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Easy discrimination of hematogones from lymphoblasts in B-cell progenitor acute lymphoblastic leukemia patients using CD81/CD58 expression ratio

Carole Nagant | Daniele Casula | Anne Janssens | Vo Thanh Phuong Nguyen | Brigitte Cantinieaux

Laboratoire d'Hématologie, Réseau des Laboratoires Hospitaliers Universitaires de Bruxelles (LHUB-ULB), Bruxelles, Belgique

Correspondence: Carole Nagant, Laboratoire d'Hématologie, Réseau des Laboratoires Hospitaliers Universitaires de Bruxelles (LHUB-ULB), Bruxelles, Belgique (carole.nagant@gmail.com).

Abstract

Introduction: The discrimination of leukemia lymphoblasts (LB) in diagnosis and follow-up of B-cell progenitor acute lymphoblastic leukemia (BCP-ALL) by multiparameter flow cytometry (MFC) may be difficult due to the presence of hematogones (HG). The aim of this study was to compare lymphoblasts of BCP-ALL and HG for the expression of the most discriminating antigens.

Methods: A total of 82 bone marrow samples (39 BCP-ALL and 43 patients with HG) were analyzed using MFC. Mean fluorescence intensity (MFI) was measured for ten markers commonly used in hematology laboratories: CD45, CD19, CD10, CD34, CD38, CD20, CD22, CD58, CD81, and CD123. Statistical comparison of the MFI between LB and HG was performed. The presence on LB of aberrant expression of myeloid and/or T-cell markers was also investigated.

Results: Qualitative pattern expression of antigens showed overexpression on LB of CD58, CD22, CD34, CD10 and underexpression of CD81, CD45, CD38 when compared to HG. Expression of CD123 was positive in 34% of BCP-ALL LB and always absent on HG. Aberrant antigen expression (myeloid and/or T-cell marker) including CD123 was observed in 58% of BCP-ALL patients. The use of a MFI antigen ratio of the most discriminating markers (CD81/CD58) (analysis of variance, $P < 0.005$) increased the distinction of LB versus HG with a high specificity and sensitivity as demonstrated by the use of ROC curve analysis (AUC of CD81/CD58: 0.995).

Conclusion: We demonstrate in this study that routine use of the MFI antigen ratio (CD81/CD58) in addition to the MFC evaluation using WHO classical criteria appears to be an efficient approach to discriminate LB from HG.

KEYWORDS

BCP-ALL, CD81/CD58, hematogones, lymphoblasts, MRD

1 | INTRODUCTION

The detection of minimal residual disease (MRD) in B-cell progenitor acute lymphoblastic leukemia (BCP-ALL) has two main applications with clinical involvement for the management of patients: firstly, the evaluation of the response to initial therapy for the risk stratification and adjustment of treatment for MRD-high risk patients[1-4] and secondly, the monitoring of patients in complete remission to detect the reoccurrence of MRD and to allow the introduction of a rescue treatment.[5,6]

Immunophenotyping by multiparameter flow cytometry (MFC) is a rapid technique for detecting MRD at a 10^{-4} cut-off, with results reported within a few hours.[7] The method is based on the identification of immunophenotypic markers associated with leukemic cells (LAIP, leukemia-associated immunophenotypic pattern) which are distinct from normal hematopoietic cells. Monitoring of MRD by MFC in patients treated for BCP-ALL can be challenging because normal bone marrow B-cell precursors, HG, share many immunophenotypic characteristics with BCP-ALL blasts. The most commonly employed markers for LB overlap with those of HG.[8] In addition to phenotypic similarities, LB and their nonneoplastic counterparts share similar morphological features, making their morphologic distinction in bone marrow difficult.[9]

Increasing levels of HG have been described in different situations, particularly in the phase of bone marrow regeneration, following chemotherapy or bone marrow transplantation[10,11] or in healthy infants.[12,13] In certain conditions, they constitute 5% to more than 50% of cells.[9]

The identification of aberrant antigen expression is therefore critical to discriminate HG from residual or recurrent leukemia in patients treated for BCP-ALL.

The objective of this study was to evaluate the expression of potentially discriminating markers between LB of newly diagnosed BCP-ALL and the CD19+CD10+ HG of the bone marrow. We also aimed to evaluate the combined expression (fluorescence intensity ratio) of the most discriminating markers (CD81/CD58) to highlight the differences between HG and LB for subsequent MRD following.

2 | MATERIALS AND METHODS

2.1 | Samples

A total of 82 patients submitted for MFC analysis in the LHUB-ULB, from January 2014 through August 2017, were included in this study. Thirty-nine newly diagnosed BCP-ALL bone marrow samples were analyzed. Patients included 30 children and 9 adults (median age : 5 years, age range : 2-71 years). The diagnosis of BCP-ALL was established according to conventional WHO criteria[14] and included MFC data derived from an extensive panel of myeloid and lymphoid markers. From all patients with BCP-ALL included in the study (n = 39), 25 were of pre B category BCP-ALL and 14 were common ALL. Pro B BCP-ALL were excluded from the study. To evaluate HG, a total of 43 subjects with nonmalignant conditions were included in the study with 23 children and 20 adults (median age : 11 years, range : 0.2-77 years). Finally, 8 cases of postacute leukemia therapy were investigated.

2.2 | MFC analysis

For each bone marrow sample, three 10-colors tube (tube 1: KappaFITC-LambdaPE-CD3ECD-CD38PC5.5-CD5PC7-CD10APC-CD19AA700-CD20AA750-CD22PB-CD45KO; tube 2 : CD138FITC-CD13PE-HLAECD-CD33PC5.5-CD34PC7-CD56APC-CD19AA700-CD117AA750-CD38BV421-CD45KO; tube 3: CD3FITC-CD56/16PE-CD4ECD-CD14PC5.5-CD8PC7-CD2APC-CD19AA700-CD7AA750-CD57PB-CD45KO), that are used for routine panel in hematological disorders, were acquired on a Navios flow cytometer (Beckman Coulter). Two 5-colors tubes (CD123FITC-CD10PE-CD45ECD-CD38PC5.5-CD19PC7 and CD58FITC-CD81PE-CD45ECD-CD10PC5.5-19PC7) were analyzed on a FC500 flow cytometer (Beckman Coulter). Data were acquired using CXP and Navios multiparameter Kaluza program (Beckman Coulter, Miami, FL). The flow cytometers were subjected to daily quality control using the fluorescent beads flow-check, flow-check pro and flow-set pro (Analis-Beckman Coulter, Ireland).

To determine the positivity criteria for marker expression, other cell populations like mature T- and B- lymphocytes were used as reference. Positive expression was defined when ≥20% of the population expressed the marker.

TABLE 1 Differential expression of phenotypic markers on HG versus LB

	MFI				% of positivity (n/total n)		
	HG	LB	Mann–Whitney test	Kruskal–Wallis test	HG	LB	Fisher test
CD58	0.3 (0.2-0.5)	3.5 (1.7-5.0)	<0.0001	$P < 0.01$	53% (23/43)	90% (35/39)	0.0005
CD81	54.7 (40.4-69.2)	5.7 (4.3-11.7)	<0.0001	$P < 0.005$	100% (43/43)	100% (39/39)	ns
CD123	0.2 (0.2-0.3)	0.5 (0.4-1.2)	<0.0001	ns	0% (0/22)	34% (13/38)	0.0011
CD10	6.7 (4.7-7.9)	16.1 (8.0-25.6)	<0.0001	ns	100% (25/25)	100% (19/19)	ns
CD38	22.2 (18.0-26.2)	11.5 (4.6-16.9)	0.0002	ns	100% (19/19)	100% (14/14)	ns
CD22	1.4 (1.3-1.6)	2.9 (1.9-6.3)	0.0027	ns	100% (19/19)	100% (14/14)	ns
CD20	0.6 (0.4-0.9)	0.4 (0.2-3.3)	ns	ns	63% (12/19)	71% (10/14)	ns
CD19	5.2 (4.5-6.0)	6.2 (3.8-8.8)	ns	ns	100% (19/19)	100% (14/14)	ns
CD34	0.5 (0.3-2.2)	4.3 (2.1-15.9)	0.0134	ns	44% (19/43)	87% (33/38)	<0.0001
CD45	11.1 (8.0-13.5)	1.8 (0.9-2.9)	<0.0001	ns	100% (37/37)	73% (27/37)	0.0010

Median of fluorescence intensity with 25th and 75th percentiles of antigen expression for each group (IQR). Single antigen comparison between HG and LB was analyzed using Mann–Whitney test. Positive expression was defined when ≥20% of the population expressed the marker. Fisher exact test was used to compare positivity for marker expression.

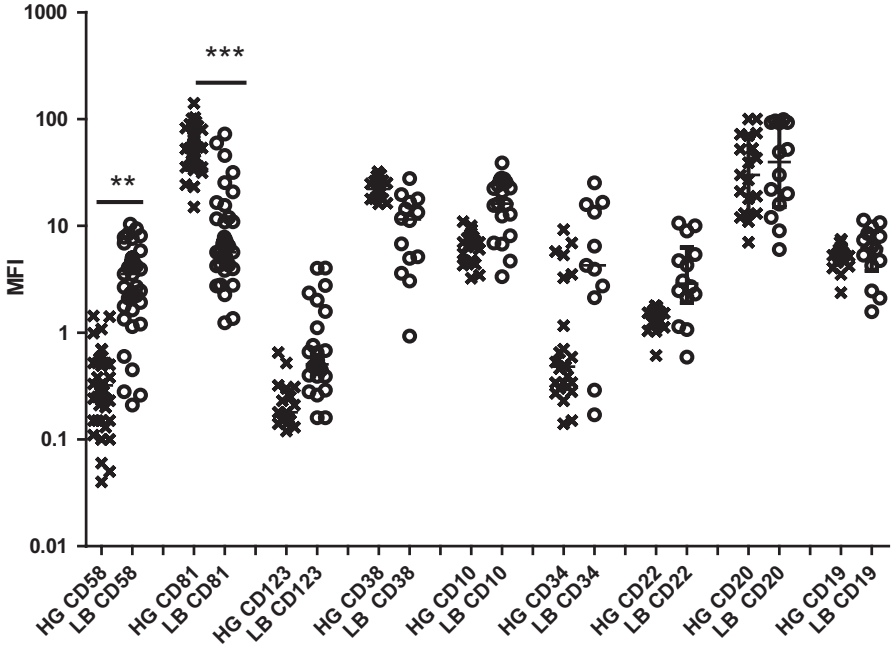

FIGURE 1 Antigen expression on HG versus LB. Fluorescence intensity of antigen expression (MFI) for each group. All markers were analyzed using the nonparametric Kruskal–Wallis analysis of variance test, followed by Dunn's multiple comparison test performed for each group

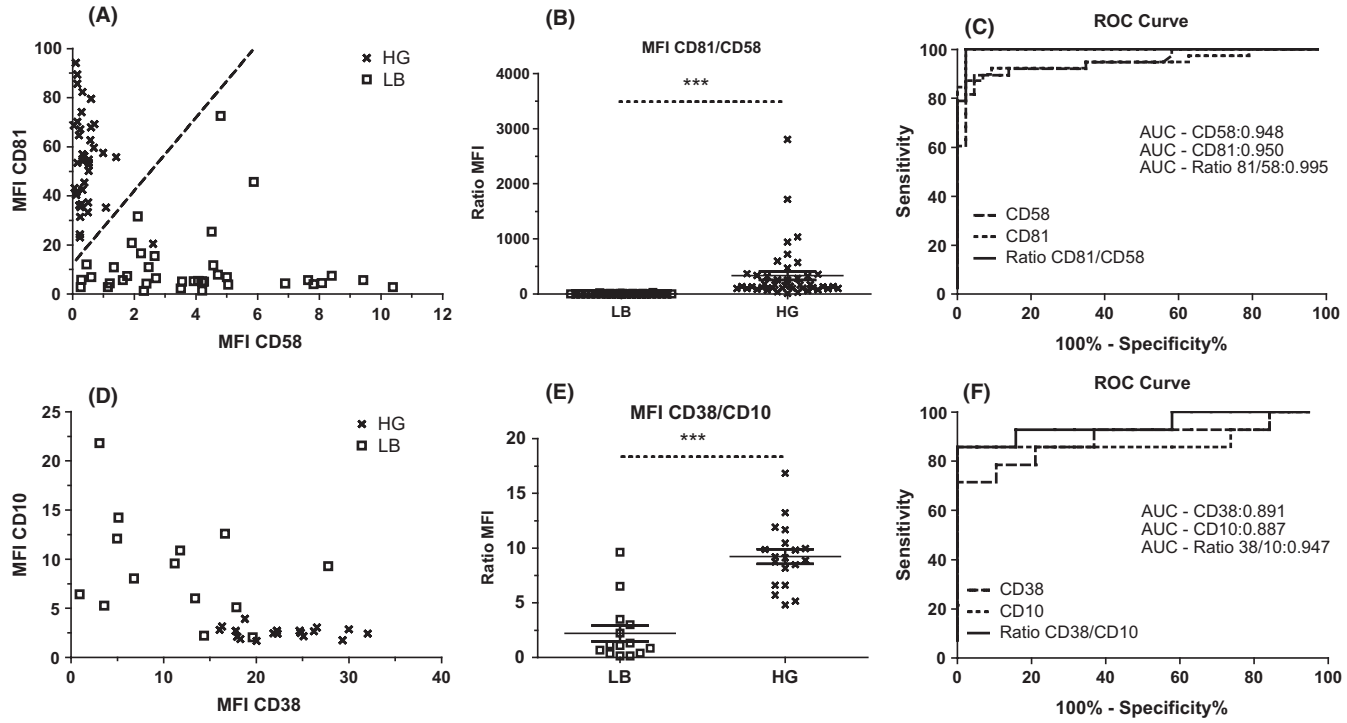

FIGURE 2 The use of a ratio to discriminate HG from LB. A, Combined expression of CD58 and CD81 on HG (cross symbol) and LB (square symbol) B, CD81/CD58 intensity of fluorescence ratio on LB versus HG C, ROC curve analysis of CD58, CD81 and CD81/CD58 D, Combined expression of CD38 and CD10 on HG (cross symbol) and LB (square symbol) E, CD38/CD10 intensity of fluorescence ratio on LB versus HG F, ROC curve analysis of CD38, CD10 and CD38/CD10

2.3 | Gating strategy

The following gating strategies were used for HG analysis. First, HG were identified by gating on the CD19+CD10+ events and then by back-gating them on the side scatter-vs-CD45 histogram. Further analysis for CD45, CD20, CD22, CD58, CD81, CD38 and finally CD123 expression were carried out on the CD45dimCD10+CD19+ gated HG or LB cells.

2.4 | Statistical analysis

Statistical analysis of data was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; ns, nonsignificant). The nonparametric Mann–Whitney test was used for the comparison of two groups and the nonparametric Kruskal–Wallis analysis of variance, followed by a Dunn's

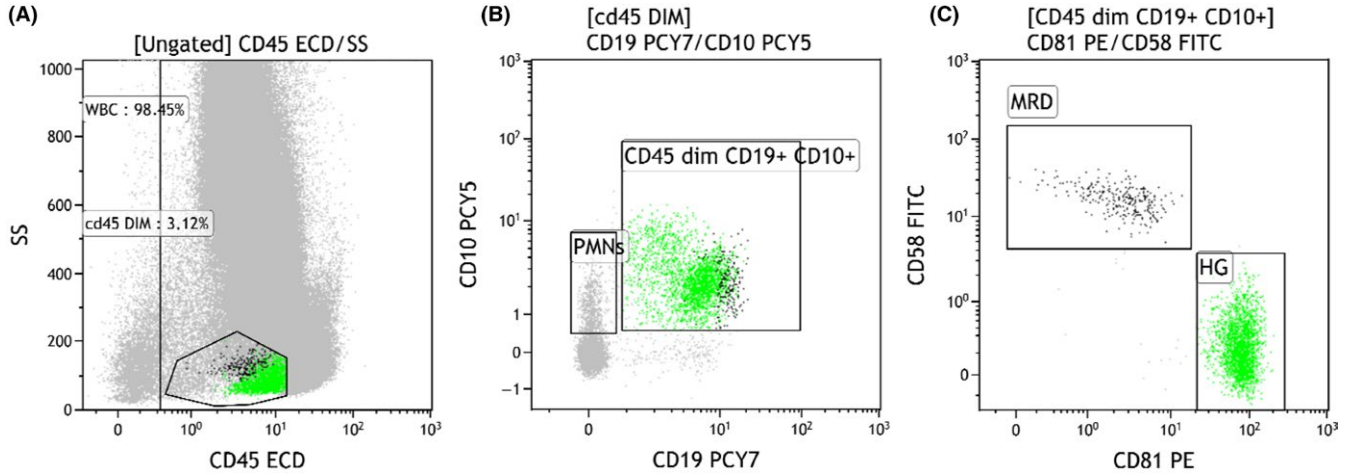

FIGURE 3 Illustrative case including both HG and LB in the same specimen. Illustrative case of MRD in BCP-ALL patient. A,B,C, Gatting strategy C, MRD cells are colored in black and hematogones are colored in gray. Leukemia lymphoblasts (MRD) had a CD81/CD58 ratio of 0.2 (pathological interval) and the hematogones had a CD81/CD58 ratio of 331 [Colour figure can be viewed at wileyonlinelibrary.com]

multiple comparison test, was performed for the comparison of more than two groups. Fisher exact test was used to compare positivity for marker expression.

3 | RESULTS AND DISCUSSION

The expression of individual antigens (CD58, CD81, CD38, CD10, CD123, CD22, CD19, CD20, CD34, CD45) on leukemic lymphoblasts (LB) and on normal precursor B-cell subpopulations (HG) was measured in terms of MFI.

CD81 was expressed on both HG and LB, with 100% of positivity for both populations (Table 1). However, HG expressed CD81 with a significantly higher fluorescence intensity than LB (HG : 54.7 MFI (interquartile range (IQR) : 40.4-69.2); LB : 5.7 MFI (IQR 4.3-11.7); $P < 0.0001$) (Table 1). Aberrant underexpression of CD81 on LB of BCP-ALL when compared to benign precursors has been previously reported,[15] making CD81 a potential marker in BCP-ALL diagnosis.

Veltroni et al[16] showed that CD58 expression was significantly higher in BCP-ALL blasts than in HG. Our results are in accordance with this observation: 90% (35/39) of patients with BCP-ALL expressed CD58 and only 53% of subjects with HG expressed CD58 (Fisher test, $P = 0.0005$). Additionally, the intensity of CD58 expression by HG was significantly lower than on LB (HG: 0.3 MFI (IQR 0.2-0.5); LB : 3.5 MFI (IQR 1.7-5.0); $P < 0.0001$).

The 34% (13/38) of patients with BCP-ALL were positive for CD123 while the expression of CD123 was absent on normal hematopoietic precursors B-cells (Fisher test, $P = 0.0011$). These data are in accordance with Djokic et al[17] who reported strong expression of CD123 in 34% of BCP-ALL patients. The persistence of LB with aberrant expression of CD123 after induction therapy is therefore an important contribution for the follow-up of MRD and relapse in these CD123 positive BCP-ALL patients. The clinical relevance of CD123 is of particular interest as targetable therapy with anti-CD123 is in development.[18]

The intensity of CD10 and CD34 expression was higher in LB when compared to HG (CD10, HG : 6.7 MFI (IQR 4.7-7.9); LB : 16.1 MFI (IQR 8.0-25.6); $P < 0.0001$) (CD34, HG: 0,5 MFI (IQR 0,3-2,2); LB : 4,3 MFI (IQR 2,1 -15,9); $P = 0,0134$). In contrast, CD38 and CD45 were underexpressed in LB versus HG (CD38, HG: 22.2 MFI (IQR 18.2-26.2); LB : 11.5 MFI (IQR 4.6-16.9); $P = 0.0002$) (CD45, HG: 11,1 MFI (IQR 8,0-13,5); LB : 1,8 MFI (IQR 0,9-2,9); $P < 0,0001$).

Although high differences in antigen expression were observed among the different populations, an overlap in the values of fluorescence intensity (MFI) between HG and LB was observed for all markers with the exception of CD123. Expression of CD123 was consistently negative on HG. As an overlap in the expression of CD38, CD10, CD58, CD19, CD20 CD34, CD45, CD81, and CD22 was observed between HG and LB, a cut-off value for these markers could not be determined as a discriminator between these populations. Monitoring of aberrant antigen expression can be very helpful for detecting relapse or MRD. At diagnosis, 58% (22/39) of BCP-ALL patients presented CD123 or aberrant markers (CD13, CD33, CD117, CD7, CD2, CD5, CD4, CD56) on LB. For the cases (42%, 17/39) where no aberrant markers could be noted, MRD follow-up can be really challenging using standard MFC approaches. We therefore further investigated the interest of using a combination of markers to allow a more objective and easy discrimination of the two populations.

An analysis of variance for all antigens enabled identification of the most discriminating markers (CD58 $P < 0.01$; CD81 $P < 0.005$, Figure 1). Two combinations (CD81/CD58 and CD38/CD10) were evaluated, both including one underexpressed and one overexpressed surface marker. The results herein show a significant difference in fluorescence intensity ratio CD81/CD58 between LB and HG (LB : 2.1 (IQR 0.8-7.5); HG : 141.6 (IQR 101.3-334.2); $P < 0.0001$) (Figure 2B). Using receiver operating characteristic (ROC) curve analysis, this data highlights the interest of employing a combination of the two markers compared to the use of markers CD58 and CD81 individually for discriminating HG from LB (AUC of CD58 alone: 0.948;

AUC of CD81 alone: 0.950; AUC of CD81/CD58 : 0.995) (Figure 2C). LB could be distinguished from HG with a sensitivity of 100% and a specificity of 97.7% when CD81/CD58 ratio (MFI) was lower than 29.7. The CD38/CD10 ratio was also investigated to distinguish LB from normal bone marrow precursors. The MFI ratio CD38/CD10 was significantly higher on HG when compared to LB (HG : 9.1 (IQR 6.6-10.5); LB : 1.1 (IQR 0.4-3.1); $P < 0.0001$) (Figure 2E). The use of ROC curve analysis demonstrated that the combined use of two markers (ratio CD38/CD10) allowed both a higher sensitivity and specificity for the discrimination of the two populations than the use of both markers separately (AUC of CD38 alone: 0.891; AUC of CD10 alone : 0.887; AUC of CD38/CD10 : 0.947). A ratio inferior to 4.2 strongly indicates LB with a specificity of 100% and a sensitivity of 85.7%. To our knowledge, this is the first study reporting the interest of a combined use of markers (fluorescence intensity ratio) to rapidly differentiate malign and benign B precursors.

Finally, 8 cases with HG of postacute leukemia therapy (4 cases from post-therapy for BCP-ALL and 4 cases from post-therapy for acute myeloid leukemia (AML)) were investigated for the CD81/CD58 ratio expression and compared to normal bone marrow. The median MFI ratio of the 8 cases of HG from post-therapy was not significantly different from the median MFI ratio of HG from normal bone marrow (nonmalignant condition) (HG post-therapy : 164 (IQR 98-331); nonmalignant HG condition : 141.6 (IQR 101.3-334.2), *P* ns). This observation underlines the absence of treatment impact on the measured ratio. One case of postacute leukemia therapy was positive for BCP-ALL MRD (Figure 3). Both HG and LB were present in the same specimen. Leukemia lymphoblasts (MRD) had a CD81/CD58 ratio of 0.2 (pathological interval) and HG had a CD81/CD58 ratio of 331 demonstrating the interest of the CD81/CD58 ratio approach.

4 | CONCLUSION

It is of great importance to recognize immunophenotypic deviation of neoplastic immature B-lineage cells from HG in initial diagnosis of BCP-ALL and in monitoring of MRD. This work highlights qualitative patterns of antigen expression that may improve the sensitivity of MRD detection by MFC. LB showed overexpression of CD123, CD58, CD10, CD22, CD34, underexpression of CD38, CD81, CD45 and aberrant expression of CD13, CD33, CD117, CD7, CD2, CD5, CD4, CD56 when compared to HG.

However, in some difficult MRD cases, routine MFC analysis using qualitative patterns may fall short to successfully discriminate LB from HG. We propose in this work an alternative manner for representing flow data by the use of a MFI ratio that could help for discrimination between these populations. We showed that using MFI antigen ratio in routine with the most discriminating antigens (CD81/CD58) allows a clear-cut distinction of LB versus their normal immature B-lineage counterparts (HG). The use of this MFI ratio in conjunction with a qualitative analysis would be beneficial in many laboratories that perform standard gating analysis to set objective criteria for MRD. These markers should be included in flow cytometry panels at the time of diagnosis of BCP-ALL patients and could allow reliable and rapid MRD monitoring, especially for patients with no aberrant markers on their LB.

CONFLICT OF INTEREST

None to declare.

ORCID

Carole Nagant http://orcid.org/0000-0001-9755-5069

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