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Research paper

Differential expression of CD73, CD86 and CD304 in normal vs. leukemic B-cell precursors and their utility as stable minimal residual disease markers in childhood B-cell precursor acute lymphoblastic leukemia

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ABSTRACT

Background: Optimal discrimination between leukemic blasts and normal B-cell precursors (BCP) is critical for treatment monitoring in BCP acute lymphoblastic leukemia (ALL); thus identification of markers differentially expressed on normal BCP and leukemic blasts is required.
Methods: Multicenter analysis of CD73, CD86 and CD304 expression levels was performed in 282 pediatric BCP-ALL patients vs. normal bone marrow BCP, using normalized median fluorescence intensity (nMFI) values.
Results: CD73 was expressed at abnormally higher levels (vs. pooled normal BCP) at diagnosis in 71/108 BCP-ALL patients (66%), whereas CD304 and CD86 in 119/202 (59%) and 58/100 (58%) patients, respectively. Expression of CD304 was detected at similar percentages in common-ALL and pre-B-ALL, while found at significantly lower frequencies in pro-B-ALL. A significant association ($p = 0.009$) was found between CD304 expression and the presence of the *ETV6-RUNX1* fusion gene. In contrast, CD304 showed an inverse association with *MLL* gene rearrangements ($p = 0.01$). The expression levels of CD73, CD86 and CD304 at day 15 after starting therapy (MRD15) were stable or higher than at diagnosis in 35/37 (95%), 40/56 (71%) and 19/41 (46%) cases investigated, respectively. This was also associated with an increased mean nMFI at MRD15 vs. diagnosis of +24 and +3 nMFI units for CD73 and CD86, respectively. In addition, gain of expression of CD73 and CD86 at MRD15 for cases that were originally negative for these markers at diagnosis was observed in 16% and 18% of cases, respectively. Of note, CD304 remained aberrantly positive in 63% of patients, despite its levels of expression decreased at follow-up in 54% of cases.
Conclusions: Here we show that CD73, CD86 and CD304 are aberrantly (over)expressed in a substantial percentage of BCP-ALL patients and that their expression profile remains relatively stable early after starting therapy, supporting their potential contribution to improved MRD analysis by flow cytometry.

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1. Introduction

Acute leukemias (AL) are a heterogeneous group of hematological malignancies, characterized by the neoplastic expansion of immature precursor cells (blasts) of lymphoid, myeloid or mixed lymphoid/myeloid lineages (Jaffe et al., 2017). The most frequent type of AL in children is acute lymphoblastic leukemia (ALL) which results from the malignant transformation of B- or T- lymphoid precursor cells. Among the two subtypes, B-cell-precursor (BCP) ALL is more prevalent and represents around 85% of all pediatric ALL patients (Mirkowska et al., 2013; Onciu, 2009; Szczepański et al., 2003). BCP (morphologically recognized as hematogones) represent a normal bone marrow (BM) cell compartment which typically forms a continuous maturation pathway consisting of three major maturational stages, all of which show CD19 expression: pre-B-I-, pre-B-II- and immature/transitional B-cells, respectively. Leukemic blasts in BCP-ALL share many phenotypic features with normal BCP (Lucio et al., 1999). Despite this, the expression levels of BCP-associated antigens commonly assessed in BCP-ALL (e.g. CD10, CD20, CD34, TdT, CD22, CD38, CD45) are frequently altered at variable degree, as detected by multiparameter flow cytometry (FC) (Sędek et al., 2014; van Dongen et al., 2015). In the past decades, it has been shown that immunophenotypic differences between normal BCP and leukemic blasts -(i.e., the so-called leukemia-associated immunophenotypes (LAIPs)-, can be found in virtually all (~98%) BCP-ALL patients (Campana, 2009). Despite such high frequency, these differences may be either very subtle or modulated by further which may induce either up- or downregulation of unstable markers such as CD10, CD20 and CD34 (Dworzak et al., 2010; Gaipa et al., 2005; Slamova et al., 2014). Furthermore, administration of different types of chemotherapy might also induce significant and variable changes in the proportion of normal BCP in BM, which might increase the difficulty in distinguishing small numbers of normal regenerating BCP from persisting leukemic cells – minimal residual disease (MRD) (Gaipa et al., 2013; Mejstrikova et al., 2010; Sędek et al., 2014). Proper FC-based distinction between leukemic blasts and normal BCP is critical for reliable treatment decisions in BCP-ALL, which justifies the need for identifying new, stable markers which are 1) differentially expressed on leukemic blasts vs. normal BCP in a significant fraction of BCP-ALL patients, and 2) that are persistently aberrant during/after therapy (van Dongen et al., 2015).

Based on the screening of the leukemia cell surface proteome (Mirkowska et al., 2013) and genome-wide gene expression vs. normal BCP (Coustan-Smith et al., 2011), novel candidate markers for more efficient MRD monitoring in ALL have been recently identified. Here, we evaluated i) the utility of three of such candidate markers (CD73, CD86 and CD304) to discriminate leukemic blasts from normal BCP, and ii) their stability early after starting therapy (day +15 of induction therapy), and thereby, their potential utility for MRD monitoring in BCP-ALL. For this purpose, we performed a multicenter, standardized FC validation study based on the analysis of expression of these three markers on leukemic blasts from a large series of pediatric patients with different immunological and genetic subtypes of BCP-ALL vs. normal/reactive BM BCP.

2. Materials and methods

2.1. Patients and controls

The overall study group comprised 282 children (male/female ratio of 1.06; median age of 4 years) with newly-diagnosed BCP-ALL at 6 different pediatric hemato-oncology centers that are members of the EuroFlow consortium: Medical University of Silesia in Katowice (SUM, Zabrze, Poland), Erasmus Medical Center (EMC, Rotterdam, The Netherlands), Dutch Childhood Oncology Group (DCOG, The Hague, The Netherlands), Federal University of Rio de Janeiro (UFRJ, Rio de Janeiro, Brazil), Charles University (CU, Prague, The Czech Republic) and University of Milan-Bicocca (UMB, Monza, Italy). The entire study, including data analysis strategies and interpretation of the results was collectively designed by all involved EuroFlow centers, during regular EuroFlow meetings. All involved centers stained and provided FCS 3.0 data files of BCP-ALL patients, as well as normal BM samples which were analyzed centrally at SUM. The potential association between the expression profiles observed for each marker evaluated and both the different EGIL BCP-ALL subtypes (i.e., pro-B-, common- and pre-B –ALL) and distinct genetic subgroups of BCP-ALL (e.g., *MLL, ETV6-RUNX1*, *BCR-ABL1*, *TCF3-PBX1* gene rearrangements, hyperdiploidy and hypodiploidy) was also investigated. Not all studied antigens were assessed in every patient (Table 1) and genetic data was also not available for every case, which explains differences in the total number of cases per specific genetic subgroup (Table 2). A control group of 19 BM samples from children with benign hematologic conditions such as thrombocytopenia and neutropenia, subjected to BM aspiration for diagnostic purposes at all involved centers, was studied in parallel.

Table 1
Overall expression profile of CD73, CD86 and CD304 on leukemic BCP-ALL blasts at diagnosis expressed both as nMFI values and classical 3-step notation (square parentheses).

	CD73	CD86	CD304
Marker expression notation	(n = 108)	(n = 100)	(n = 202)
Negative (nMFI: 0–1) [negative]	37 (34%)	42 (42%)	83 (41%)
Overall positive (nMFI ≥ 2)	71 (66%)	58 (58%)	119 (59%)
Heterogeneous-negative-to-dim expression (nMFI: 2–3) [dim]	27 (25%)	28 (28%)	41 (20%)
Dim expression (nMFI: 4–6) [dim]	16 (15%)	13 (13%)	22 (11%)
Strong/bright expression (nMFI: 7–10) [bright]	12 (11%)	9 (9%)	22 (11%)
Overexpression (nMFI ≥ 11) [bright]	16 (15%)	8 (8%)	34 (17%)

Results expressed as number of cases (percentage).

The current study was approved by the local ethics committees and informed consent was given by each child and/or their parents/guardians, following the Declaration of Helsinki.

2.2. Sample preparation and quality control

BM samples were subjected to the EuroFlow standardized stain, lyse and wash sample preparation protocols (van Dongen et al., 2012) and stained with a 2-tube, 8-color panel of fluorochrome-conjugated mouse anti-human antibodies, containing a backbone of 6 common markers: CD34/PerCP-Cy5.5, clone 8G12 (Becton Dickinson Biosciences (BD), San Jose, CA, USA); CD19/PE-Cy7, clone J3-119 (Beckman Coulter, Brea, CA, USA); CD10/APC, clone HI10a (BD); CD38/APC-Ax750, clone LS198-4-3 (Beckman Coulter); CD20/Pacific Blue, clone 2H7 (Biolegend, San Diego, CA, USA); and CD45/Pacific Orange, clone HI30 (Life Technologies, Carlsbad, CA, USA). Additionally, tube 1 contained the CD86/FITC, clone FUN-1 (BD) and CD304/PE, clone 12C2 (Biolegend) antibody reagents, while tube 2 included the CD73/PE, clone AD2 (BD) antibody. Erythrocyte lysis was performed after staining the samples, using the FACS Lyse solution (BD), according to the recommendations of the manufacturer. Stained samples were measured in FACSCanto II or LSR II flow cytometers (BD) available at each participating center, which had been set-up and calibrated following the standardized EuroFlow guidelines and protocols (Kalina et al., 2015; Kalina et al., 2012). During the study, each flow cytometer was subjected to daily quality assessment using fluorescent beads of the same lot (Sphero Rainbow Calibration Particles, Spherotech, Lake Forest, IL, USA) and the EuroFlow guidelines for monitoring instrument performance, in order to ensure the reproducibility of the obtained stainings (Kalina et al., 2012). In addition, all contributing centers were subjected to regular external quality assessment (EQA) trials for standardized instrument settings, reagent panels, and sample preparation protocols during the entire period of the study, which ensured high degree of

Table 2
Expression of CD73, CD86 and CD304 in different genetic subgroups of BCP-ALL.

Genetic subgroup	CD73-pos (%)	CD73-pos median nMFI (range)	CD86-pos (%)	CD86-pos median nMFI (range)	CD304-pos (%)	CD304-pos median nMFI (range)
MLL-rearranged						
Neg	50/74 (68%)	5.5 (2–49)	46/77 (60%)	3 (2–24)	93/152 (61%) *	7 * (2–60)
Pos	2/6 (33%)	2.5 (2–3)	5/7 (71%)	7 (5–44)	2/9 (22%) *	3 * (2–4)
ETV6-RUNX1						
Neg	37/58 (64%)	6 (2–49)	40/62 (65%)	5 (2–44)	62/117 (53%) *	5 * (2–60)
Pos	12/18 (67%)	3.5 (2–15)	5/8 (63%)	2 (2–9)	21/25 (84%) *	7 * (2–26)
BCR-ABL1						
Neg	52/80 (65%)	5 (2–49)	48/81 (60%)	4 (2–44)	92/157 (59%)	6 (2–60)
Pos	0 (0%)	–	3/3 (100%)	3 (2 – 10)	3/3 (100%)	10 (4–14)
TCF3-PBX1						
Neg	44/64 (69%)	4.5 (2–49)	33/52 (64%)	3 (2–44)	70/116 (60%)	7 (2–60)
Pos	0/2 (0%)	–	0 (0%)	–	0/2 (0%)	–
Hyperdiploidy						
Neg	27/44 (61%)	5 (2–34)	25/47 (53%)	3 (2 – 20)	57/93 (61%)	8 (2 – 33)
Pos	17/25 (68%)	4 (2–49)	14/18 (78%)	3 (2–44)	24/44 (55%)	4 (2–60)
Hypodiploidy						
Neg	41/67 (61%)	5 (2–49)	37/64 (58%)	3 (2–44)	79/131 (60%)	7 (2–60)
Pos	1/1 (100%)	9 (9)	1/1 (100%)	2 (2)	0/2 (0%)	–

pos – positive, neg – negative; * corrected $p < 0.05$ for both ways of determination of antigen expression between positive vs. negative cases for individual genetic aberrations: nMFI-based and classical 3-step (negative/dim/bright) notation (Mann-Whitney *U* test and Pearson chi-square test, respectively). Results expressed as number of cases /total cases (percentage) unless otherwise specified.

comparability of the data generated (Kalina et al., 2015). For data analysis the Infinicyt software was used (Cytognos SL, Salamanca, Spain).

2.3. Determination of reference populations and calculation of antigen expression levels in nMFI units

The levels of expression of CD73, CD86, CD304, CD34, CD10 and CD20 were assessed on leukemic blasts, the three major stages of maturation of normal BCP (pre-B-I, pre-B-II and immature B-cells) and mature B/T/NK lymphocytes, and results expressed as normalized MFI (nMFI) values, calculated as previously reported (Sędek et al., 2014) and further summarized below. Antigen-specific negative and positive reference cell populations (internal controls) were defined as follows: i) positive reference cell populations for CD73, CD86 and CD304 were the subset of CD73-positive mature B lymphocytes, plasma cells (PC) and plasmacytoid dendritic cells (pDC), respectively; and ii) mature B/T/NK lymphocytes and the CD73-negative subset of B/T/NK lymphocytes were used as negative reference cell populations for CD86, CD304 and CD73, respectively (Fig. 1 and Table 3). Reference cell populations for CD34, CD10 and CD20 were selected as previously reported (Sędek et al., 2014): i) positive reference cell populations included pre-B-I cells for CD34 and CD10, and immature B-cells for CD20, while ii) mature B/T/NK lymphocytes were used as the negative reference cell population for all three markers (Sędek et al., 2014).

To calculate nMFI values, the median MFI of the negative reference populations was subtracted from the median MFI of the corresponding

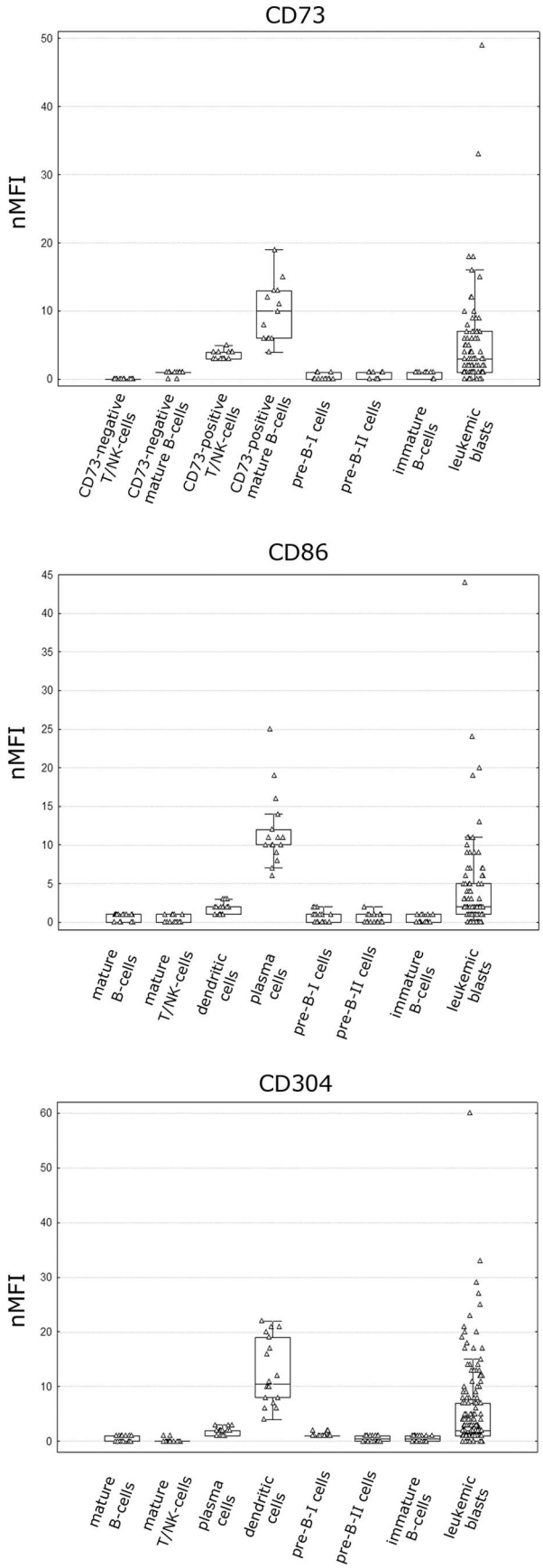

Fig. 1. Normalized median fluorescence intensity (nMFI)-based expression levels of CD73, CD86 and CD304 on normal BM cells vs. BCP-ALL leukemic blasts.

Table 3
Pattern of expression of CD73, CD86 and CD304 on different subsets of normal bone marrow cells.

	CD73		CD86		CD304	
	MFI range	Median nMFI (IQR)	MFI range	Median nMFI (IQR)	MFI range	Median nMFI (IQR)
Mature B-cells	N/A	N/A	**118–159**	**1 (0–1)**	**20–94**	**0 (0–1)**
T/NK cells	N/A	N/A	**107–151**	**0 (0–1)**	**19–49**	**0 (−)**
CD73-negative T/NK-cells	**20–44**	**0 (−)**	N/A	N/A	N/A	N/A
CD73-negative mature B-cells	**44–128**	**1 (−)**	N/A	N/A	N/A	N/A
CD73-positive T/NK-cells	366–699	3 (3–4)	N/A	N/A	N/A	N/A
CD73-positive mature B-cells	**656–3047**	**10 (6–13)**	N/A	N/A	N/A	N/A
Plasma cells (PC)	N/A	N/A	**553–1063**	**10 (10−12)**	38–256	2 (1–2)
Plasmacytoid dendritic cells (pDC)	N/A	N/A	157–264	2 (−)	**364–1968**	**10 (8–18)**
Pre-B-I cells	16–50	0 (0–1)	112–225	1 (0–1)	44–161	1 (−)
Pre-B-II cells	36–143	1 (0–1)	99–210	0 (0–1)	17–83	0.5 (0–1)
Immature B-cells	34.8–178.9	1 (0–1)	102.5–158.0	0 (0–1)	21.6–73.8	0.5 (0–1)

MFI – median fluorescence intensity, IQR – interquartile range. Negative and positive reference cell populations for each of the three markers investigated are highlighted in bold.

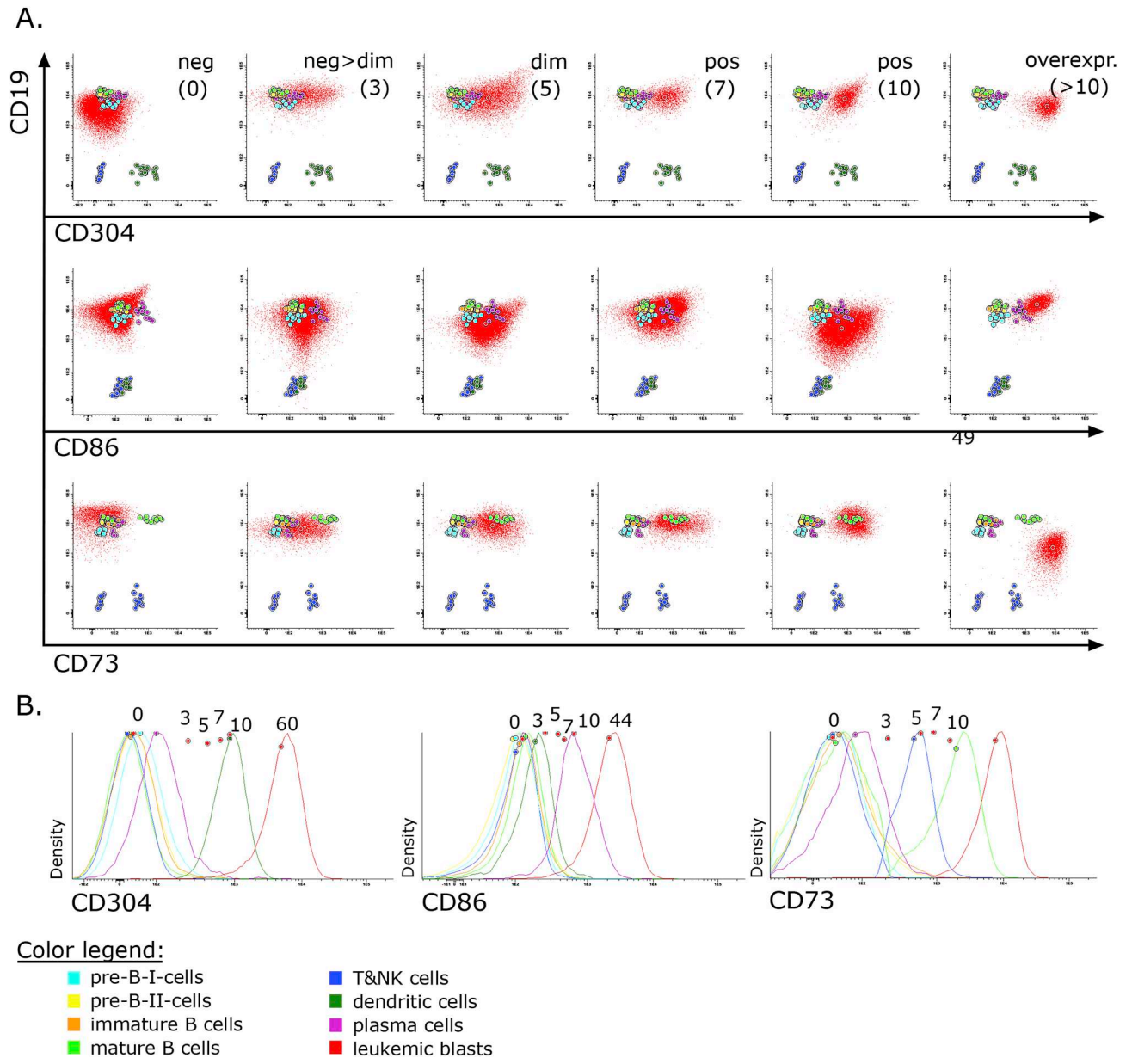

Fig. 2. Illustrating bivariate dot plots (A) and single parameter histograms (B) of the different patterns of expression (nMFI scores) of CD73, CD86 and CD304 observed on BCP-ALL blast cells. Red dots in panels A and B represent BCP-ALL blasts of different nMFI scores on the background of different reference cell types; for clarity, the histograms in panel B were drawn only for the highest nMFI values of BCP-ALL blasts. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

positive reference populations, per marker. Subsequently, the median MFI value obtained for each marker for the negative reference cell population was assigned an nMFI score of 0, while the median MFI value obtained for the corresponding positive reference cell population was arbitrarily assigned an nMFI score of 10. Then, the MFI range of values between those of the negative (0) and positive (Dworzak et al., 2010) reference cell populations, were divided into 10 equal (linear scale) intervals, corresponding to a single step on the nMFI scale. Subsequently, nMFI scores of 0–1 were classified as negative (absence of) expression, nMFI values of 2–3 were equivalent to heterogeneous negative-to-dim expression, and nMFI scores of 4–6 and 7–10 were classified as dim and strong positive expression levels, respectively; nMFI values > 10 reflected antigen overexpression levels vs. the corresponding positive reference cell population (Fig. 2). In parallel, a classical 3-step semi-quantitative annotation was used for determination of antigen expression levels, where cases with heterogeneous negative-to-dim and dim expression were classified altogether as "dim" expression and cases with strong expression and overexpression were classified as having "bright" antigen expression levels (Table 1).

2.4. Stability of CD73, CD86, CD304, CD34, CD10 and CD20 at early follow-up time points

CD73 (n = 37), CD86 (n = 56), CD304 (n = 41), CD34 (n = 55), CD10 (n = 56), CD20 (n = 53) were each evaluated for the stability of their expression levels on leukemic blasts at day +15 of induction therapy (MRD15) vs. initial diagnosis (Dx). For this purpose, absolute difference in nMFI values was assessed in paired MRD15/Dx cases, and mean differences in nMFI calculated per marker and patient. Additionally, the rates of loss of antigen expression at MRD15 (for cases that were positive at Dx), gain of expression at MRD15 (for cases that were negative at Dx) and persistently negative cases (both at MRD15 and Dx) were calculated (Fig. 5, Table 4 and Supplementary Fig. 1).

2.5. Statistical methods

The non-normal distribution of nMFI values obtained for each marker analyzed was confirmed by the Shapiro-Wilk's test. To determine the statistical significance of differences in the expression levels (nMFI values) of the markers evaluated, within the different immunological and genetic subgroups of BCP-ALL, the Mann-Whitney U test was applied. Corrected p values (Bonferroni test) < 0.05 were considered to be associated with statistical significance. For the expression levels given in the classical 3-step notation (negative/dim/bright), the Pearson chi-square test was used with statistical significance being set at $p \leq 0.05$. All p values cited throughout the text, figure legends and tables are corrected p values. All statistical analyses were performed centrally at the Medical University of Silesia in Katowice (Zabrze, Poland) using the Statistica 8.0 software (Statsoft, Tulsa, OK, USA).

3. Results

3.1. Expression of CD73, CD86 and CD304 on the positive and negative reference cell populations

Among all cell populations identified in normal BM, PC expressed the highest levels of CD86: median nMFI of 10 -(interquartile range (IQR): 10–12)-. These levels were higher than those detected for normal pDC (median nMFI: 2). In contrast, mature lymphocytes (B and T/NK cells) showed very low CD86 expression levels (median nMFI of 1 and 0, respectively); thereby, they were assigned as negative reference cell populations for CD86. As could be expected, the highest CD304 expression levels were found among pDC, for which CD304 (BDCA4) is a lineage-specific marker: median nMFI of 10 (IQR: 8–18). None of the mature BM lymphocytes showed CD304 expression (median nMFI of 0), while dim CD304 expression was observed on PC -median nMFI of 2 (IQR: 1–2)-. Expression of CD73 was observed in a distinct subset of mature B- and T/NK lymphocytes -mean percentage of CD73-positive cells (range) of 74% (41%–88%) and 26% (10%–39%), respectively-, with significantly higher CD73 expression levels on the CD73-positive B-cell subset (median nMFI of 10 (IQR: 6–13) vs. 3 (IQR: 3–4) for the CD73-positive T/NK-cell subset). Based on these results, the subset of CD73-positive mature B-cells was selected as the positive reference population for this marker (Figs. 1 and 2 and Table 3).

3.2. Expression of CD73, CD86 and CD304 on normal BM BCP

Based on the above defined criteria for positivity (nMFI ≥ 2, or dim plus bright expression using the classical notation), normal BCP either did not express or expressed very low levels of the CD73, CD86 and CD304 antigens (Fig. 1). In detail, CD86 and CD304 were expressed at low levels only by pre-B-I cells ($CD34^+$) and only in a fraction of all control BM samples analyzed (median nMFI of 1 for both markers). Pre-B-II and immature B-cells did not express CD86 and CD304 (median nMFI of 0 (IQR: 0–1) and 0.5 (IQR: 0–1), respectively). In contrast, CD73 was expressed at very low levels only on pre-B-II and immature B-cells -median nMFI of 1 (IQR: 0–1)-, which were therefore, consistently classified as CD73-negative cells (Figs. 1–3, and Table 3).

3.3. Expression of CD73, CD86 and CD304 on leukemic blasts at diagnosis

CD73 was the most frequently expressed antigen in BCP-ALL blast cells, being positive (nMFI ≥ 2, or dim and bright expression levels in the classical notation) in 71/108 (66%) patients. This percentage reflects also the percentage of cases with higher CD73 expression than that of all pooled normal BM BCP populations (Fig. 3 and Table 3). In detail, in most cases (43/108 (40%) patients), CD73 was expressed at rather low levels: heterogeneous negative-to-dim and dim CD73 expression (nMFI range: 2–6, i.e., dim using the classical notation), whereas strong positivity and overexpression of CD73 (nMFI ≥ 7, or

Table 4
Expression of distinct markers on blast cells from BCP-ALL cases at day +15 of induction treatment (MRD15) vs. diagnosis (Dx).

Marker expression pattern at MRD15 vs. Dx	CD73 (n = 37)	CD86 (n = 56)	CD304 (n = 41)	CD34 (n = 55)	CD10 (n = 56)	CD20 (n = 53)
Overall stable or increased expression	35 (95%)	40 (71%)	19 (46%)	21 (38%)	8 (14%)	52 (98%)
Overall decreased expression	2 (5%)	16 (29%)	22 (54%)	34 (62%)	48 (86%)	1 (2%)
Negative both at Dx and MRD15	1 (3%)	6 (11%)	6 (15%)	8 (15%)	3 (5%)	36 (68%)
Gain of expression at MRD15 from negative at Dx	6 (16%)	10 (18%)	4 (10%)	5 (9%)	0 (0%)	13 (25%)
Loss of expression at MRD15 from positive at Dx	0 (0%)	3 (5%)	9 (22%)	5 (9%)	4 (7%)	1 (2%)
Difference in median nMFI (MRD15 vs. Dx)[a]	+20	+3	−2	−6	−23	0
Mean nMFI difference (MRD15 vs. Dx)[b]	+24	+3	−3	−6	−26	+1

nMFI – normalized median fluorescence intensity. Results expressed as number of cases (percentage) unless otherwise specified; positive values indicate higher antigen expression levels at day +15 than at Dx; positive values indicate higher antigen expression levels at day +15 than at Dx.

[a] Absolute difference between median nMFI value achieved at MRD15 and Dx.

[b] Mean of differences between nMFI at MRD15 and Dx of paired cases.

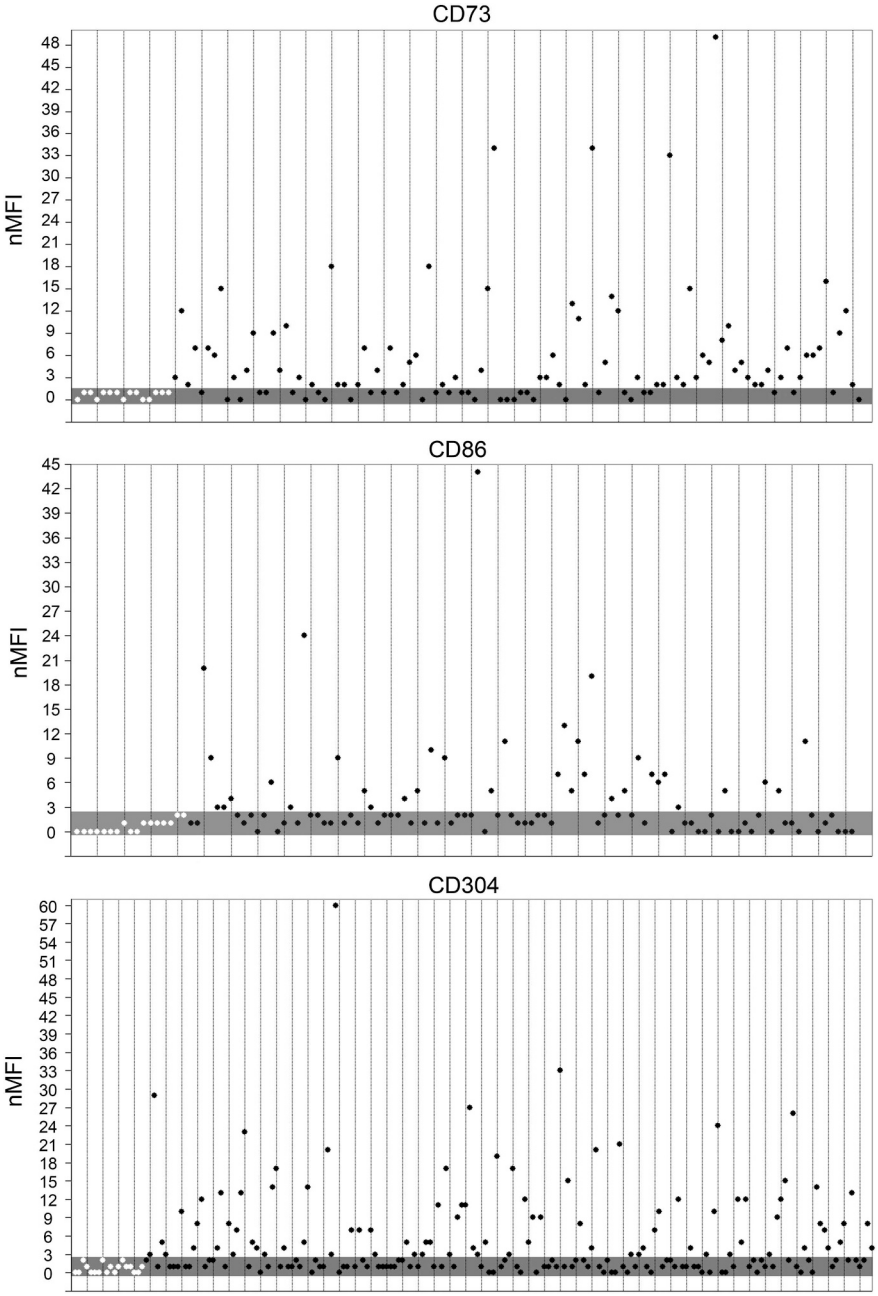

Fig. 3. Expression of CD73, CD86 and CD304 on normal BCP (white dots) vs. leukemic blasts (black dots) expressed in nMFI units. The shaded areas correspond to the nMFI spread detected among normal bone marrow BCP.

bright expression using the classical notation) was identified in 28/108 (26%) patients (Figs. 1–3 and Table 1).

CD304 and CD86 were expressed in a slightly lower percentage of BCP-ALL cases than CD73: 119/202 (59%) and 58/100 (58%) patients, respectively. Due to the fact that CD304 and CD86 exhibited low expression levels (nMFI range: 0–2) on pre-B-I and pre-B-II cells from a fraction of all control BM samples analyzed, expression of these markers on leukemic blasts was considered to be greater than normal only when nMFI values were ≥3. Thus, in 94/202 (47%) and 35/100 (35%) patients CD304 and CD86 were expressed at higher levels than in normal pooled BM BCP (Fig. 3). However, CD304 more frequently showed strong positivity and overexpression (nMFI ≥ 7, or bright expression) than CD86: 56/202 (28%) vs. 17/100 (17%) patients, respectively (Figs. 1–3 and Table 1).

3.4. Association between CD73, CD86 and CD304 expression and different immunological subgroups of BCP-ALL

Data on the EGIL immunological subtype of BCP-ALL was available for 274 (97%) patients, out of which 18 (7%) had pro-B-ALL, 184 (67%) common-ALL and 72 (26%) had pre-B-ALL. Overall, similar median nMFI values, median nMFI values of positive cases, expression rates (nMFI ≥ 2, or dim plus bright using the classical notation) and overexpression rates (i.e., nMFI ≥ 11) were observed for CD73 and CD304 between common- and pre-B-ALL cases. From these two markers, only CD304 showed significantly higher expression levels in common- and pre-B-ALL vs. pro-B-ALL (nMFI of CD304-positive cases of 5 and 7 vs. 2, respectively, $p = 0.0007$ and $p = 0.002$, respectively; Fig. 4, Supplementary Table 1). Regarding CD86, a similar percentage of positive cases was observed among all three immunological subgroups of BCP-ALL, the highest median nMFI of CD86-positive cases being observed

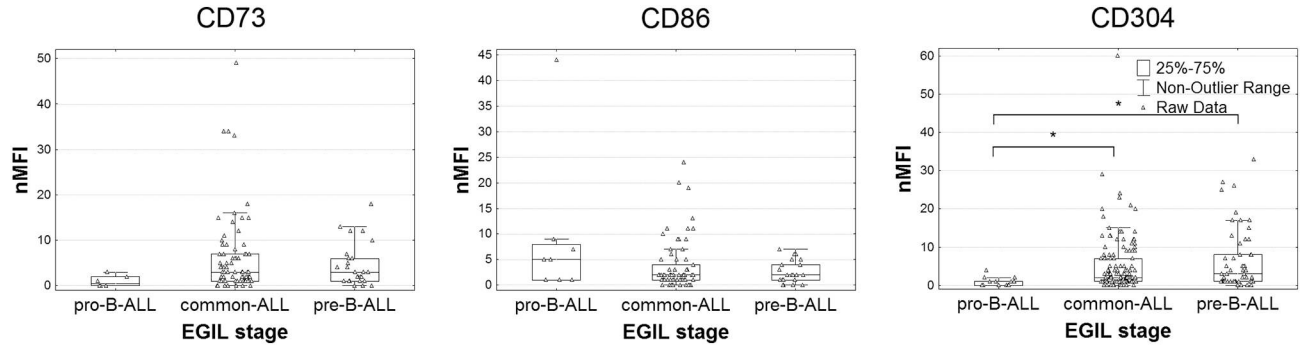

Fig. 4. Expression levels (nMFI values) of CD73, CD86 and CD304 in different immunological subgroups of BCP-ALL as defined by the EGIL classification. * corrected $p < 0.05$ for both ways of determination of antigen expression between positive vs. negative cases for individual genetic aberrations: nMFI-based and classical 3-step (negative/dim/bright) notation (Mann-Whitney *U* test and Pearson chi-square test, respectively).

among pro-B-ALL cases (nMFI of 7 vs. 3 and 4 for common-ALL and pre-B-ALL, respectively), but these differences did not reach statistical significance (Fig. 4, Supplementary Table 1).

3.5. Expression of CD73, CD86 and CD304 in different genetic subgroups of BCP-ALL

Overall, a significant association ($p = 0.009$) was found between CD304 expression and the presence of the *ETV6-RUNX1* fusion gene: CD304 was positive in 21/25 (84%) cases carrying the *ETV6-RUNX1* gene rearrangement with a median nMFI of 7 (range: 2–26) vs. 62/117 (53%) *ETV6-RUNX1*-negative cases (median nMFI of 5; range: 2–60) (Table 2).

In addition, CD304 expression also showed a negative association ($p = 0.01$) with *MLL* gene rearrangements: expression of CD304 was observed in 93/152 (61%) *MLL*-negative patients (nMFI of 7; range: 2–60), but in only 2/9 (22%) *MLL*-rearranged patients (nMFI of 3; range: 2–4). In turn, *MLL*-rearranged BCP-ALL patients showed slightly higher expression ($p > 0.05$) of CD86 vs. *MLL*-negative cases: 5/7 (71%) *MLL*-rearranged patients were CD86-positive (median nMFI of 7; range: 5–44), vs. 46/77 (60%) *MLL*-negative patients (median nMFI of 3; range: 2–24). No statistically significant association was observed between the patterns of antigen expression of hyperdiploid BCP-ALL cases vs. the remaining genetic subgroups of BCP-ALL (i.e., *BCR-ABL1*-positive, *TCF3-PBX1*-positive, hypodiploidy) which were individually too small (1–3 cases (1.5–3.6%) per subgroup) to draw any reliable conclusions on potential associations with specific expression patterns for any of the antigens investigated. Despite this, it should be noted that both CD86 and CD304 were positive in all 3 *BCR-ABL1*-positive cases, with a median nMFI of 3 (range: 2–10; dim expression) and 10 (range: 4–14; bright expression), respectively (Table 2). Finally, no statistically significant association was revealed as regards antigen overexpression (exceeding the expression observed on the relevant positive reference cell type, i.e., nMFI ≥ 11) and any specific genetic subgroup of BCP-ALL (data not shown).

3.6. Stability of CD73, CD86, CD304 and other antigens at early follow-up MRD time points

Out of the six markers evaluated for the stability of their expression levels at MRD15 vs. Dx, three showed stable or higher median nMFI in the majority of cases: CD73 in 35/37 (95%), CD86 in 40/56 (71%) and CD20 in 52/53 (98%) patients. The mean nMFI increase at MRD15 (vs. Dx) was of +24 nMFI units for CD73, and of +3 and +1 nMFI units for CD86 and CD20, respectively, pointing out that CD73 was considerably upregulated during follow-up. Gain of aberrant marker expression at MRD15 for cases that were originally negative at Dx was also most frequently observed for CD73 (6/37; 16% of cases), CD86 (10/56; 18% of cases) and CD20 (13/53; 25% of cases), proving that expression of these markers can be upregulated at MRD15 in a significant proportion of cases, even when absent at Dx.

In contrast, CD304 levels at MRD15 remained stable in only 19/41 (46%) cases with a mean nMFI decrease at MRD15 of −3 nMFI units. Of note, even though CD304 expression decreased in more than half of the cases, of which 9/41 (22%) showed complete loss of expression, CD304 still remained aberrantly positive at MRD15 in 26/41 (63%) cases (Table 4 and Fig. 5). The levels of expression of the remaining two markers (i.e., CD34 and CD10) decreased in the majority of cases after therapy (mean nMFI decrease at MRD15 of −6 and −26 nMFI units, respectively). In addition, these two markers also showed a moderate rate of complete loss of expression at MRD15 among those cases that were $CD34^+$ and $CD10^+$ at Dx (5/55 (9%) and 4/56 (7%) cases, respectively) (Table 4).

4. Discussion

MRD evaluated during early phases of treatment currently represents the most significant predictive factor for relapse, and thereby, forms the basis for patient risk stratification in most ALL treatment protocols which are contemporarily used worldwide, particularly in childhood ALL (Basso et al., 2009; Brüggemann et al., 2012; Campana, 2012; Kusenda et al., 2014; Pui and Evans, 2006; van Dongen et al., 2015). Therefore, it is critical to optimize currently used MRD detection techniques, particularly FC-based MRD approaches. In this regard, identification of new candidate markers for an improved distinction between normal BCP and leukemic blasts, still remains a challenge in order to increase the applicability, as well as both the specificity and sensitivity of the assay. An ideal candidate marker should exhibit differential expression on normal BCP and leukemic blasts and remain stable during treatment, in order to overcome the immunophenotypic switches frequently observed for the most commonly used MRD markers (Chen et al., 2001; Coustan-Smith et al., 2011; Dworzak et al., 2010; Gaipa et al., 2013) as also confirmed here for e.g., CD10 and CD34. Further optimization of the FC assay can be achieved by increasing the number of markers combined in a tube and improved data analysis software enabling assessment of multiple (≥12) antigens simultaneously (Denys et al., 2013; Flores-Montero et al., 2017; Theunissen P, 2017).

Analysis of the leukemia cell surface proteome and genome-wide gene expression screening of leukemic cells against normal BM BCP, have both proven to be powerful techniques to search for potential MRD candidate markers in BCP-ALL (Coustan-Smith et al., 2011; Mirkowska et al., 2013). Thus, these studies have highlighted the potential utility of tens of new markers, three of which, i.e., CD73, CD86 and CD304 were investigated in detail here, as potentially useful targets for MRD monitoring in childhood BCP-ALL. For this purpose we used a prospective, multicenter and standardized FC approach based on the EuroFlow techniques, protocols and tools (Kalina et al., 2015; Kalina

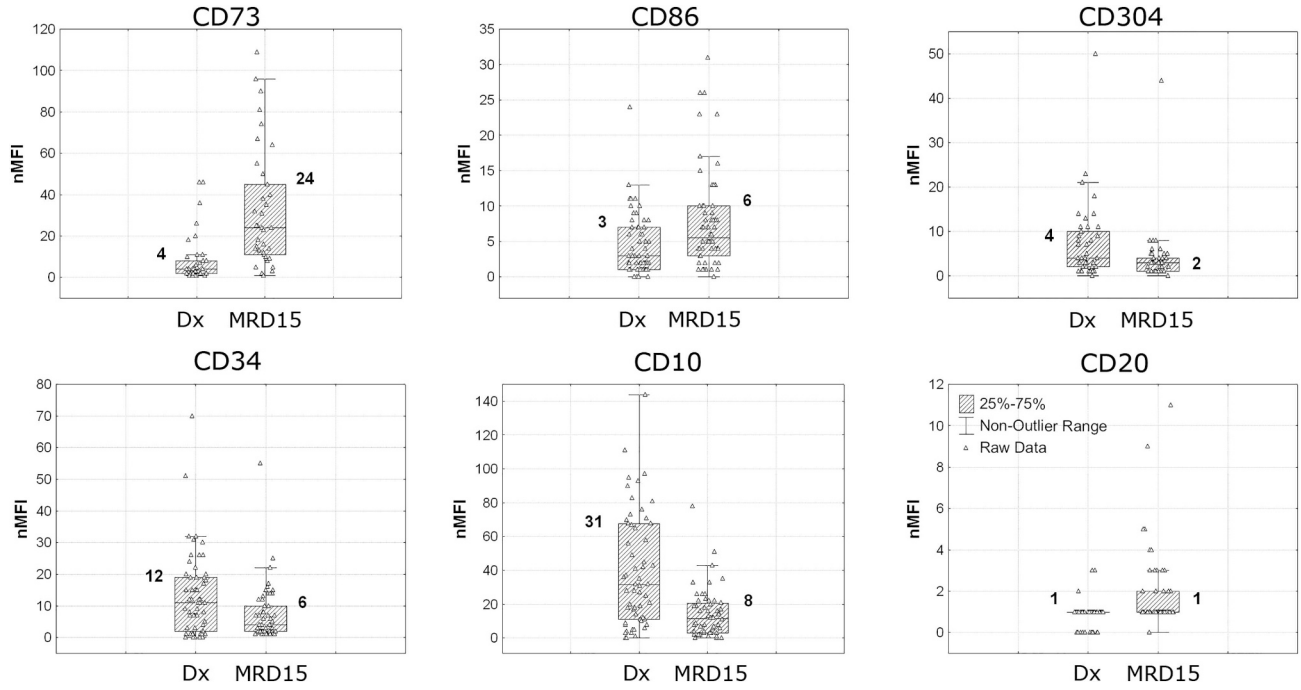

Fig. 5. Levels of expression of the different markers evaluated in BCP-ALL blast cells at diagnosis (Dx) and at day +15 of induction treatment (MRD15). The boxes cover the interquartile range (25%–75%) and whiskers non-outlier range. The numbers above the boxes correspond to median nMFI values obtained by particular antigens at Dx and MRD15.

et al., 2012; van Dongen et al., 2012). Since the same standardized EuroFlow instrument settings, reagent panels, sample preparation and data acquisition protocols (Kalina et al., 2012) were followed at each participating center, we were able to apply highly standardized approaches for data analysis, including normalization of MFI values on a pooled group of patients and controls, with the use of the innovative Infinicyt software tools (Denys et al., 2013; Flores-Montero et al., 2017; Kalina et al., 2015; Kalina et al., 2012).

Overall, our results are consistent with previously reported data on the differential expression patterns of CD73, CD86 and CD304 in normal BCP vs. leukemic blasts from BCP-ALL patients. Thus, the pattern of expression of CD304 on normal BCP from our cohort of control BM was concordant to that reported by Meyerson et al., (2012) and Solly et al., (2012); in this regard, our results confirmed variably weak/partial CD304 expression on pre-B-I cells, which progressively decreased with BCP maturation, becoming negative on mature B-cells. Around one-fourth of the cases showed strong expression and even overexpression of CD304 on leukemic blasts, in line with previous results reported by Coustan-Smith et al. (2011) and Meyerson et al. (2012). In contrast, Solly et al. (2012) reported that CD304 was overexpressed in a significantly higher proportion-24/50 (48%)- of patients, including > 80% of $CD45^{low}CD19^{+}$ leukemic cells from 18/50 (36%) of BCP-ALL patients (Solly et al., 2012). These differences might be due to the fact that the patient group studied by Solly et al. (2012) consisted of both pediatric and adult BCP-ALL patients, while both our and the other two former studies (Coustan-Smith et al., 2011; Meyerson et al., 2012), were all based on childhood ALL patients.

Regarding CD73, two-thirds of our cases showed either dim or bright expression of this marker. This frequency of CD73-positive cases is slightly greater than that previously reported by others (42–55% of cases) (Denys et al., 2013; Wang et al., 2016), with a tendency to higher CD73 expression rates among the EGIL common- and pre-B-ALL subgroups vs. pro-B-ALL, as also found by Wang et al. (2016) and Wieten et al. (2011). Interestingly, Wang et al. (2016) observed increasing CD73 expression levels along normal BCP maturation, giving rise to a bimodal expression profile with highest CD73 expression levels on mature B-cells. Even if such increased expression of CD73 along normal BCP maturation was not clearly visible in our study, we confirmed the bimodal expression pattern of CD73 on mature B-lymphocytes, in addition to BM T/NK lymphocytes.

Data available on CD86 expression is currently more limited. Despite this, Coustan-Smith et al. (2011) have previously reported expression of this marker in around half of all childhood BCP-ALL patients, as also confirmed here.

The overall pattern of expression of CD73, CD86 and CD304 showed limited associations with the genetic subgroups of BCP-ALL, except for: i) greater CD304 expression among cases with *ETV6-RUNX1* gene rearrangements as also reported by Solly et al. (2012) but not by Coustan-Smith et al. (2011) and, ii) the inverse relationship between *MLL* gene rearrangements and CD304 expression reported here for the first time. In contrast, Coustan-Smith et al. (2011) reported a marginal association ($p = 0.09$) between CD86 expression and hyperdiploidy, which could not be confirmed in our series. Of note, relatively high expression levels of CD86 and CD304 were detected in our three *BCR-ABL1*-positive cases, but differences vs. *BCR-ABL1*-negative patients did not reach statistical significance, probably due to the low number of *BCR-ABL1*-positive cases. In this regard, it should be also noted that Solly et al. (2012) and Meyerson et al. (2012) have also found a higher frequency of *BCR-ABL1*-positive cases among $CD304^{+}$ (vs. $CD304^{-}$) patients, however these differences only showed a marginal statistical significance in both studies (*p*-values of 0.08 and 0.09, respectively).

In order to further investigate the potential utility of the three markers evaluated here for MRD monitoring, we assessed their stability together with other frequently used MRD markers such as CD34, CD10 and CD20 during the early phases of therapy (i.e., day +15 of therapy). As previously shown (Basso et al., 2009; Brüggemann et al., 2012; Campana, 2012; Kusenda et al., 2014; Pieters and Carroll, 2008), MRD-positivity levels > 0.1% are frequently observed at this time in the majority of patients, which ensures more reliable comparative analyses than at later time points with lower levels and lower rates of MRD-positivity. Of the three markers evaluated, CD73 turned out to be the most stable, being expressed at equal or higher levels in virtually all (95%) cases, without complete loss of CD73 expression being observed among our cases. In turn, CD86 expression levels remained stable in around 70% of cases, with only three patients showing complete loss of CD86 expression at MRD15. In contrast, CD304 expression levels

decreased in around half of the cases at MRD15; despite this, in the majority of them, leukemic blasts remained aberrantly positive for CD304 at this time point.

To date, there is only a limited number of studies in which the stability of CD73, CD86 and CD304 has been investigated in detail during follow-up of BCP-ALL. Thus, Solly et al. (2012) previously reported stability of CD304 expression on leukemic blasts after therapy, however, in this study, the levels of CD304 at diagnosis were compared with those of relapsed samples in a relatively limited patient cohort (Solly et al., 2012). In turn, Meyerson et al. (2012) and Coustan-Smith et al. (2011) concluded that CD304 might potentially represent an useful MRD marker, based on its relatively high overexpression rate at diagnosis, but they did not investigate the stability of CD304 during therapy. In this regard, our results show that CD304 overexpression at diagnosis does not always predict for stable expression levels of this aberrant marker, even at early treatment time points (day +15 of induction therapy). Despite the fact that decreased expression of CD304 was observed in some cases, this does not rule out the potential utility of this antigen for MRD monitoring, particularly because expression of CD304, as well as of CD86, was also found to increase in cases in which leukemic blasts were $CD304^-$ and $CD86^-$ at diagnosis. This is also true for CD73, a marker for which we proved the highest stability and the highest mean nMFI increase at day +15, in line with what has also been previously reported by Wang et al. (2016). Altogether, these findings point out the great potential of this marker, since the increase on the level of expression of this aberrant marker may considerably facilitate MRD monitoring in BCP-ALL.

Modulation (switch) of antigen expression is a relatively common finding during treatment, and might occur in up to 70% of all BCP-ALL patients (Chen et al., 2007; Mejstrikova et al., 2010; Slamova et al., 2014). Down- or upregulation of well-established MRD markers, is mainly due to steroid-induced blast cell maturation. Thus, decreased expression of "immaturity" markers like CD34 and CD10, together with upregulation of differentiation-associated antigens such as CD20, have been recurrently reported during the early phases of treatment of BCP-ALL patients (day +15 and day +33) (Basso et al., 2009; Coustan-Smith et al., 2002; Dworzak et al., 2010; Gaipa et al., 2013; Gaipa et al., 2005; Lucio et al., 2001; Mirkowska et al., 2013; Szczepański, 2007) as also confirmed here. In order to overcome this problem, it is recommended that multiple aberrant markers, found to represent LAIP at diagnosis are followed, so that at least one LAIP will remain stable.

The above findings on LAIP marker stability assessment led us to evaluate more in-depth the stability of CD73 and CD304, vs. that of CD86 (Theunissen P, 2017). Thus, a study on 319 pediatric BCP-ALL patients was conducted, in parallel with molecular IG/TR gene PCR-based MRD monitoring. Both markers were combined in the same tube with the same fluorochrome which did not result in an increase in the background staining. The use of the antibody combination containing CD73 and CD304, together with a bulk lysis standardized sample preparation protocol enabling collection of ≥4 million cells/tube, which resulted in comparable MRD-positive rates to those obtained by conventional allele-specific oligonucleotide (ASO)-PCR IG/TR gene rearrangement-based MRD. Moreover, the inclusion of combined CD73/CD304 significantly improved the distinction between normal BM BCP and leukemic blasts in around one-third of the patients, vs. other aberrant markers such as CD66c and CD123 (Theunissen P, 2017).

In summary, here we confirm and extend on previous findings about the potential utility of the CD73, CD86 and CD304 as candidate and reliably stable MRD markers in childhood BCP-ALL.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2018.03.005.

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