Received: 3 June 2016 | Revised: 5 August 2016 | Accepted: 6 September 2016 | Published on: 17 October 2016

DOI: 10.1002/pbc.26285

RESEARCH ARTICLE

WILEY Pediatric Blood & Cancer

SIOP INTERNATIONAL SOCIETY OF PAEDIATRIC ONCOLOGY

aspho The American Society of Pediatric Hematology/Oncology

Characteristics of the phenotypic abnormalities of bone marrow cells in childhood myelodysplastic syndromes and juvenile myelomonocytic leukemia

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Abstract

Background: Immunophenotyping of bone marrow (BM) hemopoietic precursors is useful for diagnosis of adult myelodysplastic syndrome (MDS), but data concerning pediatric patients are limited. We analyzed immunophenotypic features of BM cells at diagnosis of children who were referred to the Brazilian Pediatric Cooperative Group of Myelodysplastic Syndromes.

Methods: Diagnosis was based on clinical information, peripheral blood counts, BM cytology and cytogenetics. Patients with Down syndrome were excluded. Children with deficiency anemias or transitory neutropenias were used as controls (CTRLs). Immunophenotyping was performed on an eight-color antibody platform evaluating myelomonocytic maturation and progenitor cells.

Results: A total of 32 patients were examined: 6 refractory cytopenia of childhood [RCC]; 5 refractory anemia with excess of blasts [RAEB]; 8 refractory anemia with excess of blasts in transformation [RAEB-t]; 13 juvenile myelomonocytic leukemia [JMML] and 10 CTRLs. Median age was 66 months (RCC), 68 months (RAEB/RAEB-t), 29 months (JMML) and 70 months (CTRLs). Median number of phenotypic alterations was 4 (range 1–6) in RCC; 6 (range 2–11) in RAEB/RAEB-t and 6 (range 2–11) in JMML ($P = 0.004$). The percentage of $CD34^+/CD117^+/CD13^+$ cells was 0.5% (range 0.1–2.8) in RCC; 4.2% (range 0.3–10.1) in RAEB/RAEB-t and 3.7 % (range 0.5–8.6) in JMML cases, compared with 0.7% (0.5–1.2) in CTRLs ($P < 0.0005$). Aberrancies in antigen expression of myeloid progenitors were seen in 63% of JMML and in 45% of RAEB/RAEB-t. $CD34^+/CD19^+/CD10^+$ cells were decreased or absent in patients compared with age-matched controls. T lymphocytes were decreased in JMML.

Conclusions: Phenotypic abnormalities were similar to those found in adult MDS. A decrease in B-cell precursors was observed especially in RAEB/RAEB-t. JMML and RAEB showed a similar pattern.

KEYWORDS

B-cell precursors, flow cytometry, juvenile myelomonocytic leukemia, myelodysplastic syndromes

1 | INTRODUCTION

Myelodysplastic syndromes (MDS) comprise a heterogeneous group of bone marrow clonal disorders characterized by abnormal maturation, proliferation and apoptosis of bone marrow (BM) hemopoietic precursors which result in peripheral blood cytopenias. They vary in clinical presentation, severity and risk of transformation to acute myeloid leukemia (AML). They are rare in children, with an annual incidence of 0.8 to 1.8 per million patients from 0 to 15 years old.[1–5] Childhood

Abbreviations: AML, acute myeloid leukemia; BCG-MDS-PED, Brazilian Pediatric Cooperative Group of Myelodysplastic Syndromes; BM, bone marrow; JMML, juvenile myelomonocytic leukemia; MDS, myelodysplastic syndromes; MFC, multiparameter flow cytometry; RAEB, refractory anemia with excess of blasts; RAEB-t, refractory anemia with excess of blasts in transformation; RCC, refractory cytopenia of childhood; SSC, sideward light scatter; WHO, World Health Organization

MDS differs from the diagnostic categories of the adult classification by including refractory cytopenia of childhood, presenting a hypocellular BM to distinguish from severe aplastic anemia.[5,6] The cut-point of BM blasts is 30% to distinguish MDS from AML, thereby so including refractory anemia with excess of blasts in transformation (RAEB-t) in the MDS category.[1] Juvenile myelomonocytic leukemia (JMML) is included in the group of myelodysplastic/myeloproliferative neoplasms as a separate entity, due to its myeloproliferative behavior.[6] The frequency of each entity within MDS may vary depending on the inclusion or exclusion of patients with Down syndrome, cytopenias caused by an inherited genetic abnormality, and post-chemotherapy MDS.[2,4,6]

Multiparameter flow cytometry (MFC) is an ancillary, standardized analytical tool, frequently employed for immunophenotyping as part of the diagnostic work-up of adult MDS. It is useful for differentiating clonal from non-clonal cytopenias, especially in cases with a normal karyotype and few cell atypias in BM precursors.[7–12] However, there are few studies on the immunophenotypic characteristics of childhood MDS,[13–16] and due to the difficulty of obtaining normal controls in the pediatric population, these distinctive disease features are often compared with findings in adult BM.

In the present study, MFC analysis of BM aspirates was included in the diagnostic assessment of patients registered in the Brazilian Pediatric Cooperative Group of Myelodysplastic Syndromes (GCB-SMD-PED). We describe the immunophenotypic characteristics of these patients and compare the observed abnormalities with pediatric age-matched controls.

2 | METHODS

2.1 | Patients

We analyzed retrospectively BM samples collected for multiparametric flow cytometry analysis during the diagnostic work-up of children included in the Registry from January 2013 until April 2016. Diagnosis of MDS was based on clinical data, peripheral blood (PB) counts, BM cytology and histology as well as cytogenetics as previously described.[1,2] In addition, molecular diagnosis was performed in the cases of JMML.[6] Patients with myeloid proliferation related to Down's syndrome were excluded. Classification of the cases was made according to the criteria of the updated World Health Organization (WHO) Pediatric Classification.[1,6] The immunophenotypic features found in the patients were compared with those of cases of non-clonal PB cytopenias such as deficiency anemias (n = 5), benign familial neutropenias (n = 3) and transitory post-viral cytopenias (n = 2).

Written informed consent was given by the parents or legal guardians before sample collection according to local laws and the Declaration of Helsinki (Proc. 54386316.0.0000.5437— Ethics' Committee of the Cancer Center, Barretos).

2.2 | Flow cytometric analysis

At least 1 mL of BM was collected in ethylenediaminetetraacetic acid (EDTA). Immunophenotyping of BM was performed with two tubes of eight-color monoclonal antibody combinations within 24 hours after BM aspiration using a standardized "direct lyse-wash" technique. BM samples were diluted to a concentration of 2–5×10^6 cells/μL. A panel of antibody combinations was used aiming to evaluate myelomonocytic maturation and progenitor cell populations (Table 1). Antibodies and suppliers are shown in Supplementary Table S1.

TABLE 1 Panel of antibody combinations used

Monoclonal antibodies and fluorochromes
FITC PE PERPCy5.5 PECy7 APC APC-H7 V450 V500
CD7 / CD56 / CD34 / CD117 / CD10 / CD19/ CD38/ CD45
CD16 / CD13 / CD34 / CD117 / CD11b / CD14 / HLA - DR / CD45

The samples were acquired in a FACSCanto II flow cytometer (Becton Dickinson—BD Biosciences) using the FACSDiva software (BD Biosciences), which was calibrated at a daily basis. At least 100,000 nucleated cells were acquired. Analysis was made with FACSDiva Software.

The several BM cell subsets were identified using a dot plot CD45/sideward light scatter (SSC) defining mature myeloid precursors as $CD45^{dim}/SSC^{high}$; monocytes $CD45^{bright}/SSC^{int}$ in combination with CD14 expression. Myeloid progenitors were $CD45^{int}/SSC^{int}$ in combination with the expression of CD34, CD117 and/or CD13 (myeloblasts). Early B-cell precursors (hematogones stage I) were defined as $CD45^{int}/SSC^{low}$ and $CD34^+/CD19^+/CD10^+$.[17–19]

We assessed 18 alterations.[10–12] In granulocytic precursors we assessed decreased SSC, abnormal maturation pattern in the CD16/CD13 and CD16/CD11b combinations (deficiency or increase in antigen expression or asynchronous maturation) and abnormal expression of CD7, CD56 or CD34 in >10% of maturing granulocytes.

In the monocytic population we assessed increase in number, increase in the percentage of $CD16^+$ monocytes, abnormal expression of HLA-DR and expression of CD7 or CD34 in >10% of the cells.

Concerning $CD34^+$ cells (Fig. 1), the following features were assessed: percentage of total $CD34^+$ cells, percentage of myeloblasts ($SSC^{int}/CD13^{\pm}/CD117^+/CD34^{\pm}$), deficiency in one of these antigens, abnormal expression of CD7 or CD56 in $CD34^+$ cells (percentage of abnormal cells among all nucleated cells compared with the controls), as well as a decrease in B-cell precursors ($CD34^+/CD19^+/CD10^+$), which were quantified among all nucleated cells.

$CD34^-/CD19^+/CD10^+$ and mature B lymphocytes ($CD34^-/CD19^+/CD10^-$) were also calculated but not considered as MDS-linked abnormalities. In addition, the percentage of mature T lymphocytes among all nucleated cells was estimated quantifying the $CD7^+/CD56^{neg}$ cells among $CD45^{high}/SSC^{low}$ (lymphoid) population.

2.3 | Statistical analyses

Since only a few cases were included in WHO categories, we grouped them together into: (1) patients with BM blasts <5% (RCC), (2) cases with BM blasts >5%: RAEB and RAEB-t (RAEB), and (3) patients with JMML. All variables were tested for normal distribution by the

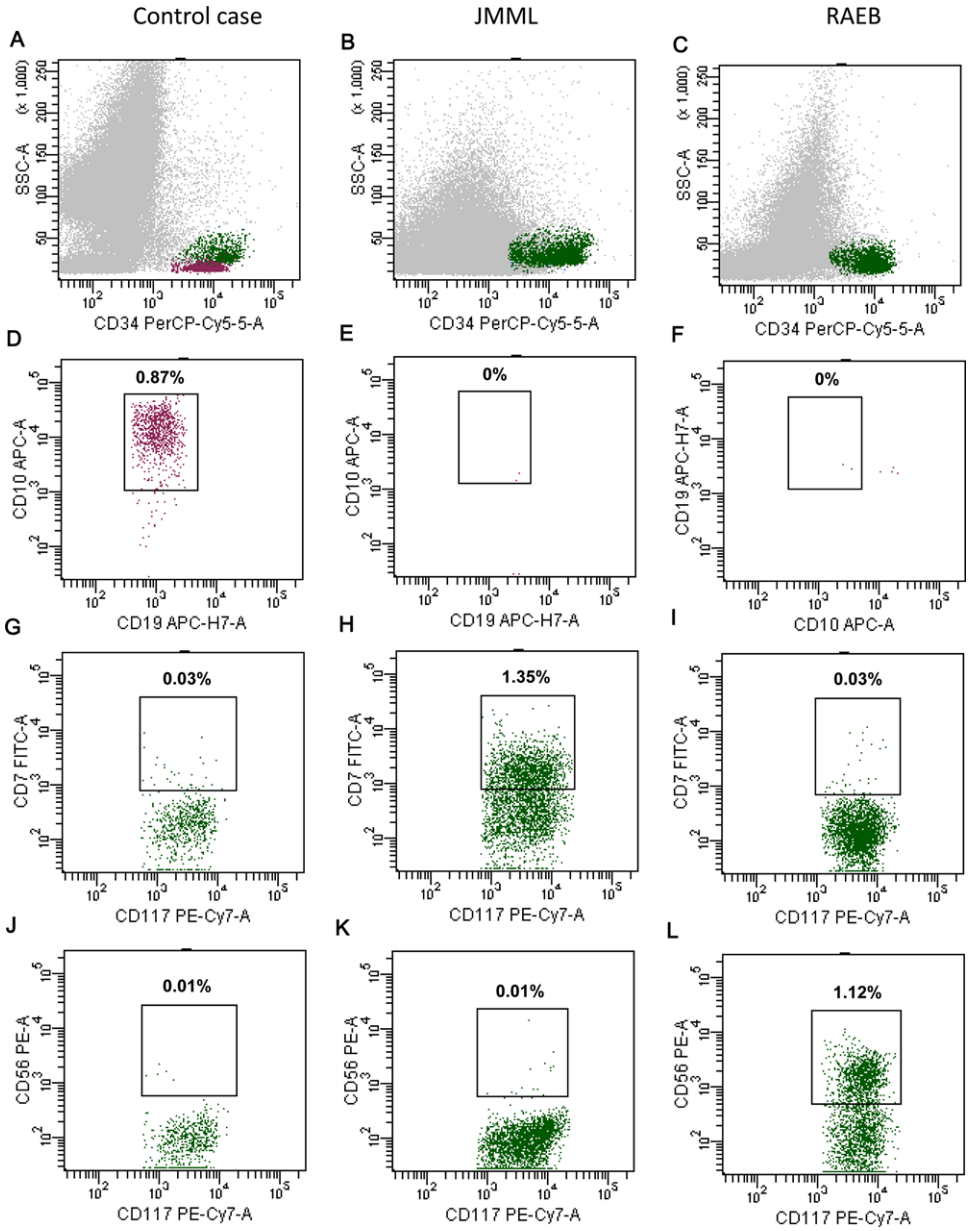

FIGURE 1 Gating strategy for the analysis of the CD34$^+$ cell subsets. First, CD34$^+$ cells were separated according to their SSC (A–C). In those with a low SSC, the proportion of those expressing CD19 and CD10 was assessed (D–F). Among those with a high SSC, abnormal co-expression of CD7 (G–I) and CD56 (K, L) in cells CD34$^+$/CD117$^+$ was assessed. The case with JMML was a 29-month-old girl with a normal karyotype. The RAEB case was a 31-month-old girl with no available cytogenetics.

Kolmogorov–Smirnov method, and those with a non-normal distribution were log transformed. Groups were compared by analysis of variance followed by the Tukey post hoc test. The correlation between logarithm transformed age and flow cytometric variables was tested using the Pearson correlation.

Lastly, a linear discriminant analysis was used to assess which MFC characteristics would best distinguish the groups of children examined.[10,20,21] The diagnostic category was used as a dependent variable, which was subsequently tested against the values of all features that were significantly different between the groups. Values were considered significant when $P < 0.05$. WINSTAT 3.1 and SPSS 15.0 software were used for all calculations.

3 | RESULTS

A total of 32 cases were examined: 13 JMML, 5 RAEB, 8 RAEB-t and 6 RCC, of whom two had received previous chemotherapy for a solid tumor but with a normal karyotype. They were compared with 10 controls. Age distribution, peripheral blood (PB) counts and cytogenetic findings are shown in Table 2. There was a significant inverse correlation between PB lymphocyte counts and age in all groups studied. Patients with JMML were the youngest. Among them, three had at least two features of neurofibromatosis type 1 according to the NIH Consensus Development Conference[22] and five had mutation of PTPN11. Mutations of N-RAS, K-RAS and CBL were present in one

TABLE 2 Age, peripheral blood counts (median and range) and cytogenetic findings of controls and patients

	Controls N = 10	RCC N = 6	JMML N = 13	RAEB N = 13	*P* value
Age (months)	70 (2–206)	66 (23–266)	29 (4–49)	68 (16–195)	0.01
Hemoglobin g/dL	13.8 (9.2–16.5)	11.2 (8.2–15.2)	10.1 (7.7–12.9)	10.1 (7.7–12.9)	0.07
Leukocytes $\times 10^9$/L	4.4 (2.9–6.0)	4.1 (2.4–5.1)	25.6 (10.2–113.7)	4.1 (1.6–13.6)	<0.0001
Lymphocytes $\times 10^9$/L	2244 (1218–4028)	2334 (1147–3740)	7138 (3570–22,000)	2116 (1170–4746)	
Monocytes $\times 10^9$/L	0.23 (0.19–0.48)	0.32 (0.1– 0.43)	4.5 (1.48–110.0)	0.24 (0.1– 1.80)	<0.0001
Platelets $\times 10^9$/L	210 (48–322)	53 (24–129)	46 (5–158)	24 (3–60)	0.003
Circulating blasts %	0	0	0 (0–10)	1 (0–8)	
Karyotype					
Normal	10	5	10	3	
Monosomy 7	0	1	2	2	
Trisomy 8	0	0	1	4	
Not available	0	0	0	3	

case each, and in two patients no mutation was found. In RCC, karyotype was normal for all patients except one case with monosomy 7. In JMML, monosomy 7 and trisomy 8 were found, but abnormal karyotypes were more frequent in RAEB and RAEB-t (Table 2).

The MFC analysis (Tables 3 and 4) showed no maturation abnormalities in myelomonocytic precursors in any of the controls. The total number of $CD34^+$ cells showed a negative correlation with the child's age ($r = -0.66$; $P = 0.018$) due to the variation in the percentage of B-cell precursors ($CD34^+/CD19^+/CD10^+$) ($r = -0.52$; $P = 0.05$) (Fig. 2). The number of T lymphocytes showed no variation with age.

In RCC, at least one abnormality was found in two cases, but several alterations were seen in the myelomonocytic maturation in the other four cases. Only few abnormalities were found in the $CD34^+$ cell subsets (Table 4).

Patients with RAEB, RAEB-t and JMML had a similar pattern of abnormalities in hemopoietic precursors and progenitors (Tables 3 and 4). There was a frequent increase in $CD34^+/CD117^+/CD13^+$ cells which had no significant correlation with the blast count in cytology in both groups. Aberrant cross-lineage antigen expressions in myeloid progenitors were found in 63% of the cases of JMML and in 45% of RAEB/RAEB-t, but in none of the RCC cases.

B-cell precursors were frequently decreased especially in RAEB/RAEB-t, being absent in 1 of 6 cases of RCC, in 4 of 13 patients with JMML and in 6 of 13 patients with RAEB/RAEB-t (Table 4). The significant difference found among the groups was due to a decrease of these cells in children with RAEB (Fig. 2).

T lymphocytes were increased in RCC and RAEB, and decreased in JMML (Table 3). In this last group, there was a positive correlation between T lymphocytes and hemoglobin value ($r = 0.63$; $P = 0.009$).

We performed a linear discriminant analysis with the features that were different in all the groups studied, and the best model could be obtained using "percentage of $CD34^+/CD117^+/CD13^+$ cells," "hematogones stage I," "percentage of T lymphocytes" ($CD7^+/CD56^{neg}$) and "total number of phenotypic abnormalities." This set of variables was able to correctly classify 83% of the cases. This percentage dropped to 72% after cross-validation in the leave-one-out procedure.

4 | DISCUSSION

Immunophenotyping by MFC has proven to be a useful ancillary tool for diagnosis in adult MDS,[7–12] but has seldom been used for this purpose in children.[13–16] In the present work we employed MFC to analyze the cellular characteristics in children with confirmed MDS and JMML who had entered the Registry of GCB-SMD-PED in the last 3 years. As expected, the youngest patients were those with JMML, whereas patients with RCC and RAEB were older and in the same age range as the control group. The distribution of the MDS types was similar to that found in a larger survey of Brazilian children with MDS.[23]

The inclusion of an eight-color MFC platform in the diagnostic work-up of patients with childhood MDS in our Cooperative Group was feasible in all patients, cost-effective and disclosed several interesting aspects. This platform allowed for analysis of up to 18 phenotypic features concerning the maturation pattern of the myelomonocytic series and the evaluation of myeloid and B lymphoid progenitors and enabled an estimation of the proportion of T-lymphocytes. Taking into account the current results, wealth of data, and success of analysis, the scope of future study can be extended to antibody combinations to assess T-cell subsets, using CD3 as a backbone, especially since immunophenotyping can be used to monitor treatment response with 5-azacytidine, which has an immunomodulatory effect.[24]

Our control group was small and age-matched with patients with RCC and RAEB and RAEB-t, but somewhat older than the patients with JMML. These children had received BM morphological and MFC evaluation for cytopenias related to deficiency anemias and transitory neutropenias. All of them showed normalization of PB counts after appropriate treatment. No myeloid maturation abnormalities were found in this group and $CD34^+$ cells were not increased according to reference values from the literature[11,16,18,19] so they were considered suitable for comparison with our patients with MDS and JMML.

We found abnormalities in myeloid maturation and increased $CD34^+$ myeloid progenitors similar to those already described in adult MDS. In addition, we could analyze in more detail the B-cell precursors that are usually decreased or absent in adults, and have been used in diagnostic scores.[8,15] These cells are much more numerous in

TABLE 3 FCM characteristics found in the control group and children with MDS and JMML (median and range)

	Controls N = 10	RCC N = 6	JMML N = 13	RAEB N = 13	*P* value[a]
% blasts in BM cytology	0.3 (0–1.0)	0 (0–1)	4.8 (0–13)	16.2 (7–27)	<0.0005
MFC features					
% monocytes	2.7 (2.0–3.5)	3.2 (2.4–8.0)	9.0 (2.5–20.6)	3.0 (0–27.3)	0.02
% cells in blast gate	1.2 (0.7–2.7)	2.1 (0.1–14)	5.2(0.3–9.8)	2.7(0.5–14.9)	<0.0005
% total $CD34^+$ cells	1.6 (0.8–4.7)	0.9 (0.3–6.3)	4.7 (0.5–8.6)	3.4(0.6–10.2)	0.003
$CD34^+/CD117^+/CD13^+$	0.71 (0.5–1.2)	0.5 (0.1–2.8)	3.7 (0.5–8.6)	4.2 (0.3–10.1)	<0.0005
$\%CD34^+/CD19^+/CD10^+$	0.83 (0.1–3.0)	0.28 (0–2.4)	0.5 (0–6.4)	0.03 (0–0.6)	0.01
$\%CD34^-/CD19^+/CD10^+$	4.1 (0.6–12.9)	1.6 (0–15.5)	0.8 (0–6.2)	0.02 (0–2.1)	0.04
mature B lymphocytes	2.6 (0.1–18.2)	6.1 (1.1–10.3)	2.6 (0.5–14.8)	2.2 (0–9.4)	0.56
T lymphocytes	5.3 (3.7–16.8)	10.5 (3.8–22.2)	2.9 (1.0–8.8)	9.2 (3.8–31.0)	<0.0005
Number of maturation abnormalities	0 (0–1)	1.5 (0–4)	4 (2–5)	3 (0–6)	<0.0005
Number of abnormalities in $CD34^+$ cell subsets	0	2 (1–3)	3 (1–5)	3 (1–6)	<0.0005
Total alterations	0 (0–1)	4 (1–6)	6 (2–11)	6 (2–11)	0.004

[a]Comparing all groups.

TABLE 4 Types of flow cytometric abnormalities found in the patients with MDS and JMML

	RCC N = 6	JMML N = 13	RAEB N = 13
Granulocytic precursors			
Decreased SSC	0	10	9
Abnormal maturation pattern	3	8	9
Aberrant CD7	0	1	0
Aberrant CD56	0	0	4
Monocytes			
% monocytes >3.5%[a]	2	11	5
Abnormal HLA-DR expression	2	0	0
Aberrant CD7	0	0	1
Aberrant CD56	0	1	3
CD34+ cells			
% total $CD34^+$ cells >5%[a]	1	4	6
$CD34^+/CD117^+/CD13^+$ >2%[a]	1	11	12
Aberrant CD7	0	8	6
Aberrant CD56	0	0	4
Absence of $CD34^+/CD19^+/CD10^+$	1	4	6

[a]Compared with normal range.

children, and vary with age, especially in children <5 years old.[11,17–19] So, it would be interesting to examine their behavior in children with MDS or JMML.

We confirmed the relationship between age and percentage of lymphoid progenitors in our control group. Concerning patients with MDS, Veltroni et al.[14] and Aalbers et al [13] have described a decrease in B-cell precursors in cases with RCC and RAEB. In the present work, we confirmed this finding. In patients with JMML, a decrease was also found compared with the six control cases in the same age range, as well as with the reference values from the literature.[18] So, we could confirm the great importance of this feature for the diagnosis of pediatric MDS, as it has been repeatedly shown in adults,[8–12] and previously highlighted in children.[6,13,14]

Recent studies have shown a decrease in B-cell precursors in patients with a GATA2 mutation in several pediatric disorders such as immunodeficiency syndromes and MDS, as well as in mycobacterial infections with monocytopenia (MonoMAC), hereditary lymphedema (Emberger syndrome) and familial MDS-related AML.[16,25–28] Nováková et al.[16] reported that 14% of all patients with advanced myelodysplastic syndromes (RAEB, RAEB-t and MDS-related AML) and 17% of cases with RCC had GATA2 mutations. These patients showed an important decrease of B cells in PB and BM. Also, Wlodarski et al.[26] found a high prevalence of a germline GATA2 mutation in patients with MDS presenting a deletion of chromosome 7. Therefore, the search for this mutation should be included in the diagnostic screening of children with MDS with monosomy 7.

In our study we found an important decrease of B-cell precursors, especially in children with RAEB, RAEB-t, and JMML, independent of age, type of cytogenetic abnormality and immunodeficiency. It will be important to include the detection of germline GATA2 mutation in future studies of the patients of the Brazilian Registry.

In all patients, except for two with RCC, at least two abnormalities were found, particularly involving altered antigen expression in maturing myelomonocytic precursors. Immunophenotyping was useful to establish the differential diagnosis between reactive and clonal cytopenias. These abnormalities were not seen in the control group, although several of them had PB cytopenias.

In RAEB cases the frequency of aberrant cross-lineage antigen expression in myeloid progenitors was similar to that seen in adults. An increase in $CD34^+$ myeloid progenitors and the presence of aberrant co-expression in myelomonocytic progenitors have been associated with more aggressive forms of MDS and a higher frequency of progression to acute leukemia not only in adults but also in children.[9,10,14]

The patients with JMML presented a high number of phenotypic abnormalities including an increase in myeloid progenitors similar to RAEB, even in cases with a low BM blast count in cytology. It was the

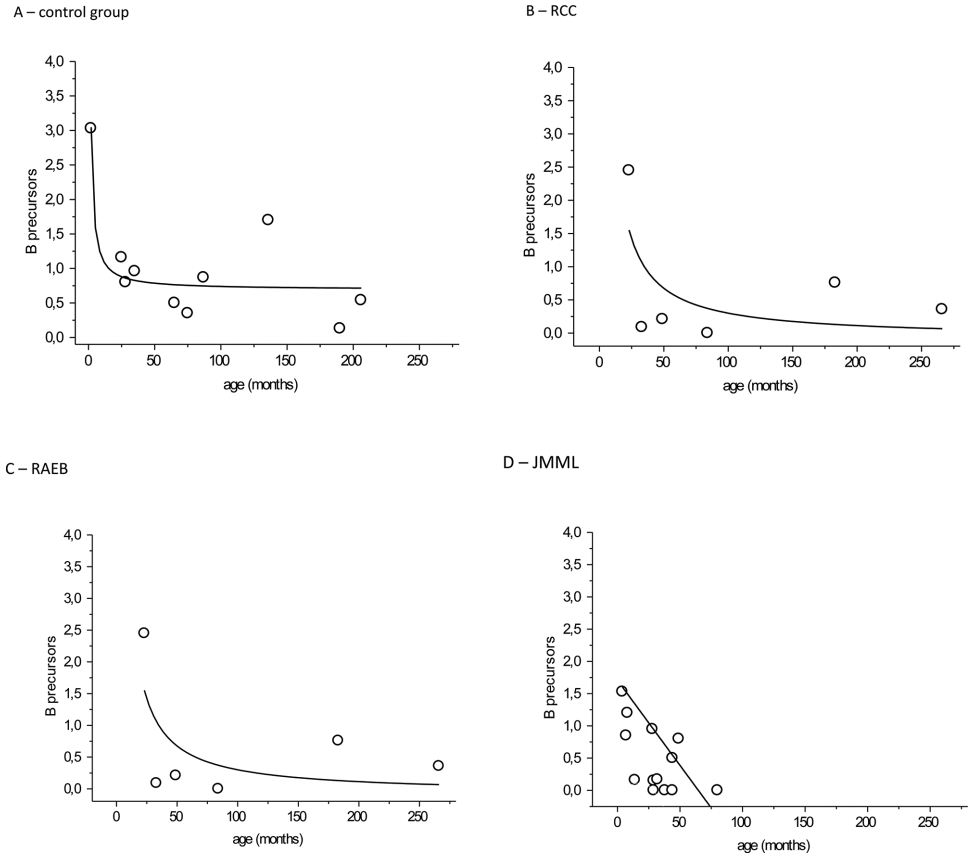

FIGURE 2 Relation between age and percentage of $CD34^+/CD19^+/CD10^+$ cells within the groups studied. ANOVA global test: $P < 0.0001$. In the post hoc test, comparing controls with the other groups, there was only a significant difference between controls and RAEB. In control cases (A) a negative Pearson's correlation was found between log age and B precursors in controls ($R = -0.79$; $P = 0.006$). The distribution can be best approximated by a hyperbolic equation: % B precursors: $y = 0.629 + 4.71/(\text{age in months})$; $R^2 = 0.736$.

only group presenting a decrease in T-lymphocytes, which correlated with the degree of anemia and thus may serve as a marker for disease severity.

On the basis of these observations, we can conclude that the inclusion of immunophenotyping in the diagnostic work-up of childhood MDS, even with a limited eight-color screening panel, gives useful diagnostic information as observed in adults.

ACKNOWLEDGMENTS

The authors would like to acknowledge the Barretos Pediatric Cancer Center, Brazil, for their financial support. Drs. Konradin Metze and Irene Lorand-Metze have a research grant from the Brazilian Research Council. All the patients in this study had a diagnostic revision from the Brazilian Cooperative Group in Pediatric MDS. The authors would like to extend our gratitude to all the children and their parents who agreed in participating. They thank Maressa Ferreira Neto for technical assistance in performing and interpreting the cytogenetic data of the patients. They would also like to thank Dr. Eszter Nagy, King's College, London, for the revision of the English style of the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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SUPPORTING INFORMATION

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