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HAEMATOLOGY

Myeloblasts in normal bone marrows expressing leukaemia-associated immunophenotypes

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Summary

Measurable residual disease (MRD) status of patients undergoing treatment for acute myeloid leukaemia (AML) is important for prognosis and guides treatment. Multicolour flow cytometry (MCF) is a sensitive MRD method. The current approach relies on identification of blasts expressing leukaemia-associated immunophenotypes (LAIP) or by blasts expressing aberrant differentiation/maturation profiles compared to that seen in normal haematopoietic precursor cells at follow-up, i.e., different from normal (DFN). However, expression of LAIP on normal myeloblasts affects the specificity of the result, and the understanding of what is normal is important. Limited published data are currently available. We report findings from 14 normal adult bone marrows.

MCF was performed on the residual normal marrow specimens from 14 adults. Expression of CD15, CD11b, CD7, CD4, and CD56 on CD34+ myeloblasts was assessed. Analysis of samples was performed using 4-colour flow cytometry which was the methodology used when this work was done, and is still being used in many clinical flow laboratories worldwide. LAIP is defined by lineage infidelity or asynchronous expression of differentiation markers.

The cases of normal myeloblasts with LAIP involving the markers used and above the cut-off levels for MRD detection (0.01%) varies between 43% and 100%, limiting the specificity of the results for MRD. Even if the threshold is raised to 0.1%, there will still be false positive cases using aberrant CD15 or CD7.

Our work provided useful information for AML MRD determination in our laboratory. A collaborative database of LAIP on normal myeloblasts using standardised analysis should be useful to determine the optimal diagnostic cut-off for AML MRD using LAIP.

Key words: Normal myeloblasts; leukaemia-associated immunophenotype; AML; residual disease; flow cytometry.

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INTRODUCTION

In healthy individuals, normal maturation and differentiation of haematopoietic cells is associated with reproducible, sequentially occurring patterns of antigen expression in the majority of the cells. Deviation from this pattern is seen in a minute population of normal myeloblasts, and also in neoplastic conditions including leukaemia. The resulting phenotypic aberrancy is commonly termed 'leukaemia-associated immunophenotype' (LAIP). Evaluation of the LAIP expression on myeloid blasts is important in confirming the diagnosis of acute myeloid leukaemia (AML) and is the first approach used in the assessment of measurable residual disease (MRD) following leukaemia treatment by multicolour flow cytometry (MFC).[1–5]

Even patients who fall into favourable risk groups based on genetic and molecular findings at time of diagnosis relapse due to the re-expansion of malignant clones that are resistant to treatment. The amount of resistant malignant cells present at different time points during therapy is a major determinant of outcome, and MRD detected by flow cytometry provides prognostic information and helps guide clinical treatment decisions.[2–5] LAIP is commonly defined by lineage infidelity or cross-lineage antigen expression, asynchronous expression of differentiation markers (co-expression of antigens that are not concomitantly present during normal differentiation), lack of antigen or antigen overexpression.[5–7] Change in the intensity of cell marker expression on the leukaemic blasts compared to the normal myeloblasts can also be assessed, but is technically more demanding, hence less commonly used. The 'stability' of LAIP is also an important variable. The intensity of expression of markers that comprise a LAIP may vary between time of diagnosis and follow up, and may lead to a false negative or overestimated result.[1,2] For AML, the use of LAIP in detecting MRD is complicated by the phenotypic heterogeneity of normal myeloblasts, which includes very small populations of cells with the LAIPs. These are in fact small subsets of normal myeloblasts at different stages of differentiation.

The identification of LAIPs depends on the panel of surface marker and processing reagents used, settings of the flow cytometer (compensation, laser stability, and resolution), gating strategies and operator experience.[7] For MFC MRD results to be meaningful, the definition of LAIPs needs to be standardised, and the sensitivity and specificity of the analytical assay should be determined.

Sensitivity refers to the ability of the MFC analysis to correctly identify cells with LAIPs and this depends on the percentage of blasts that bear the aberrancy at diagnosis, the number of cells counted and the antibody markers used. Sensitivity of 0.1% is routinely achieved but lower levels down to 0.01% may be attained.[3,5]

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Specificity of a LAIP depends on the frequency of expression of the aberrant markers on normal myeloid progenitors. The lower the frequency of background LAIP expression, the higher the specificity.[1–4] There are limited studies reported in the literature regarding the proportion of normal bone marrow blasts which express LAIP. Here we report the findings from flow cytometric analysis of myeloid blasts collected from 14 patients with normal or reactive marrows.

MATERIALS AND METHODS

Flow cytometry analysis was performed on the residual normal marrow aspirate specimens obtained from 14 adult patients after the intended investigations were completed. These analyses were carried out as standard test development/validation and test improvement. The residual specimens were de-identified in accordance with the institutional policy on use of residual patient specimens. Patient informed consent and formal ethics approval are not required for this purpose according to the New Zealand Human Tissue Act 2008.

Samples were collected in EDTA tubes and processed within 24 hours of collection. Samples were pre-lysed as described by Ogata *et al.*[8] and stained using the following reagent combinations:

Tube 1 – CD15 / CD117 / CD45 / CD34.
Tube 2 – CD4 / CD56 / CD45 / CD34.
Tube 3 – CD11b / CD7 / CD45 / CD34.

Fluorochromes and clones for each marker are summarised in Table 1.

The bone marrow samples were processed individually and analysed separately. No data files were merged. Data were acquired using a FacsCanto cytometer (Becton Dickinson, USA). At least 100,000 cells with the exception of one case were acquired and analysed using CellQuest software (median 197,167, mean 185,285, range 85,791–262,813).

The blast population was defined as clustered events following Boolean gating performed as previously described by Ogata *et al.*[8] and illustrated in Fig. 1. Briefly, on the forward scatter (FSC) versus side scatter (SSC) display, R1 was set to delimit all bone marrow nucleated cells. All cells with relatively low SSC were selected (R2). The mononuclear cells were then plotted on a CD45 versus CD34 display, and CD34+ cells with intermediate CD45 expression were gated (R3). The blasts in gate R3 were plotted on a CD45 versus SSC display allowing B cell progenitors to be excluded as an easily identifiable cluster and the remainder of CD34+ cells consistent with CD34+ myeloblasts (R4).

The percentage of CD34+ myeloblasts from all CD45+ cells was determined and expressions of asynchronous (CD15, CD11b) or lineage infidelity (CD4, CD7, and CD56) markers on myeloid blasts were assessed. The mean and median percentage of LAIP positive myeloid blasts present in normal bone marrow samples was calculated.

RESULTS

The 14 bone marrow biopsies were taken from seven male and seven female patients with a median age of 58 years (mean 56 years, range 17–85 years). The bone marrow examinations were performed for investigation of anaemia (n=3), splenomegaly of unknown cause (n=1), staging of patients with lymphoma (n=9) and the evaluation of plasma cell burden in a patient with monoclonal gammopathy of undetermined significance (n=1). All 14 marrow samples were morphologically normal or showing only reactive features.

Myeloblasts constituted on average 1.136% (range 0.137–2.800%) of CD45+ cells in the marrow samples which is within the published normal ranges.[9,10] Myeloblasts with LAIP were present in 71–100% of the bone marrow samples, though the percentage of myeloblasts with each aberrancy differed (see Table 2).

Table 3 summarises the size of the normal myeloblast population with LAIP. At the usual MRD sensitivity cut-off of 0.01%[11,12] LAIP expression was detected on 43–100% of normal myeloblasts.

Even if the threshold for MRD positivity is raised to 0.1% as has been suggested[1] and generally used in studies as a clinically relevant threshold for AML MRD, asynchronous CD15 or aberrant CD7 expression on the normal myeloblasts is still present in the majority of cases (86% and 64%, respectively). This indicates there is a significant risk of a false positive result using these as the MRD markers (see Fig. 2).

Table 4 compares the results of this study with the published findings of normal myeloblasts with LAIP from selected studies. The studies including this one showed presence of LAIP on normal myeloblasts, but the findings were derived from a variable number of normal marrows, with marked variation of results.

DISCUSSION

MRD is an important prognostic factor in the outcome of AML in both adults and children.[1–5,13–15] Current AML studies are underway to incorporate MRD into AML treatment to optimise the outcome. Optimal methods for MRD detection must have specificity for the leukaemia cell population and be sensitive enough to detect a small number of leukaemic cells in a background of normal cells.[16] It is beyond the scope of this article to discuss MRD detection techniques in detail, and the reader is referred to recent excellent reviews.[4,5]

Assessment of bone marrow morphology has long been considered the standard approach to evaluate treatment response in AML.[6] However, inter-observer variability and limitations of light microscopy renders the sensitivity of this method low.[12] Cytogenetic analysis/karyotyping is useful if a cytogenetic abnormality is detected at diagnosis; however, the sensitivity is relatively low and the method requires

Table 1 Details of immunophenotyping reagents used

Phenotypic marker	Fluorochrome	Clone	Supplier catalogue number
CD15	FITC	MMA	BD347323
CD117	PE	104D2	BD340529
CD45	PerCP	2D1	BD347464
CD34	APC	8G12	BD340933
CD4	FITC	SK3	BD340133
CD56	PE	MY31	BD347747
CD11b	FITC	D12	IM0530
CD7	PE	8H8.1	IM14294

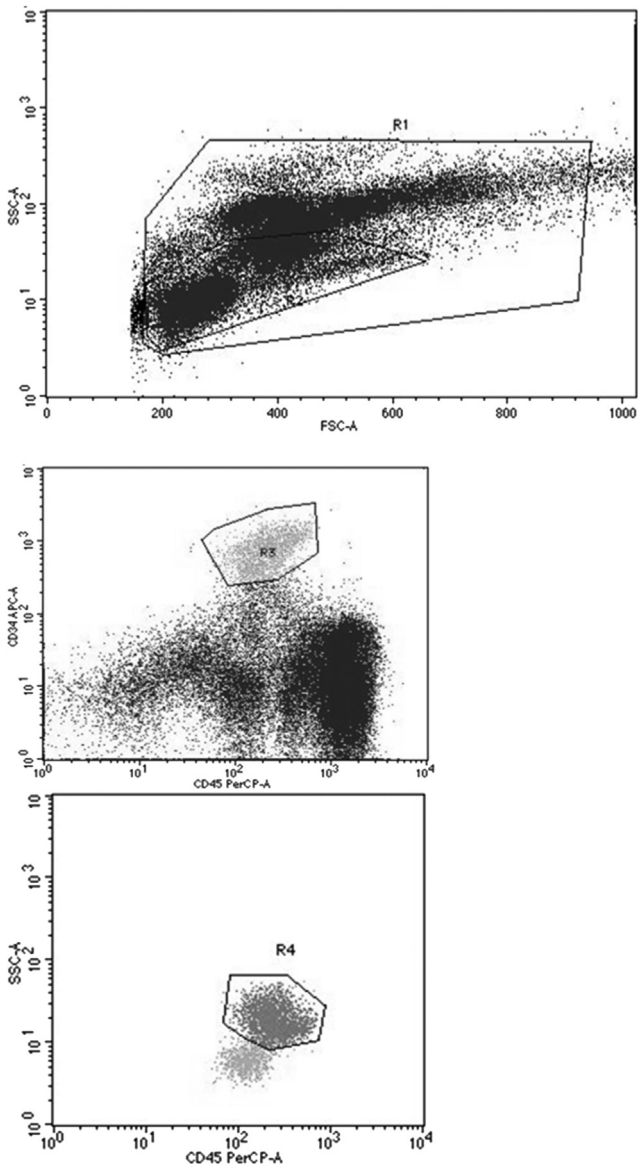

Fig. 1 Gating strategy. Bone marrow nucleated cells were identified on a FSC × SSC dot plot (R1). Low SSC MNC were gated (R2), and plotted on a CD45 × CD34 dot plot. CD34+ CD45 dim cells were gated (R3), and plotted on a CD45 × SSC dot plot. CD34+ myeloblasts were differentiated from B cells progenitors based on their relatively high SSC (R4). Co-expression of the following markers was examined on the CD34+ myeloblasts: CD15, CD11b, CD4, CD7 and CD56.

dividing cells in order to generate metaphase spreads, which can be challenging.[5,16] Fluorescent *in situ* hybridisation (FISH) is more sensitive than karyotyping (10^{-2}) and does not need cells to be dividing; however, this method is only applicable if a cytogenetic abnormality is present.[5]

Molecular studies such as polymerase chain reaction analysis (PCR) or next generation sequencing (NGS) are useful in detecting recurrent mutational targets in AML. The sensitivity and specificity for molecular techniques is high (10^{-3} to 10^{-5}), though the method is only applicable for 30–60% of AML in which an informative leukaemia-specific molecular marker is identified.[4–6,12]

Aberrant expression of surface antigens (LAIP) is common in leukaemic blasts at diagnosis. LAIP provides a marker for MRD assessment and multicolour flow cytometry detects both surface and intracellular markers with sensitivities ranging from 10^{-3} to 10^{-5} and is applicable to >90% of cases.[5,12,16,17]

Two approaches to MRD assessment by MFC are currently employed, although in reality the dichotomy is artificial. The first approach, which is still being commonly used, relies on the identification of LAIP on myeloid blasts at the time of AML diagnosis. Patient-specific antibody panels can then be constructed for post-treatment evaluation, with MFC tracking the persistence or disappearance of the diagnostic LAIPs. This approach is limited by the stability of the LAIP and the phenomenon of 'immunophenotypic shift', i.e., changes in antigen expression which can occur due to instability in the original leukaemic clone, expansion of a pre-existing small subclone and/or emergence of a new clone. The immunophenotypic changes can occur due to new mutations or be due to drug effect.[4,5,18]

The second approach is identifying blasts that are 'different from normal' (DFN). This focuses on normal blasts and the subsequent identification of blasts which deviate from normal. The approach relies on the observation that when myeloid cells mature, the change in immunophenotype is very consistent, both in the array of antigens expressed and the intensity of antigen expressions. The consistency of antigen expression, including the intensity of expression, is disturbed in leukaemic blasts, providing a platform to distinguish leukaemic blasts from normal blasts. The DFN approach requires access to a 'normal template', derived from the full characterisation of normal cells. Such a template should ideally take into consideration the presence of LAIP on normal myeloblasts. Using the 'normal template', phenotypic result from MRD assessment can be superimposed, enabling assessment of whether or not the immature population detected is 'different from normal', hence residual leukaemic blasts.[5,6,12]

Both the LAIP and DFN approaches require a solid and accurate database of what is normal as well as the use of standardised panels and analytical methods to allow inter-laboratory comparability. There are limited published data on normal myeloblasts expressing LAIP, and to date a standardised normal template of myeloid cell maturation is not available despite several groups working on this.

Our results demonstrate that the definition of aberrant markers on myeloid blasts is not clear cut. The percentages of normal myeloid blasts expressing LAIP involving the markers used in our study and that are above the MCF detection sensitivity of 0.01% range from 43% to 100%. This significantly limits the specificity of the results for AML MRD assessment. Even if the threshold for MRD positivity is raised to 0.1%, there will still be false positive cases if CD15 or CD7 are used as aberrant markers. The large differences in specificity are problematic when cut-off levels have to be defined which alter risk group stratification and prognostication of individual patients and thus impact on clinical decision making and patient management.[11]

There is only limited published information on background LAIP expression on normal myeloblasts. Kern *et al.*[17] reported the findings on normal bone marrow cells carrying a LAIP from MFC analysis of 26 normal adult bone marrow samples. The results showed aberrant expression of CD15, CD11b, CD7 and CD56 at a level ranging from 0.0% to 0.47% in the majority of samples analysed. However, a study by Al-Mawali *et al.*[19] showed a much lower level for the same markers. A smaller data set for paediatric population

Table 2 LAIP expression on normal CD34+ myeloid blasts

	CD15	CD11b	CD4	CD7	CD56
No. cases with aberrant marker	14/14 (100%)	13/14 (93%)	10/14 (71%)	14/14 (100%)	10/14 (71%)
Mean	18.36%	1.65%	1.13%	9.93%	1.61%
Median	19.88%	1.21%	1.04%	8.13%	1.52%
Range	6.75–25.15%	0.16–4.33%	0.72–2.00%	3.37–23.78%	0.45–3.32%

LAIP, leukaemia-associated immunophenotypes.

Table 3 Normal CD34+ myeloid blasts expressing LAIP as percentage of CD45+ cells and above cut-off levels commonly used for MRD detection

	CD15+	CD11b+	CD4+	CD7+	CD56+
No. with LAIP at any level	14/14 (100%)	13/14 (93%)	10/14 (71%)	14/14 (100%)	10/14 (71%)
>0.01%	14/14 (100%)	8/14 (57%)	6/14 (43%)	14/14 (100%)	8/14 (57%)
>0.1%	12/14 (86%)	0	0	9/14 (64%)	0

LAIP, leukaemia-associated immunophenotypes; MRD, measurable residual disease.

showed aberrant expression of the same markers present at levels ranging from 0.01% to 0.09%,[20] and further data has been extracted from a study by Xu *et al.*,[21] in which immunopenotypes of normal bone marrow myeloid blasts were evaluated as part of development of benchmark/reference values. Table 4 summarises the findings in these papers including the results from this study. The cause of the difference is not immediately clear but can possibly be from the lack of standardisation of the analysis procedures, including the pre-analytical preparation of the specimen (washing, lysing, staining), choice of reagents and fluorochrome, instrument setting, number of events analysed, and gating and analysis algorithm.

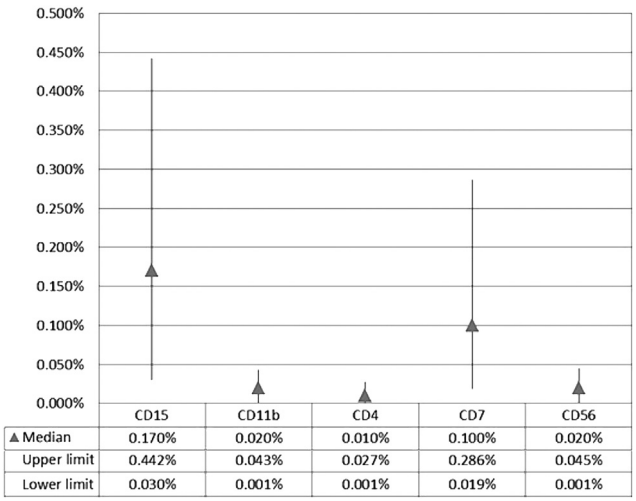

Fig. 2 Normal CD34+ myeloid blasts with aberrant marker expression as a percentage of all CD45+ cells.

Our study provided useful information for AML MRD determination in our laboratory based on a limited number of LAIPs. The work was based on four-colour flow analysis, which is a limitation of this study, using standard approach and software available in many flow cytometry laboratories. The limited number of markers used in the four-colour flow analysis does not allow for finer definition of LAIP expression through co-expression of aberrant markers, which can be determined through actual high order flow analysis or virtual high order analysis through data merging. The combination of high order flow analysis with data merging that can be carried out in advanced software such as Infinicyt (Cytognos, Spain) originally developed by the EuroFlow consortium, has opened up MCF into what has been termed 'next generation flow cytometry' with sensitivity approaching molecular analysis, and can be particularly useful for research and clinical studies. The four-colour flow cytometry approach which is still used in many clinical laboratories, however, can be performed in more clinical flow laboratories, is still being standardised across laboratories,[22] and should provide useful information for clinical

Table 4 Comparison of LAIP expression in normal bone marrow myeloblasts

LAIP	This study		van der Velden *et al.*[20]		Kern *et al.*[17,a]		Al Mawali *et al.*[19]		Xu *et al.*[21,a]	
	Median (range)	*n*	Median (range)	*n*	Median (range)	*n*	Median (range)	*n*	Benchmark (mean + 2 SD)	*n*
CD15+	0.17 (0.03–0.442)	14	0.02 (<0.01–0.03)	3	0.02 (0.00–0.09)	26	0.004 (0.00–0.024)	10	0.651	21
CD11b+	0.02 (0.001–0.043)	14	0.06 (0.05–0.09)	3	0.33 (0.01–0.81)	26	0.002 (0.00–0.022)	10	0.15	21
CD7+	0.1 (0.019–0.286)	14	0.01 (<0.01–0.04)	3	0.09 (0.02–0.09)	26	0.002 (0.00–0.009)	10	N/A	
CD56+	0.02 (0.001–0.045)	14	0.02 (<0.01–0.04)	3	0.02 (0.00–0.17)	26	0.001 (0.00–0.039	10	0.178	21
CD4+	0.01 (0.001–0.027)	14	0.01 (<0.01–0.07)	3	N/A		N/A		0.215	21

LAIP, leukaemia-associated immunophenotypes; SD, standard deviation.
[a] Modified from the published paper.

patient management. A database of LAIP expression on normal myeloid blasts, ideally from multicentre collaboration using standardised flow protocol including maturation markers used, antibody clones, fluorochromes, and preparation and analysis steps, will enhance the use of this approach in determining the optimal diagnostic cut-off for AML MRD for everyday patient management. This can also contribute to the DFN analysis in improving the sensitivity and specificity of AML MRD detection.

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References

1. Al-Mawali A, Gillis D, Lewis I. The role of multiparameter flow cytometry for detection of minimal residual disease in acute myeloid leukaemia. *Am J Clin Pathol* 2009; 131: 16–26.
2. Freeman SD, Virgo P, Couzens S, *et al*. Prognostic relevance of treatment response measured by flow cytometric residual disease detection in older patients with acute myeloid leukaemia. *J Clin Oncol* 2013; 31: 4123–31.
3. Feller N, van der Pol MA, van Stijn A, *et al*. MRD parameters using immunophenotypic detection methods are highly reliable in predicting survival in acute myeloid leukaemia. *Leukaemia* 2004; 18: 1380–90.
4. Schuurhuis GJ, Heuser M, Freeman S, *et al*. Minimal/measurable residual disease in AML: consensus document from ELN MRD Working Party. *Blood* 2018; 131: 1275–91.
5. Ravandi F, Walter RB, Freeman SD. Evaluating measurable residual disease in acute myeloid leukaemia. *Blood Adv* 2018; 2: 1356–66.
6. Zeijlemaker W, Gratama JW, Schuurhuis GJ. Tumour heterogeneity makes AML a "moving target" for detection of residual disease. *Cytometry Part B* 2014; 86B: 3–14.
7. Slobodnyuk K. Detection of minimal residual disease in patients with acute myeloid leukaemia with multicolour flow cytometry. *Cell Ther Transplant* 2011; 3: 1–4.
8. Ogata K, Della Porta MG, Malcovati L, *et al*. Diagnostic utility of flow cytometry in low-grade myelodysplastic syndromes: a prospective validation study. *Haematologica* 2009; 94: 1066–74.
9. van de Loosdrecht AA, Alhan C, Bene MC, *et al*. Standardization of flow cytometry in myelodysplastic syndromes: report from the first European Leukaemia Networking conference on flow cytometry in myelodysplastic syndromes. *Haematologica* 2009; 94: 1124–34.
10. Brooimans R, Kraan J, van Putten W, *et al*. Flow cytometric differential of leukocyte populations in normal bone marrow: influence of peripheral blood contamination. *Cytometry Part B* 2009; 76B: 18–26.
11. Feller N, van der Velden VH, Schuurhuis GJ. Defining consensus leukaemia-associated immunophenotypes for detection of minimal residual disease in acute myeloid leukaemia in a multicenter setting. *Blood Cancer J* 2013; 3: e129.
12. Grimwade D, Freeman S. Defining minimal residual disease in acute myeloid leukaemia: which platforms are ready for "prime time"? *Blood* 2014; 124: 3345–55.
13. Chen X, Xie H, Wood BL, *et al*. Relation of clinical response and minimal residual disease and their prognostic impact on outcome in acute myeloid leukaemia. *J Clin Oncol* 2015; 33: 1258–64.
14. Loken M, Alonzo T, Pardo L, *et al*. Residual disease detected by multidimensional flow cytometry signifies high relapse risk in patients with de novo acute myeloid leukaemia: a report from Children's Oncology Group. *Blood* 2012; 120: 1581–8.
15. Tierens A, Bjorklund E, Siitonen S, *et al*. Residual disease detected by flow cytometry is an independent predictor of survival in childhood acute myeloid leukaemia; results of the NOPHO-AML 2004 study. *Br J Haematol* 2016; 174: 600–9.
16. McCann SR, Lawler M. The value of molecular monitoring in haematological malignancy; minimal residual disease (MRD), relapse and chimerism. In: Apperley J, Carreras E, Gluckman E, Gratwohl A, Masszi T, editors. *Hematopoietic Stem Cell Transplantation*. 5th ed. Paris: ESH-EBMT, 2008.
17. Kern W, Danhouser-Riedl S, Ratei R, *et al*. Detection of minimal residual disease in unselected patients with acute myeloid leukaemia using multiparameter flow cytometry to define leukaemia-associated immunophenotypes and determine their frequencies in normal bone marrow. *Haematologica* 2003; 88: 646–53.
18. Jaso JM, Wang SA, Jorgensen JL, Lin P. Multi-colour flow cytometric immunophenotyping for detection of minimal residual disease in AML: past, present and future. *Bone Marrow Transpl* 2014; 49: 1129–38.
19. Al-Mawali A, Gillis D, Hissaria P, Lewis I. Incidence, sensitivity, and specificity of leukaemia-associated phenotypes in acute myeloid leukaemia using specific five-color multiparameter flow cytometry. *Am J Clin Pathol* 2008; 129: 934–45.
20. Van der Velden VH, van der Sluijs-Geling A, Gibson BE, *et al*. Clinical significance of flowcytometric minimal residual disease detection in paediatric acute myeloid leukaemia patients treated according to the DCOG ANLL97/MRC AML12 protocol. *Leukaemia* 2010; 24: 1599–606.
21. Xu F, Li X, Wu L, *et al*. Flow cytometric scoring system (FCMSS) assisted diagnosis of myelodysplastic syndromes (MDS) and the biological significance of FCMSS-based immunophenotypes. *Br J Haematol* 2010; 149: 587–97.
22. Brooimans RA, van der Velden VHJ, Boeckx N, *et al*. Imunophenotypic measurable residual disease (MRD) in acute myeloid leukemia: is multicentric MRD assessment feasible? *Leuk Res* 2019; 76: 39–47.