Identification of residual leukemic cells by flow cytometry in childhood B-cell precursor acute lymphoblastic leukemia: verification of leukemic state by flow-sorting and molecular/cytogenetic methods

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Online Supplementary Appendix
Standard FC-MRD procedures and cytogenetic analyses in NOPHO

Standard FC-MRD

Cell staining

Briefly, membrane markers were stained by incubation with fluorochrome-coupled antibodies (BD Biosciences, San Jose, CA, US (BD)) and subsequently erythrocytes were lyzed with EasyLyse (Dako, Glostrup, Denmark) under non-fixating conditions. After washing, the cells were resuspended in FACSflow (BD). Intracellular markers were stained after fixation and permeabilization using IntraStain (Dako).

Antibody MRD panels

After BCP-ALL diagnosis, patients from NOPHO ALL-2000 were analyzed with the protocol-defined four-color MRD panel: CD45/CD34/CD19/CD10; CD10/CD20/CD19/CD45; CD34/CD22/CD19/CD45; CD34/CD38/CD19/CD45; TdT/CD10/CD19/CD45. Fluorochromes in the four channels were: FITC/PE/PC5/APC, respectively. Patients from NOPHO ALL-2008 protocol were analyzed with the protocol-defined six-color MRD panel: CD10/CD20/CD34/CD19/CD38/CD45; CD66c/CD123 /CD34/CD19/CD22/CD45; TdT /CD58/CD34/CD19/CD10/CD45. Fluorochromes in the channels were: FITC/PE/PerCP-Cy5.5/PE-Cy7/APC/APC-Cy7, respectively. Additionally, patient-specific antibody combinations were used for MRD detection in some cases.

Data analysis at follow-up

Data analysis was performed using the following steps: a) exclusion of dead cells and debris in forward scatter (FSC)/side scatter (SSC) dot plots based on propidium-iodide staining in a separate tube; b) exclusion of aggregated cells based on FSC-height *versus* FSC-area dot plots; c) gating on B-lineage in CD45/CD19 plots; d) gating on events with LAIP in dot plots of the antibodies in the respective combinations; and e) verification that the expected MRD cells form a cluster (containing at least 10 events) in all relevant plots, and locate in the relevant region in the FSC/SSC dot plot. Normal cells present in BM were used as positive and negative controls of antibody-reagent performance.

Standard cytogenetic analyses

As part of the diagnostics for childhood ALL, chromosome analyses (G-banding) and FISH were performed on diagnostic BM samples of all ALL patients. The applied FISH probes were MLL dual color, break apart rearrangement probe, TCF3/PBX1 dual color, dual fusion probe, BCR/ABL dual color, dual fusion probe, EVT6/RUNX1(TEL/AML1) ES dual color probe (all probes from Vysis, Abbott, Weisbaden, Germany). Patients with a normal or failed karyotype were in addition screened with FISH probes for chromosomes 4, 10 and 17 (CEP4, CEP10, CEP17, Vysis, Abbott).

Details from flow-sorting experiments

Patient numbers marked by #

'Gray zone' cell populations

'Gray zone' cell populations, i.e. normal cell populations potentially containing minor LAIP fractions, were analyzed in 12 BM samples. PCR/FISH-negative results in such populations were found in 4 of these samples (obtained from 4 patients), of which 3 were from late follow up (day 79 or 92) and one was from day 29.

In 3 of these BM samples (#77/00 day 79, #20/08 day 92 and #22/08 day 29), the standard MRD by FCM was reported as negative with sensitivities of from 5.0E-5 to 1.0E-4. In patient #77/00, we observed the presence of normal precursor B cells. In these cases, there remained, during the *a posteriori* evaluation, a suspicion of a possible minor fraction of malignant cells in the sort gate. In the fourth BM sample (#76/00 day 79, same patient as in Case 2), the standard FCM-MRD was negative (sensitivity of 1.0E-5). However, the used sort gate covered the CD19pos/CD34pos/CD38dim empty space. The *a posteriori* inspection showed that most cells in the gate derived from normal neighboring populations, but it could not be excluded that a minor fraction of the cells might be malignant.

The suspicions of possible minor fractions of LAIP in the sorted populations in these 4 BM samples were invalidated by the PCR/FISH-negative results.

BM samples with high MRD levels

In 4 BM samples (from 4 patients) with relatively high FCM MRD-values by standard analyses (MRD=1.3E-2, 4.3E-2, 7.8E-

2 and 1.5E-1, respectively) and in which the MRD cell populations overlapped with the applied 'normal-cell sort gate', 1-4% PCR-positive cells were detected in the sorted presumed normal cell populations (mature B cells or CD45pos/CD19neg cells). These PCR positive signals in the sorted cell populations would correspond to overall underestimations of the final MRD-values of approximately 3.0E-3, 1.2E-3 and 3.0E-3, respectively. But due to the much higher standard MRD values in these cases, the PCR-positive cells detected in the normal cell populations would be without any consequence for the final result (*Online Supplementary Table S2*).

Review of Cases 1 and 2: presumed normal cell populations with PCR/FISH-positive signals

In 2 BM samples from 2 patients, with lower standard FC-MRD values (no detectable MRD with a sensitivity of 1.0E-3 and MRD=1.4E-4, respectively), PCR-positive cells were detected in the sorted presumed normal cell populations (*Online Supplementary Table S2*).

Case 1

One sample was a day 58 BM sample from patient #81/00 who had an only partly informative LAIP at diagnosis due to broad CD34 expression (both CD34positive and CD34negative cells) combined with low CD38 expression. Consequently, the patient was followed by both PCR-MRD and FC-MRD. The standard FC-MRD results were 1.4E-4, and the PCR-positive cells detected in presumed normal populations (isolated from three distinct antibody-combinations using sort gates: CD19pos/CD45pos/CD22pos/CD34neg, CD19pos/CD45pos/CD10neg/CD34pos, and CD19pos/CD45pos/CD38neg/CD34neg) correspond to possible MRD underestimation of up to 3.0E-4 (9-39% PCR-positive cells in cell populations comprising 0.01-0.1% of the total blast count). Therefore, here there might be an MRD underestimation if based on FC with only partly informative markers, illustrating the importance of primarily evaluation by PCR in such cases. This finding would not have had any clinical importance for treatment stratification in the NOPHO ALL-2008 protocol using cut-off threshold 1.0E-3. But at MRD levels close to the cut-off limit, such an underestimation could be clinically important.

Case 2

The other sample was a day 15 BM sample (patient #2/08), in which the standard FC-MRD was "negative" with a sensitivity of only 1.0E-3 due to very poor BM sample quality with very few cells in total. One to 2% PCR-positive cells were detected in two presumed normal mature B-cell populations sorted from two antibody-combinations, using the sort gates: CD19pos/CD45bright/CD20bright/CD10neg and CD19pos/CD45bright/CD34neg/CD10neg. This would correspond to an MRD underestimation of approximately 1.4E-3 (1-2% PCR positive cells in cell populations comprising 7-13% of the total blast count). In the former case, a part of a potential LAIP population with CD20bright/CD10dim was identified *a posteriori* contaminating the used sort gate, which could explain the positive cells among the presumed normal mature B cells. This indicates significant immunophenotypic modulation with downregulation of CD10 and upregulation of CD20 on the leukemic cells at this early follow-up time point. The patient case was further complicated since it was suspected that the used clone-specific PCR primers detect a subpopulation, which potentially might have a LAIP similar to the immunophenotype of normal mature B cells.

Review of Cases 3 and 4: PCR/FISH-negative presumed malignant cell populations

In 2 BM samples from 2 patients, sorted cell populations with presumed presence of LAIP by FC were PCR/FISH-negative (*Online Supplementart Table S2*).

Case 3

One of these BM samples was a day 138 BM sample from an infant ALL patient (#62/00) monitored by FC-MRD according to the NOPHO ALL-2000 protocol. The standard FC-MRD result was maximum 7.0E-4 reported with reservation. PCR-negative results were found in three cell populations with LAIP sorted from two distinct four-color antibody-combinations. Sort gates were: CD19pos/CD45pos/CD34neg/CD22neg, CD19pos/CD45pos/CD10neg/CD20neg and CD19pos/CD45pos/CD34pos/CD22neg, respectively. Due to the PCR-negative results, we presume that these CD19dim/CD45dim/CD20neg/CD10neg cells are normal plasma cells surviving chemotherapy, and that these could have resulted in false-positive MRD-counts by FC. However, it is possible that the clone-specific PCR marker used was lost.

Case 4

The other sample was a day 29 BM sample (#76/00) with standard MRD-result of 1.1E-4. Cells with LAIP sorted were negative by FISH, shown by sequential hybridization with two different FISH probes (cep-4 (centromere, chromosome 4) probe followed by an AML-1 probe (chromosome 21)). The used sort gate covered the CD19pos/CD34pos/CD38dim informative empty space, according to the LAIP at diagnosis. Re-inspection of the sort gate revealed presence of cells from normal neighboring cell populations but also the presence of some dead cells (events in diagonal regions) which clearly reduced the discriminatory power.

Our dead cell discrimination was guided by propidium-iodide staining in a separate tube, clearly indicating that in addition to dead granulocytes located in the low FSC/high SSC region, dead lymphoid cells and/or debris were located in the low FSC/low SSC region. Some dead cells overlap into the FSC/SSC lymphoid region and cannot be excluded without losing potential MRD events. Consequently, the events defined in the diagonal region in fluorescence dot plots cannot be completely avoided by FSC/SSC gating.

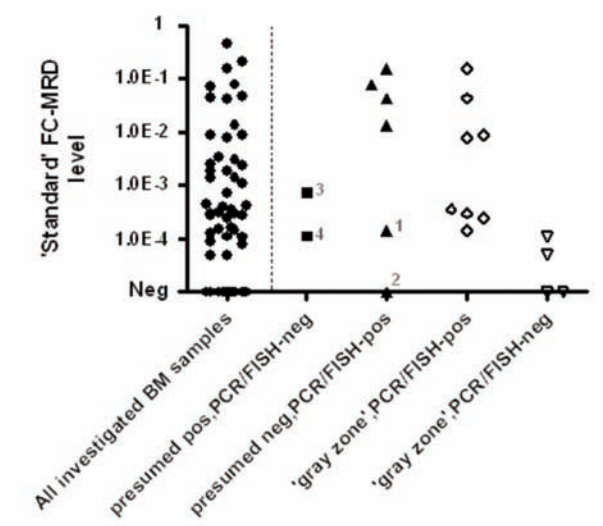
Online Supplementary Table S1. G-band karyotypes and FISH results of diagnostic BM samples as well as FISH markers and/or IG/TCR PCR markers applied for exploration of flow-sorted cell populations in follow-up BM samples (28 patients).

Patient ID	G-banding chromosome analysis (diagnostic BM sample)	Interphase FISH findings in diagnostic BM sample	Used FISH marker in flow-sorted cell populations (probe)	Used PCR marker in flow-sorted cell populations
#61/00	46,XX,qdp(21)(q22)[25]	iamp21q22	**NT**	TCRB-J2.7C
#62/00	46,XX,t(9;11)(p22;q23)[3]/46,XX[24]	MLL rearranged	**NT**	IGH-J4C
#64/00	53,XX,+X,+4,+12,+14,+17,+21, +21[25]	+21,+21	**NT**	IGH-J6A
#67/00	Failed karyotype	Positive for ETV6/RUNX1	**NT**	IGH-J4B
#74/00	46,XX,t(4;11)(q21;q23)[25]	MLL rearranged	MLL (MLL)	**NT (no PCR marker)**
#75/00	Failed karyotype	Positive for ETV6/RUNX1	ETV6-RUNX1 (ETV6/RUNX1)	**NT (no PCR marker)**
#76/00	45,XX,der(21;22)(q10;q10)[3]/46,XX[10]	+4,+10,+21,+21	+21,+21 (ETV6-RUNX1)	**NT**
#77/00	46,XY[19]	Positive for ETV6/RUNX1	ETV6-RUNX1 (ETV6-RUNX1)	**NT**
#78/00	54~56,X?,+3,+4,inc[cp6]	+4,+10,+17,+21	+21 (ETV6-RUNX1)	**NT**
#79/00	Failed karyotype	+4,+11,+21,+21,+21	+21,+21 (ETV6-RUNX1)	**NT (no PCR marker)**
#80/00	54,XX,+X,+4,+6,+8,+10,del(13) (q12q14),+18,+21,+21[25]/46,XX [1]	+21,+21	+21,+21 (ETV6-RUNX1)	TCRG-A
#81/00	Failed	Negative for ETV6/RUNX1, t(1;19),t(9;22),MLL	**NT (no FISH marker)**	IGH-J6B
#82/00	46,XY[24]	+17,+21,+21	+21,+21 (ETV6-RUNX1)	IGH-J6A
#2/08	46,XX,der(9)t(5;9)(9;p21)[28]	Positive for del(9p21)	**NT**	IGH-J6B
#3/08	Not requested	RUNX1x3-4 [91/200] Negative for ETV6/RUNX1, t(9;22), TCF3- and MLL rearrangement	**NT**	TCRD-D3B
#5/08	49,XY,+X,+17,+21 [25]	Positive for del(9)(p21) [142/200] RUNX1x3 [156/200] Negative for ETV6/RUNX1, t(9;22), TCF3- and MLL rearrangement	**NT**	IGK-KdeA
#8/08	54,XY,+X,dup(1)(q32q42),+4,+6, +10,+17,+21,+21,+3mar[11]/46, X,+X,-Y[3]/46,XY[10]	+21,+21	+21,+21 (ETV6-RUNX1)	IGH-J6A
#14/08	46,XX,t(4;12)(q21;p13)[13]/47, idem,+21[4]/46,XX[2]	Positive for ETV6/RUNX1, +21	ETV6-RUNX1 (ETV6-RUNX1)	IGH-J4B
#16/08	46,XY,t(9;11)(p22;q23)[11]/46,XY[5]	MLL rearranged	MLL (MLL)	NT (no PCR marker)
#17/08	47,XY,+21[3]/46,XY[23]	Positive for ETV6/RUNX1	**NT**	IGH-J3A
#18/08	Failed karyotype	Positive for ETV6/RUNX1	ETV6-RUNX1 (ETV6-RUNX1)	IGH-J4A
#19/08	54,XY,+X,+4,+6,+8,del(9)(p21), +14,i(17)q10),+18,+21,+21[10]/ 46,XY[15]	+21,+21	+21,+21 (ETV6-RUNX1)	IGH-J6B
#20/08	47,XY,+21[14]/46,XY[11]	Positive for ETV6/RUNX1, +21	ETV6-RUNX1 (ETV6-RUNX1)	TCRB-2.3A
#21/08	62~64,der(19)t(1;19)(q?;?p), inc[cp16]/46,XY[18].ish der(19) t(1;19)(wcp19+,wcp1+)[9]	+21,+21,+21	+21,+21,+21 (ETV6-RUNX1)	TCRD-D3B
#22/08	Failed karyotype	+4,+10,+21,+21	+21,+21 (ETV6-RUNX1)	**NT**
#23/08	46,X,del(X)(q21),t(7;12)(p13; p13)[21]/46,XX[14]	Positive for ETV6/RUNX1	ETV6-RUNX1 (ETV6-RUNX1)	IGK-KdeB
#52/08	46,XY,del(2)(q31)[7]/47,idem, +22[2]/46,XY [8]	Positive for ETV6/RUNX1	ETV6-RUNX1 (ETV6-RUNX1)	IGH-J3B
#63/08	47,XY,+8[4]/46,XY[8]	Positive for ETV6/RUNX1	ETV6-RUNX1 (ETV6-RUNX1)	TCRD-D3B

#: patient number; /00: patient from NOPHO ALL-2000; /08: patient from NOPHO ALL-2008; NT: not tested. Patients were randomly selected within the period June 2007 to January 2010.

Online Supplementary Table S2. Summary of cases with unexpected PCR/FISH-results in flow-sorted cells. Dot plots for each case are shown in Figure 2.

Case	Leukemic/ normal cells by FC	PCR/FISH-results	BM sample and patient LAIP	Standard FC-MRD result (unsorted BM sample)	Possible explanation for unexpected PCR/FISH-results	Potential effect on FC-MRD result
Case #1	Normal	PCR-positive signals	Day 58 BM sample. LAIP: CD34broad from neg. to pos.	1.4E-4	MRD cells in CD34neg cell population probably due to the only partly informative LAIP (CD34broad)	Potential underestimation of up to approx. 3.0E-4
Case #2	Normal	PCR-positive signals	Day 15 BM sample. LAIP: CD19pos/ CD10pos/CD20neg	Negative with sensitivity of only 1.0E-3 (few cells in sample)	MRD cells in CD10neg normal cell population possibly due to significant CD10 downmodulation	Potential underestimation of approx. 1.4E-3
Case #3	Leukemic	PCR-negative	Day 138 BM sample (infant patient). LAIP: CD19pos/ CD10neg/CD20neg	Max. 7.0E-4	No plasma cell marker in the used antibody-combinations. Suspected LAIP cells are possible normal plasma cells surviving chemotherapy	Potential false-positivity, but sample recognized to be sub-optimal.
Case #4	Leukemic	FISH-negative	Day 29 BM sample. LAIP: CD19pos/ CD34pos/CD38dim	1.1E-4	Suspected LAIP cells in empty space are possibly dead cells (events in diagonal regions)	Potential false-positivity, but sample recognized to be sub-optimal.
Four cases with high MRD values	Normal	1-4% PCR-positive signals	Two day 15 and two day 29 BM samples	1.3E-2 to 1.5E-1	MRD cell populations overlap into the 'normal-cell sorting gate'	Potential minor underestimation if same gates used for analysis, but no consequence for treatment stratification



Online Supplementary Figure S1. Standard FC-MRD levels in the investigated BM samples. The far left column shows the standard FC-MRD values in all the BM samples investigated (●): The investigated BM samples had standard FC-MRD results between negative and 4.4E-1. Twelve samples were scored as negative; 11 were negative with sensitivities of 1.0E-3 to 1.0E-5, while one sample was negative with sensitivity of only 1.0E-2. The remaining 42 BM samples had standard FC-MRD results between 5.0E-5 and 4.4E-1. The next two columns show the standard FC-MRD levels in the BM samples (■ and ▲), in which the flow-sorted presumed leukemic (pos) and presumed normal (neg) cell populations gave unexpected PCR/FISH-results ('presumed pos, PCR/FISH-neg' and 'presumed neg, PCR/FISH-pos', respectively). Standard FC-MRD values in BM samples in which 'gray zone' cell populations/fraction were isolated and analyzed by PCR/FISH ('gray zone, PCR/FISH-pos' and 'gray zone, PCR/FISH-neg') are shown in the last two columns (◇ and ▽). Numbers 1-4 refer to cases with 'discrepant' results in flow-sorted cell populations (described in detail in the *Online Supplementary Appendix*).