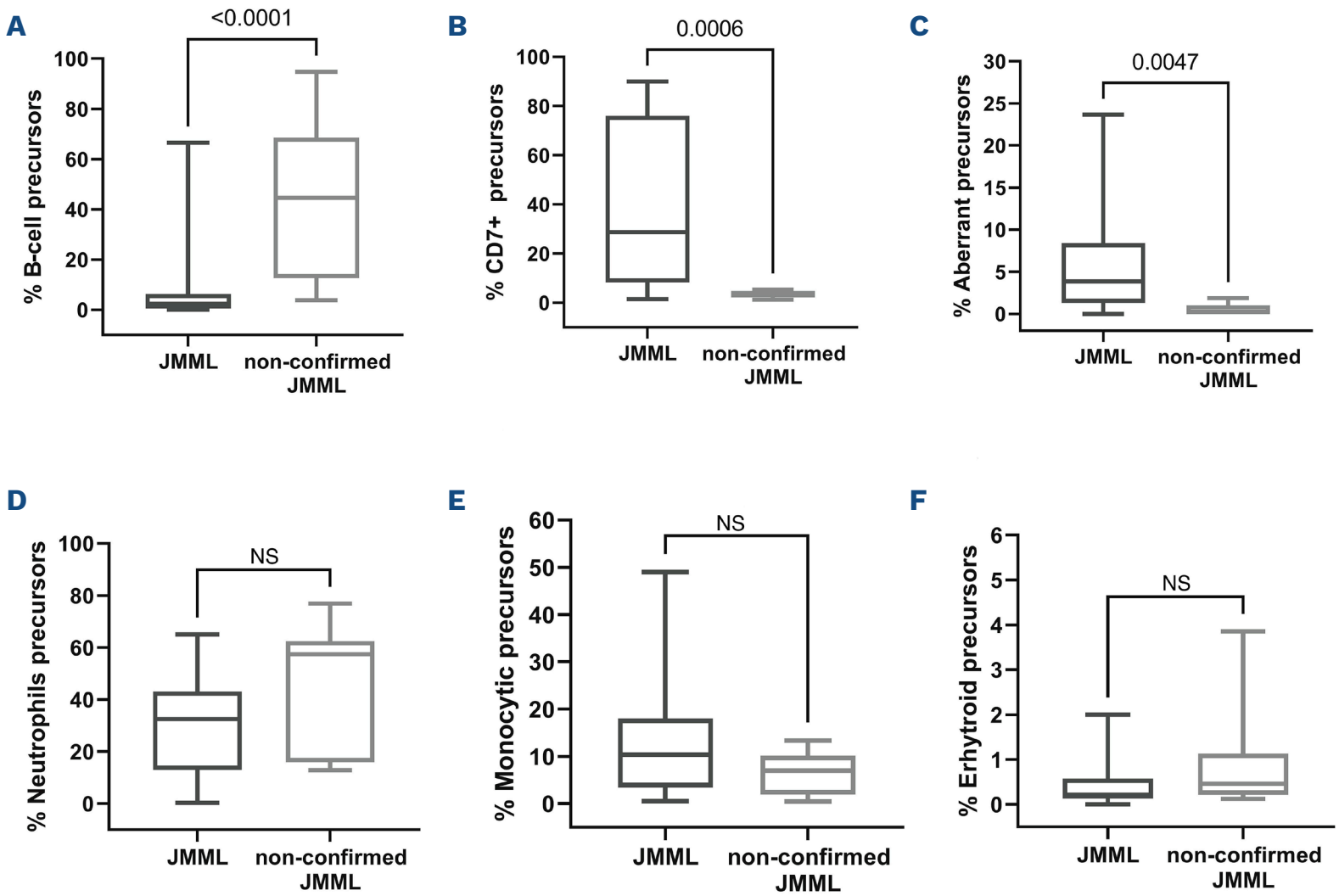


**Figure 4. Distribution of different immunophenotypic subsets in CD34⁺ hematopoietic precursor cells in juvenile myelomonocytic leukemia and non-confirmed juvenile myelomonocytic leukemia.** Box plot graphics show significantly differences in both bone marrow/peripheral blood samples for immunophenotypic parameters such as CD34⁺ B-cell precursors ($P$<0.0001), CD7⁺ precursors ($P$=0.0006) and aberrant precursors ($P$=0.0047) (A-C). Non-significant (NS) differences were found in the distribution of neutrophil, monocytic and erythroid precursors ($P$≥0.05) (D-F).

sistent plethora of other confounding malignancies, either hematological or non-hematological, mimicking JMML, can further complicate the diagnosis of this disease and delay clinical decision-making.[6,11,23,30]

Taking advantage of a fully standardized flow cytometric platform[27] herewith we investigated in depth the immunophenotypic profile of immature CD34⁺ HPC of JMML patients compared to both control children and patients with other diseases mimicking JMML. Our ultimate goal was to identify immunophenotypic alterations that could be used in a score system to discriminate between JMML and non-confirmed JMML cases.

Interestingly, an in-depth analysis of JMML CD34⁺ HPC revealed a markedly reduced percentages of both B-cell precursors and erythroid precursors, in parallel to a marked expansion of monocytic precursors and CD7⁺ lymphoid precursors. In addition, JMML CD34⁺ HPC were characterized by the systematic presence of aberrant immunophenotypes, being virtually absent in the BM of control subjects. Based on these findings we used ROC curve analysis to estimate the discriminatory potential of each individual immunophenotypic parameter. By this approach we then designed two different highly predictive score models for diagnosing JMML (score model 1 and score model 2). By applying each risk score model to an additional, albeit limited, validation series of controls and JMML samples we confirmed a high discriminatory potential with both of them. Indeed, we are aware that our control series is mainly constituted by non-healthy donor children, however we did not find any significant differences between the immunophenotypic profile of the truly healthy donors and that of children who underwent BM puncture for diagnostic purposes.

Further application of these score models in a cohort of patients with suspected diagnosis of JMML, non-confirmed after completion of the diagnostic investigations, demonstrated a greater predictive value of score model 1 as compared to score model 2. Indeed, model 1 may be a more robust tool in routine diagnostics since in some patients erythroid precursors (used in score model 2) were present at very low frequencies, which may hamper their unequivocal identification particularly in hypocellular BM specimens. Moreover, score model 1 would require cell staining only with the ALOT single-tube antibody combination whereas score model 2 would require the addition of the AML tube 3 staining.

Of note, despite the decreased number of erythroid precursors observed within CD34⁺ HPC in BM, this did not translate into a decreased (relative) production of more

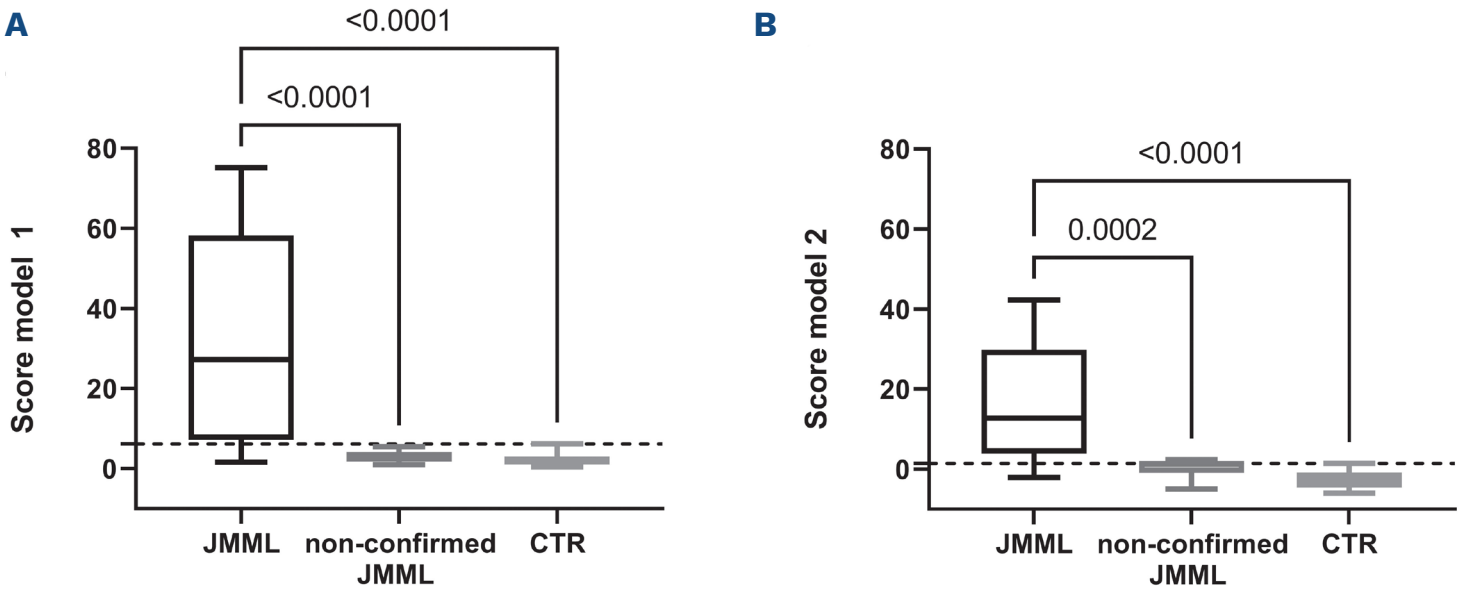


**Figure 5. Discriminatory potential of the CD34+ immunophenotypic profile in juvenile myelomonocytic leukemia patients *versus* non-confirmed juvenile myelomonocytic leukemia patients**. (A) Discriminatory potential between juvenile myelomonocytic leukemia (JMML) and non-confirmed JMML patients for the score model 1 resulted highly significant (*P*<0.0001). Dashed line at 6.178 represents the maximum score obtained in control subjects. Scores for all non-confirmed JMML patients fell below this cutoff value. (B) Discriminatory potential between JMML and non-confirmed JMML patients for the score model 2. The ability to discriminate JMML from the non-confirmed JMML was feasible. However, 3 of 9 of non-confirmed JMML samples resulted slightly above the maximum score of control subjects setting at 1.455; specifically non-confirmed JMML # 4, #8, #9 reported in *Online Supplementary Table S6*. Control samples are also reported in each graph. No differences were found between non-confirmed JMML and controls when applying both score models.

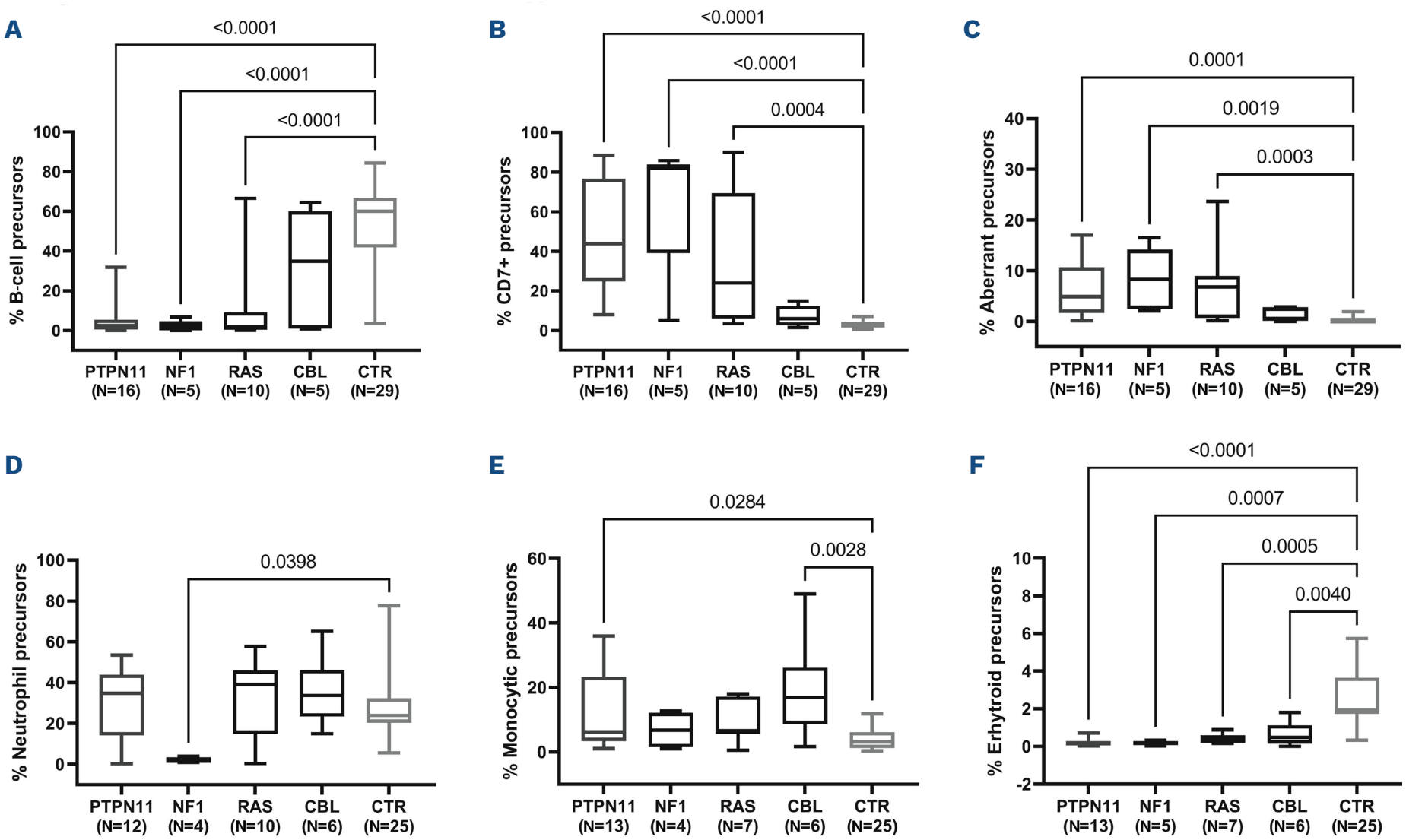


**Figure 6. Distribution of different immunophenotypic subsets in CD34+ hematopoietic precursor cells in juvenile myelomonocytic leukemia patients according to gene mutations.** All gene mutations were associated with a specific immunophenotypic parameter of CD34+ cells (indicated in the y axis). *CBL*-mutated patients showed immunophenotypic features of CD34+ hematopoietic precursor cells (HPC) more similar to those of control (CTR) cases without remarkable differences in B cell, CD7+, aberrant, and neutrophil precursors (A-D), but with significant differences in monocytic and erythroid cell precursors (*P*<0.01) (E, F). All other genetic subgroups maintained highly relevant differences compared to CTR in most of the immunophenotypic parameters including those used for score models (i.e., CD7+, aberrant and erythroid precursors).

mature CD34- nucleated red blood cells, which showed median values within the normal ranges. Whether, such apparent discrepancy is due to a greater expansion/proliferation potential of the fewer CD34+ erythroid precursors, the relative decrease of maturing neutrophil precursors and/or to the relative overall increased number of CD34+ HPC in JMML *versus* normal/reactive BM, deserves further investigations.

It is important to note that the antibody combinations proposed here are all highly validated and have proven to be easy to standardize, with highly reproducible results in different laboratories.[27,29,31]

It is known that for most patients with JMML, early allogeneic HSCT is the mandatory therapy of choice,[11,32–34] however, this indication may vary according to several risk factors to be assessed early after diagnosis, including the type of mutation and the gene methylation status.[6,35,36] Indeed, patients carrying *NF1* or *PTPN11* gene mutations are at fatal risk without rapid HSCT. In turn, children carrying *K-RAS* and *N-RAS* mutations are associated with variable clinical risk, this also considering entities such Ras-associated autoimmune leukoproliferative disorder (RALD),[37] whereas patients with germline *CBL* mutations undergo a watch-and-wait approach.[22,23,38] In this context, a goal of our study was to determine whether different gene mutations occurring in JMML were associated with specific immunophenotypic profiles of CD34+ HPC. Interestingly we observed a step-wise decline of score levels seen from highest levels in *NF1*, then *PTPN11* and *RAS* to lowest in *CBL* cases, which were almost close to normal, similarly to their well established clinical risk profile. In this regard, the sensitivity of our scoring models may be underpowered mainly by the *CBL* and *RAS* cases.

These findings suggest that the here proposed immunophenotypic score could also reflect a clinical-biological significance with potential prognostic value. These aspects are currently being investigated and they may also contribute to a better understanding of the genetic heterogeneity of JMML patients.[24,26,39]

However, given the limited sample size of our series and the possibility that RAS cases with lower scores could be RALD and not JMML, the power to assess phenotypic-genotypic associations should be considered with caution.

The novelty of our study relies on the design of a diagnostic algorithm based on immunophenotypic scoring as a new tool to be integrated in the laboratory diagnostic work-up of JMML. It allows rapid identification of children with this disease, even in those patients with confounding clinical signs, just within a few hours from sample collection while molecular tests take longer.

Further, this assay can be easily implemented worldwide being eight-color flow cytometers available in virtually every pediatric oncology center. Importantly, it can also be successfully applied in PB samples with great advantage in clinical practice, especially for infant patients.

In summary, the novel flow cytometric assay proposed here can contribute to a faster and accurate diagnosis of JMML allowing a prompt start of both treatment with demethylating agents[40] and of the search for locating a suitable HSCT donor.

## Disclosures

*JJMvD, AO, TS, and VHvdV each report being one of the inventors on the EuroFlow-owned patent PCT/NL2010/050332 (methods, reagents and kits for flow cytometric immunophenotyping of normal, reactive and malignant leukocytes). The Infinicyt software is based on intellectual property of AO and ESdC, licensed to Cytognos SL (Salamanca, Spain) which pays royalties to the University of Salamanca (Salamanca, Spain), and the scientific input of other EuroFlow members. JJMvD and AO are chairmen of the EuroFlow scientific foundation, which receives royalties from licensed patents (Cytognos - Salamanca, ES and BD Biosciences - San José, CA) that are collectively owned by the participants of the EuroFlow Foundation. These royalties are exclusively used for continuation of EuroFlow collaboration and sustainability of the EuroFlow consortium. VHvdV reports a Laboratory Services Agreement with BD Biosciences. In addition, JJMvD and AO report a Laboratory Services Agreement with BD Biosciences, an Educational Services Agreement with BD Biosciences and a Scientific Advisor Agreement with Cytognos SL and BD Biosciences; all related fees and honoraria of these agreements go to Leiden University Medical Center and University of Salamanca, respectively. AB is on the speakers bureau of Amgen and Novartis. FL has served in an advisory role for Amgen, Bellicum Pharmaceuticals, Neovii, Novarti, Sanofi and Vertex; and has served on a speakers' bureau for Amgen, Bellicum Pharmaceuticals, Bluebird bio, Gilead, Jazz Pharmaceuticals, Medac, Miltenyi Biotec, Novartis, Neovii, SOBI and Takeda. All other authors have no conflicts of interest to disclose.*

## Contributions

*CrB performed research, analyzed and interpreted the data, and wrote the paper. LA and MGV performed statistical analyses and wrote the paper. ChB collected samples and analyzed flow cytometric data. AO and GG designed the research, lead the project, and wrote the paper. FL, JJMvD and AB supervised the project. SM, TC, VHvdV, TS, AJvdS, ESdC, MN, EM, SN, FVDM, CA, PF, LS, LuS, RM and LaS collected samples and patient data. All authors reviewed and approved the final version of the manuscript.*

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**Data-sharing statement**

*The data supporting the findings of this study are available upon request to the corresponding author.*

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