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Challenges in the use of NG2 antigen as a marker to predict *MLL* rearrangements in multi-center studies

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ABSTRACT

Rearrangements in *MLL* (*MLL*-r) are common within very young children with leukemia and affect the prognosis and treatment. Previous studies have suggested the use of the NG2 molecule as a marker for *MLL*-r but these studies were performed using a small number of infants. We analyzed 148 patients (all less than 24 months, 86 less than 12 months) from various centers in Brazil to determine the predictive power of NG2 within that cohort. We show that NG2 can be used for *MLL*-r prediction; however, proper staff training and standardized sampling procedures are essential when receiving samples from multiple centers as the accuracy of the prediction varies greatly on a per center basis.

1. Introduction

Rearrangements involving the myeloid–lymphoid lineage (*MLL*) gene on chromosome band 11q23 are frequent in both lymphoid and myeloid leukemia [1,2]. These genetic aberrations constitute an indicative characteristic for etiology and prognosis as patients with *MLL-AFF1* (*MLL-AF4*) fusion are subjected to the high-risk protocol especially when the onset occurs in the first year of life (infant leukemia, IL) [3]. For this reason, the accurate detection of *MLL* rearrangements (*MLL*-r) is crucial when dealing with this aggressive type of leukemia. Currently used methods to detect these rearrangements include conventional cytogenetics, fluorescence *in situ* hybridization (FISH) and reverse transcription-PCR (RT-PCR) analyses and long distance inverse PCR [4–8]. With the discovery in humans of a homolog of the cell surface chondroitin sulfate NG2 molecule in rats, previous groups have reported that *MLL*-r may be predicted through the cell surface recognition of NG2 using the monoclonal antibody (MoAb) 7.1 [9–12]. The mechanisms underlying this association are still being examined [13] and a lack of association has been reported in certain cases [14]. Due to the fact that *MLL*-r detection using standard techniques can be costly and requires specialized skills, a rapid and cost-effective technique would be quite useful especially for developing countries [15].

Some studies, while trying to determine the optimal procedure to maximize sensitivity and specificity upon using the immunophenotipic NG2 criterion for predicting *MLL*-r, concluded that immunophenotyping is a reliable tool for the prediction of *MLL*-r [15,16]. However these results were obtained using samples where infant patients were under-represented despite the fact that this subgroup represents the vast majority of leukemia patients with *MLL*-r [1,17].

In this study, we analyzed the value of NG2 detection in predicting *MLL*-r in acute leukemia cells using a large cohort of IL patients enrolled in the Brazilian Collaborative Study Group of Infant Acute Leukemia (BCSGIAL). The data was collected by various medical centers scattered across Brazil. While using all of the patient data as a single set casts doubt on the use of NG2 as an adequate marker for *MLL*-r, an analysis performed on a per center basis indicates otherwise. We show that great discrepancies exist between the qualities of the results for each center thus showing that proper caution must be taken upon use of the NG2 marker for *MLL* diagnostic especially for countries with uneven levels of regional development. Despite this, since the technique shows great predictive power for certain centers, we can conclude that NG2 is an adequate predictor of *MLL*-r given that the sampling and handling were performed correctly.

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2. Materials and methods

2.1. Patients

Patient cohort consists of children at age ≤24 months that were diagnosed with acute lymphoblastic leukemia (ALL) or acute myeloid leukemia (AML) between January 2003 and July 2010. Initial dataset contained samples from 311 patients, all of which were diagnosed with acute leukemia during this period. Out of those, we retained 148 patients for our study based on the available results for NG2 (7.1) immunophenotyping. To examine possible bias introduced by selecting study patients, we compared the clinical characteristics for patients studied herein and all ascertainable ones. No statistical difference was found between study patients and all BCSGIAL ascertained cases as far as gender, age, and *MLL*-r are concerned ($p > 0.05$, data not shown). The laboratory received samples from 35 different medical centers scattered across Brazil [18]. The time from the samples' collection to the immunophenotypic analysis varied between 8 and 36 h depending on the distance between the sampling center and our institute. The referring physicians provided demographic and clinical information like place of birth, age, gender, white blood cell (WBC) count, sample collection date, medication use, etc. Both data collection and laboratory procedures have been evaluated and approved by the Ethics Committee of all included institutions.

2.2. Leukemia diagnosis

The diagnosis was initially established by clinical examination and morphological characteristics of lymphoid and myeloid cells according to standard criteria. The samples were collected with ethylenediaminetetraacetic acid (EDTA) and sent to the laboratory in Rio de Janeiro for immunophenotyping. Mononuclear cells were isolated from bone marrow (BM) using Ficoll–Hystopaque (Sigma, Steinheim, Germany) density gradient centrifugation. Cell counting was performed using an automated cell counter (CELM, São Paulo, Brazil), which aspirates 20 μL of WBC diluted in phosphate buffered saline (PBS) + 15% fetal bovine serum (the equipment is able to count between 0.0 and 199.9×10^6 cells/mL). The mononuclear cellular viability has been assessed through propidium iodide staining (Sigma, St Louis, USA) and the stained cells were visualized in Neubauer chamber; all samples included in this analysis presented at least 70% of viable cells. Immunophenotyping of BM aspirates was performed at diagnosis using panel of MoAbs recommended by the European Group for the Immunological Characterization of Leukemias (EGIL) [19]. Fluorochrome-labeled MoAbs were used in triplet staining experiments, using fluorescein isothiocyanate (FITC), phycoerythrin (PE) and PE-cyanine 5 (PECy5) fluorochrome conjugates in each tube. Cell samples were analyzed by flow cytometry FACSCalibur (Becton, Dickinson and Company, CA, USA) using the Cell-Quest software program. Cell surface antigens were considered positive when 20% or more cells showed fluorescence intensity greater than the negative control while the cutoff for cytoplasmic antigen aMPO was 10%. Depending on the immunophenotype of BM cells, the patients were assigned to different categories: B-lineage ALL, T-lineage ALL, AML, and biphenotypic and the presence of genetic abnormalities commonly observed in childhood leukemias (*ETV6-RUNX1*, *E2A-PBX1*, *BCR-ABL*, *AML1-ETO*, *CBFB-MYH11*) was ascertained according to BIOMED-1 Concerted Action [8].

2.3. Qualitative and quantitative NG2 immunophenotyping

The presence of NG2 chondroitin sulfate proteoglycan molecule was evaluated using the CD34FITC/7.1PE/CD45PECy5 MoAb combinations; 10 μL of monoclonal antigen NG2 (7.1) (Immunotech) 1/20 diluted was used in each tube. Qualitative analysis was based on negative or positive signals while quantitative analysis was based on quantified molecules of equivalent soluble fluorescein (MESF) values.

2.4. Cytogenetic and molecular analyses

Detection of *MLL*-r were performed either by conventional cytogenetics, reverse transcriptase PCR, and/or by FISH technique (*LSI MLL Dual Color Break Apart Rearrangement Probe*, Vysis Inc.) as previously described [5].

2.5. Statistical analysis

The database of the qualitative (negative, low, bright signals) and quantitative (MESF) data has been analyzed using standard univariate procedure. To compare the frequency of NG2 positivity between different groups (e.g. age strata, ALL vs AML patients, CD10-positive vs CD10-negative, *MLL*-r vs *MLL*-germline) the χ^2-test (two-sided) was used (or Fisher's exact *P*-values with sets with very few data points). A *P*-value of less than 0.05 was considered statistically significant. All statistical analyses were done with the package IBM SPSS Statistics 14.0 version (Chicago, Il.).

2.6. Computational methods

The accuracy scores and the receiver operating characteristic (ROC) curves were produced with the ROCR package [20] using R version 2.10.1. We manually separated our sampling centers according to the observed correlation between the quantitative and qualitative NG2 measurements and *MLL*-r into 3 groups: centers with correlated data, centers with uncorrelated data (Table 3) and centers for which too few data points to reach a conclusion (Supplementary Table 1). Data points for which the *MLL* status could not be ascertained (5 cases out of 148) were removed from the analysis.

3. Results

Eighty-five out of 148 (57.4%) were ≤12 months-old while the remaining were aged between 13 and 24 months-old. The majority of samples showed >50.000 WBC count (59.5%) as shown in Table 1. The leukemia subtype classification was successfully performed in

Table 1
Demographical and laboratorial characteristics of patients.

Categories[a]	*n*(%)	*MLL*(+)(%)				
		AFF1	*MLLT3*	*MLLT1*	NK	Total
Age (months)						
0–12	85(57.4)	19(70.4)	6(60)	6(85.7)	13(81.3)	44(73.3)
13–24	63(42.6)	8(29.6)	4(40)	1(14.3)	3(18.7)	16(26.7)
Subtype						
ALL subtype	95(64.2)					
pro-B	46(48.4)	17(63)	3(30)	2(28.6)	10(62.5)	32(53.3)
Common	36(37.9)	5(18.5)	2(20)	2(28.6)	1(6.3)	10(16.7)
pre-B	3(3.2)	0	1(10)	0	0	1(1.7)
pro-T	6(6.3)	0	0	2(28.6)	0	2(3.3)
Other[b]	4(4.2)	1(3.7)	0	0	0	1(1.7)
AML	53(35.8)	4(14.8)	4(40)	1(14.2)	5(31.2)	14(23.3)
CD10 expression[c]						
Positive	41(33.1)	6(27.3)	2(25)	2(28.6)	3(25)	13(26.5)
Negative	83(66.9)	16(72.7)	6(75)	5(71.4)	9(75)	36(73.5)
7.1 Expression[d]						
Positive	63(42.6)	20(74.1)	6(60)	2(28.6)	10(62.5)	38(63.3)
Negative	85(57.4)	7(25.9)	4(40)	5(71.4)	6(37.5)	22(36.7)
WBC count						
≤50,000	60(40.5)	6(22.2)	5(50)	1(14.3)	3(18.7)	15(25)
>50,000	88(59.5)	21(77.8)	5(50)	6(85.7)	13(81.3)	45(75)
Total	148(100)					60(100)

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; NK, not known; WBC, white blood cell.
[a] 3 Cases of ALL and 11 cases of AML could not be subclassified.
[b] Includes 3 cases with unknown subtype and one case with biphenotypic acute leukemia.
[c] In 24 samples CD10 expression was not analyzed.
[d] Qualitative analysis.

Table 2
Accuracy values for qualitative and quantitative flow cytometry.

	Qualitative FC	Quantitative FC
Overall patients ($n = 143$)		
Best cutoff	20	61.34
Accuracy	0.678	0.720
ALL ($n = 92$)		
Best cutoff	22	39.1
Accuracy	0.695	0.750
AML ($n = 51$)		
Best cutoff	Infinity[a]	Infinity
Accuracy	0.725	0.725
Time until diagnosis		
<12 h ($n = 15$)		
Best cutoff	34	92.76
Accuracy	0.93	1.00
>12 h ($n = 128$)		
Best cutoff	20	39.1
Accuracy	0.65	0.69
Previous corticoid use		
Yes ($n = 14$)		
Best cutoff	56	98.8
Accuracy	0.785	0.714
No ($n = 129$)		
Best cutoff	20	39.1
Accuracy	0.666	0.728
MLL detection by FISH ($n = 35$)		
Best cutoff	7	27.38
Accuracy	0.800	0.714

FC, flow cytometry; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; FISH, fluorescent *in situ* hybridization.

[a] An infinity value means that the algorithm labeled everything as being *MLL*-r; this indicates that the correlation is so poor that the best prediction that can be made involves marking every element in the set as being *MLL*-r.

134 out of 148 (90.5%) samples as 3 cases of ALL and 11 cases of AML could not be subclassified. Within the ALL cases ($n = 95$), Bp-ALL and T-ALL represented 89.5% and 6.3%, respectively. Forty-six ALL cases displayed the CD10-negative pro-B ALL immunophenotype (48.4%), whereas the remaining cases were common ALL ($n = 36$) or pre-B ALL ($n = 3$). AML cases represented 35.8% of the total series, and M4/M5 accounted for the majority (62%) of AML cases. A diagram representing our series of patients is shown in Supplementary Fig. 3.

In 143 out of 148 ALL cases (96.6%) the presence or absence of an *MLL*-r could be analyzed. The molecular analysis showed an *MLL*-r in 60 patients, 44 (73.3%) of which were found within infants (≤12 months). Within *MLL*-r positive cases, 27 patients (45%) had *MLL-AFF1* fusion gene transcripts, 10 (16.7%) had *MLL-MLLT3* (*MLL-AF9*) fusion gene transcripts, 7 (11.7%) had *MLL-ENL* (*MLL-MLLT1*) fusion gene transcripts, and 16 (26.6%) had unknown *MLL* partner genes. Cases with these three translocations with known partner genes accounted therefore for about 73% of all *MLL*-r leukemia samples.

Within the 27 *MLL-AFF1* cases, 4 presented the AML subtype and 23 patients had the ALL subtype. Immunophenotype analysis in the different *MLL* groups revealed that 17 out of 23 (73.9%) ALL cases with *MLL-AFF1* were pro-B-ALL (CD10-negative). The only five c-ALL (CD10-positive) *MLL-AFF1*-positive cases were found in patients who were all older than 16 months old at diagnosis. *MLL-MLLT1* and *MLL-MLLT3* infants were predominantly pro-B-ALL (CD10-negative) whereas *MLL*-germline cases were mainly CD10-positive. All patients with a fusion partner other than *AFF1*, *MLLT3* or *MLLT1* were CD10-negative. CD10-positive patients were significantly older at diagnosis as compared to CD10-negative patients (Mann–Whitney *U*-test, $p < 0.05$).

As expected from the association with *MLL*-r, NG2-positive cases were over-represented within the ≤12 months group and significantly associated with hyperleukocytosis and central nervous system involvement, with CD10-immunophenotype and myeloid-associated antigens expression ($p < 0.05$). While in ALLs, NG2 positivity correlated with pro-B immunophenotype, in AMLs, the positivity correlated with myelomonocytic cell differentiation.

Twenty out of 27 (74.1%) *MLL-AFF1* positive patients were NG2-positive (qualitative analysis, cutoff 20%) while 8 out of 17 (47%) evaluable patients with other known *MLL*-r (*MLL-MLLT1* and *MLL-MLLT3*) other than *MLL-AFF1* were NG2-positive. *MLL*-r patients had a significantly lower CD10 expression than *MLL*-germline patients (median 1.5% versus 3.5%, U test, $p < 0.0001$).

Accuracy values for the ability of NG2 to detect *MLL*-r were calculated while considering all the patients as a single set and also, by dividing this set according to a number of variables (Table 2). In the overall analysis, accuracy values were 0.67 and 0.72 for qualitative and quantitative methods, respectively (Tables 2 and 3 and Fig. 1). Although the set with previous corticoid use yielded better results

Table 3
Accuracy table for qualitative and quantitative flow cytometry separating by sets of institutes.

Center	*n*	QL FC		QT FC		QL FC with cutoff = 20	QT FC with cutoff = 400
	(total; pos; neg)	Best cutoff	Accuracy	Best cutoff	Accuracy	Accuracy	Accuracy
All uncorrelated centers	63;29;34	91.00	0.556	71.77	0.635	0.524	0.556
Goiânia, GO	6;3;3	∞	0.500	∞	0.500	0.500	0.333
Ponta Grossa, PR	2;2;0	N/A	N/A	N/A	N/A	N/A	N/A
Florianópolis, SC	5;0;5	N/A	N/A	N/A	N/A	N/A	N/A
João Pessoa, PB	8;1;7	∞	0.875	44.38	1.000	0.625	1.000
Campina Grande, PB	2;1;1	∞	0.500	∞	0.500	0.000	0.000
Recife, PE	6;4;2	2.00	0.833	1.19	0.667	0.667	0.500
Salvador, BA	29;17;12	0.00	0.586	71.77	0.621	0.517	0.448
Itabuna, BA	5;1;4	∞	0.800	∞	0.800	0.200	0.600
All correlated centers	56;22;34	34.00	0.857	39.10	0.839	0.804	0.679
Salvador, BA	3;0;3	N/A	N/A	N/A	N/A	N/A	N/A
Brasília, DF	11;5;6	8.00	1.000	40.18	1.000	1.000	0.818
Salvador, BA	8;2;6	34.00	1.000	61.34	1.000	0.750	0.875
Rio de Janeiro, RJ	6;3;3	65.00	1.000	105.92	1.000	0.667	0.667
Curitiba, PR	2;1;1	37.00	1.000	∞	0.500	0.500	0.000
Campo Grande, MS	11;3;8	46.00	0.909	41.63	0.818	0.727	0.636
São Paulo, SP	3;0;3	N/A	N/A	N/A	N/A	N/A	N/A
Teresina, PI	4;3;1	7.00	1.000	20.36	1.000	0.750	0.500
Rio de Janeiro, RJ	8;5;3	16.00	0.875	21.03	0.875	0.875	0.500

The columns labeled "best cutoff" represent the cutoff that maximizes overall accuracy while the remaining ones represent the data with the cutoffs that have been reported in previous studies. A "N/A" indicates that we were unable to find an adequate cutoff due to the scarcity or unevenness of data while an infinity symbol (∞) means that the algorithm labeled everything as being *MLL*-r. n, Number of cases; pos, positive; neg, negative; QL, qualitative; FC, flow cytometry; QT, quantitative.

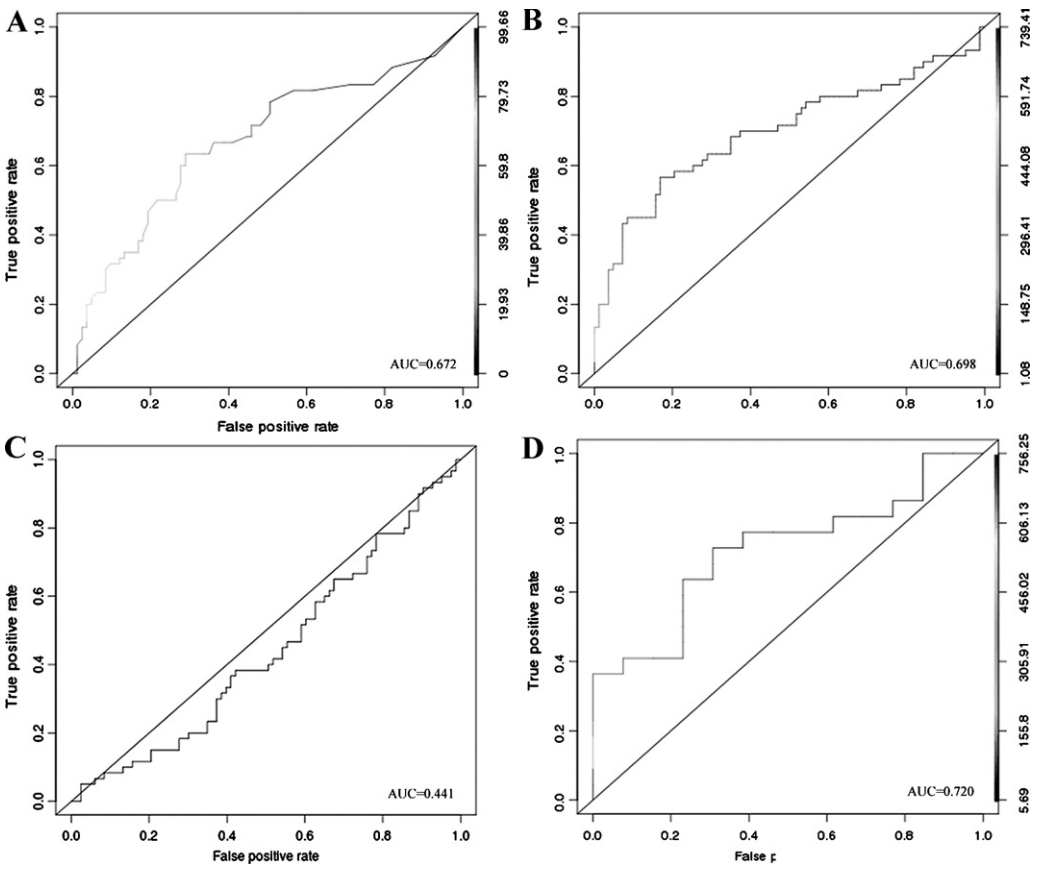

Fig. 1. ROC curves displaying the accuracy of the NG2 molecule for predicting *MLL* rearrangements. A – Qualitative analysis. B – Quantitative analysis. C – Random analysis and D – *MLL* detected by gold-standard method. AUC, area under the curve.

than the one without such use (Supplemental Fig. 1), an unequivocal conclusion cannot be made due to the scarcity of data points within that given set. While separating by leukemic subtypes (ALL vs AML), we noticed that the accuracy of NG2 in detecting leukemic cells with *MLL*-r was higher in ALL cases than AML cases (Table 2 and Supplemental Fig. 2).

To evaluate whether the time until sample analysis could influence the predictive power of NG2, we separated our dataset into the ones for which the BM aspirate collection had been processed within less than 12 h and those for which that time was greater than 12 h. We observed that, despite the small number of cases with a processing time of less than 12 h (15 in total), the accuracy of the method (0.93 and 1.00 for qualitative and quantitative methods, respectively) was significantly greater than the accuracy that had previously been obtained using the whole dataset (Table 2 and Fig. 2). However, we observed that certain geographical regions were over-represented within the dataset containing samples with less than 12 h until analysis. To ascertain whether the origin of the samples had any impact on our results, we proceeded to evaluate the predictive power of quantitative and qualitative NG2 measurements and *MLL*-r on a per center basis. The results showed that the accuracy differed significantly between them (Table 4). Despite the fact that some centers provided an uneven number of samples, we grouped data from various centers according to whether there was a significant level of correlation between NG2 measurements and *MLL*-r for the samples they provided. The results indicate that grouping centers with high levels of correlations and those poor levels of correlation and subsequently performing the analysis on the clustered data shows great disparity with the use of NG2 as a marker for *MLL*-r (Fig. 3).

Table 4
Accuracy table for qualitative and quantitative results according to time until sample processing.

	NG2[a]	
	Qualitative	MESF
Time till analysis < 12 h	*n* = 15	
True positive ratio	100	100
True negative ratio	87.5	100
False positive ratio	12.5	0
False negative ratio	0	0
Sensitivity	100	100
Specificity	87.5	100
Positive predictive value	87.5	100
Negative predictive value	100	100
Accuracy	93.3	100
Time till analysis > 12 h	*n* = 128	
True positive ratio	58.5	50.9
True negative ratio	70.6	82.6
False positive ratio	29.3	17.3
False negative ratio	41.5	49
Sensitivity	58.4	50.9
Specificity	70.6	82.6
Positive predictive value	58.4	67.5
Negative predictive value	70.6	70.4
Accuracy	65.6	69.5

MESF, molecules of equivalent soluable fluorescein.
[a] Results were generated based on best cutoff values.

4. Discussion

Solid diagnosis of *MLL*-r in pediatric patients is highly determinative in therapy stratification. The use of flow cytometry, a

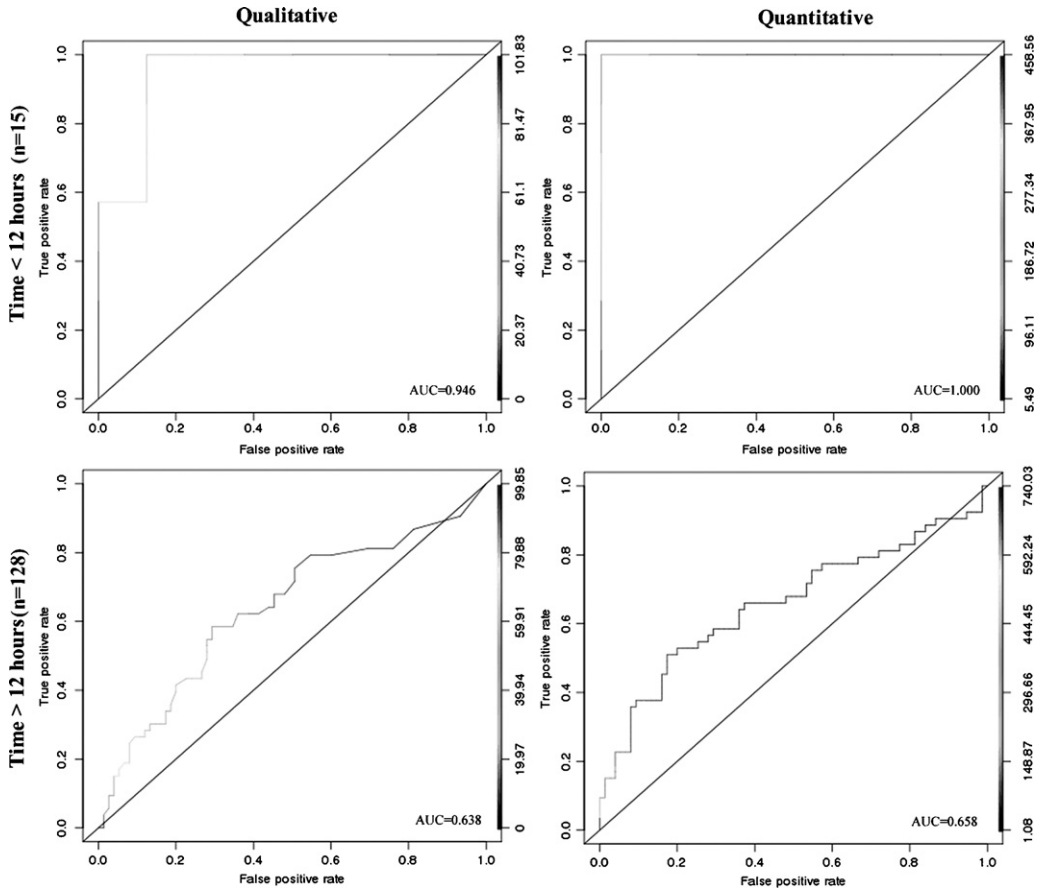

Fig. 2. ROC curves displaying the accuracy of the NG2 molecule for predicting *MLL* rearrangements while separating samples processed within <12 h from collection and those processed within >12 h. AUC, area under the curve.

widely accepted diagnostic tool to identify these rearrangements, has been pointed out to be particularly relevant in low-income countries. Worldwide efforts have focused on achieving high sensitivity and specificity values using FC to identify *MLL*-r [15,16]. However, no study has analyzed the use of this procedure in the low-income scenario for which many variables may influence the final results, especially in Brazil, a country with continental characteristics. The BCSGIAL has succeeded in collecting a substantial number of IL samples which have been well characterized through immune-molecular methods [18].

Thus, this is the first study that investigates the accuracy of NG2 in the detection of leukemic cells of both myeloid and lymphoid lineages with *MLL*-r using such a large number of early childhood cases. Furthermore, we report our efforts to determine which variables could influence the final results.

MLL-r in pediatric acute leukemias can occur with at least 64 partner genes with more than 100 possible breakpoints. These different partner genes may be categorized according to their gene ontology into functional subclasses [2] which may reflect distinct significances. We found a higher correlation between *MLL-AFF1* and the expression of NG2 than when comparing with other rearrangements. Other studies have also reported divergent NG2 reactivity in acute leukemia cells with different *MLL*-r [10–13] indicating that different NG2 expression patterns may depend on the particular translocation partner gene.

Accuracy values for our entire cohort of patient were 0.67 and 0.72 for qualitative and quantitative analysis, respectively. As expected from the results of previous studies, we also found better prediction values in ALL cases than AML [9–12]. It is known that the variability of *MLL* partner genes is more frequent among AML cases [2] therefore, this may also be an explanation for the difference in accuracy between ALL and AML samples.

The accuracy values increased significantly while using samples processed within less than 12 h and became on par with the one reported by previous studies [5–9]. Consequently, further analysis was performed by separating the data according to the institute origin of the samples. Upon noticing that the accuracy varied greatly between institutes, we compiled a list of institutes for which the samples provided adequate accuracy and those with lesser correlation. Since samples were collected in various institutes but analysis was performed in a single central institute, the observed discrepancy alludes to the inconsistency of the quality of the collection procedures on a per center basis. Ideally, both collection and analysis should occur within a core diagnostic center, however, this may not be realistic in a very large and heterogeneous country such as Brazil.

We also evaluated the city and state where the contributing institutes are located since one idiosyncratic aspect that can be observed in Brazil is the stark differences in the level of development between various regions. For instance, the Federal District with a Human Development Index (HDI) of 0.900 is on par with the Czech Republic whereas Alagoas, a small state in the north-east, has an HDI of 0.722, comparable with Equatorial Guinea [21,22]. These regional differences can be observed in our data as well. Using the list of correlated sets and uncorrelated ones, we observed that 79% of the data (stemming from 5 different centers out of a total of 8) in uncorrelated sets came from states with a medium HDI (less than 0.799). In contrast, 6 out of the 9 correlated sets (representing 73% of the data in those sets) originated from states with a high HDI (greater than 0.800). These discrepancies in accuracy

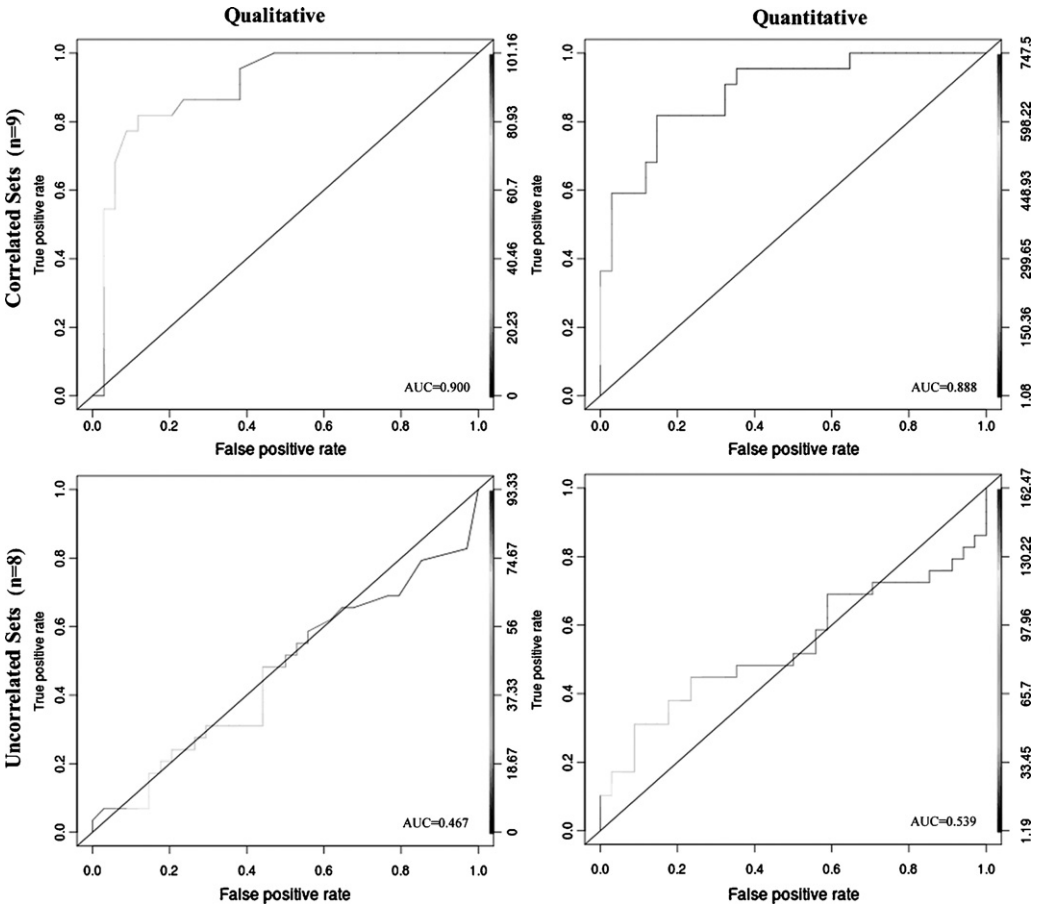

Fig. 3. ROC curves displaying the accuracy of the NG2 molecule for predicting *MLL* rearrangements while separating samples according to institute of origin. AUC, area under the curve.

can likely be explained by the differences between the quality of the reagents and staff training. As Zangrando et al. alluded to, a fast and reliable test for *MLL* is especially useful for developing countries. However, our results highlight that in developing countries with great regional differences in development like Brazil, care must be taken in predicting *MLL*-r using flow-cytometry especially when the samples come from multifarious sources stemming from regions with dissimilar levels of development. Despite this, we can also observe that the separation is somewhat ambiguous as we see sets collected in regions with a lesser levels of development within the reliable ones. This reassuring thought indicates that even centers in low-income regions can, with proper training and reagents, produce data that can be readily used for diagnostic.

Another factor to be examined is the adequate cutoff that separates patients as having an *MLL*-r from those that do not. Previous studies have reported the use of 20% for qualitative and 400 for quantitative as reasonable cutoffs for NG2 flow cytometry. In contrast, a recent study [23] evaluating the use of CD9 expression to predict *ETV6-RUNX1*-positive ALL has reported that cutoffs for immunophenotyping markers can be tailored to maximize different measures of association. Similarly, upon looking at the cutoff for maximizing the overall accuracy, we first notice that the optimal cutoff is not necessarily the aforementioned one used in the literature and, perhaps more importantly, the optimal cutoffs seem to vary from center to center. These results indicate that, despite the fact that NG2 seems an appropriate marker for *MLL*-r, greater standardization must be implemented in the collection procedure and additional work is needed to find the cutoff that minimizes as much as possible the false negative rate while keeping a relatively low false positive rate.

5. Conclusions

Our results suggest that immunophenotyping is a reliable approach to predict *MLL*-r using both subjective (gating processes by qualitative approach) and objective (MESF values by quantitative approach) FC methods on NG2 antigen data. However, in low-income regions, there might be some factors that ultimately lead to a lower accuracy.

Conflict of interest statement

Authors declare no conflicts of interest.

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Contributions. ME and GR analyzed and interpreted the data and wrote the manuscript. These authors share first authorship as they

contributed equally to this work. MS and CB acquired the laboratorial and demographical data. FP contributed to the design of the study and revised the article. MSPO contributed to the conception and the design of the study, revised the article critically, and made the final approval of the version to be submitted.

Appendix A. List of pediatricians from the BCSGIAL who contributed with samples included in completing this study.

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1. Hospital Martagão Gesteira IPPMG/UFRJ ($n = 6$); 2. Hospital Napoleão Laureano ($n = 8$); 3. Hospital Regional de Mato Grosso do Sul Pedro Pedrossian CETOHI/HRMS ($n = 12$); 4. Instituto Nacional de Câncer (n=8); 5. ONCO – Sociedade de Oncologia da Bahia LTDA ($n = 30$); 6. Hospital Universitário Oswaldo Cruz CEON ($n = 6$); 7. Hospital Araújo Jorge – Associação de Combate ao Câncer em Goiás ($n = 6$); 8. Hospital São Marcos – Associação Piauiense de Combate ao Câncer – APCC ($n = 4$); 9. Hospital Infantil Joana de Gusmão ($n = 5$); 10. Hospital de Apoio Brasília, Unidade de Onco-Hematologia Pediátrica ($n = 12$); 11. Hospital Martagão Gesteira – Instituto de Oncologia e Onco-Hematologia ($n = 8$); 12. Santa Casa – Hospital Manoel Novaes ($n = 5$);

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.leukres.2011.03.006.

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