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AIEOP-BFM consensus guidelines 2016
flow cytometric immunophenotyping in pediatric acute leukemias

Michael N. Dworzak

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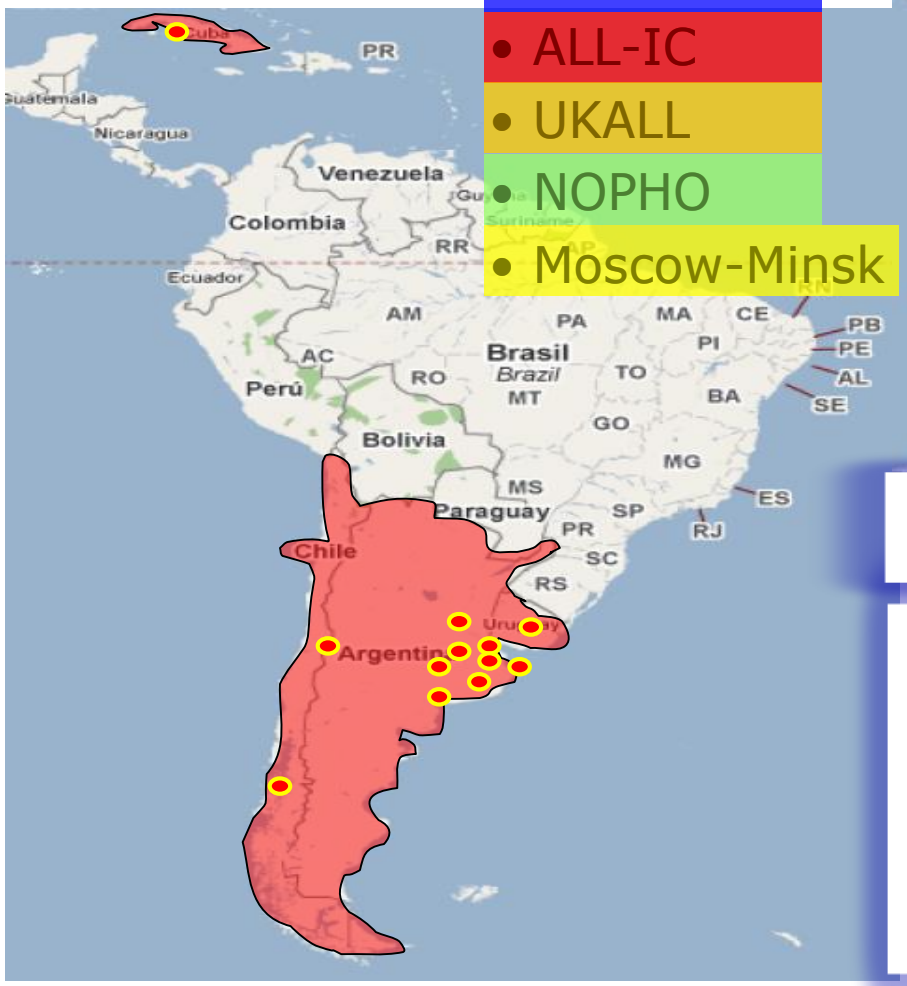
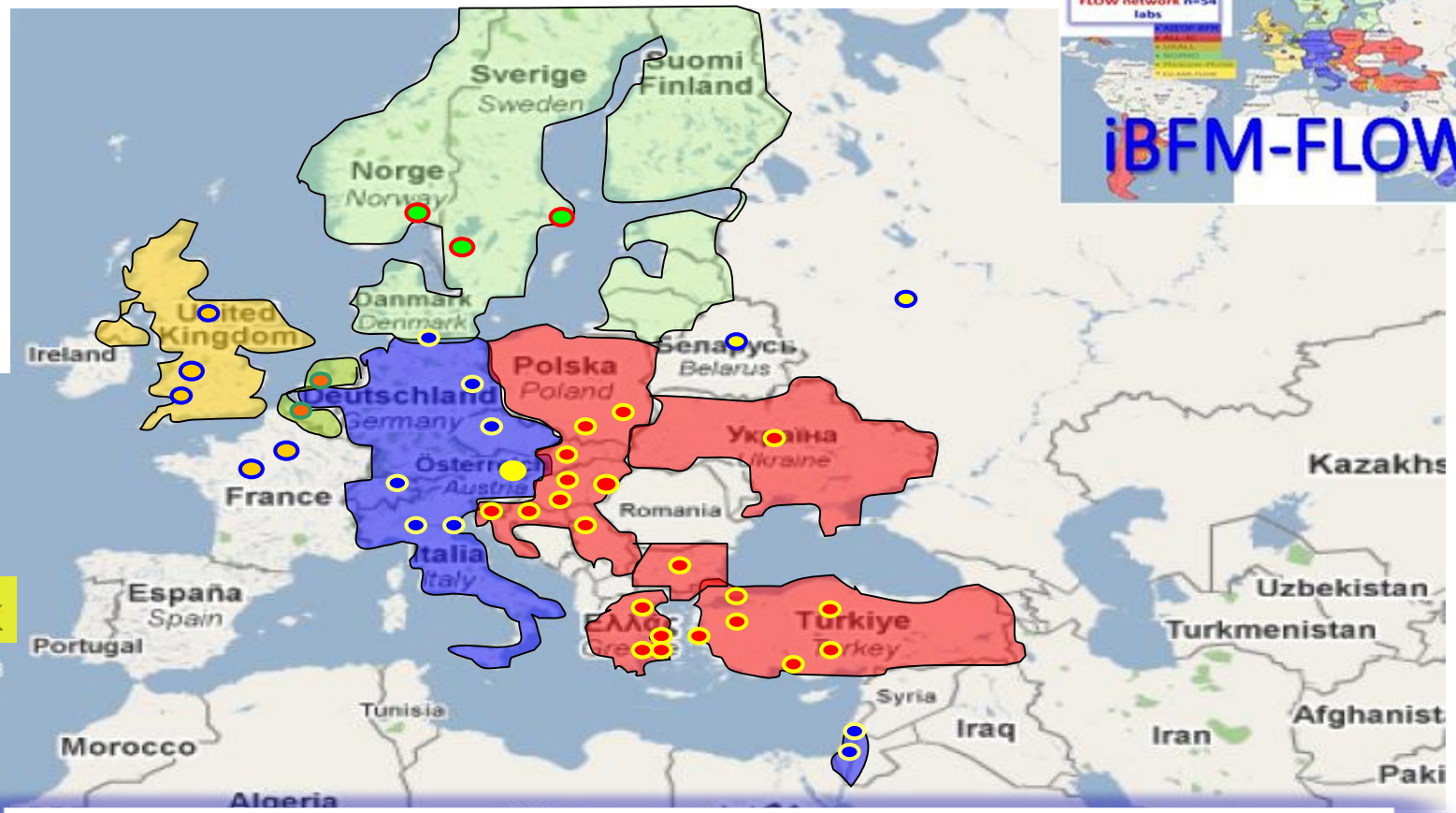
St.Anna Kinderkrebs Forschung

iBFM
FLOW network
n=56 labs

- AIEOP-BFM
- ALL-IC
- UKALL
- NOPHO
- Moscow-Minsk





Coordination: Michael N. Dworzak

- assure quality
- make results comparable
- foster collaborative research

Harmonization of leukemia immunophenotyping

AIEOP-BFM Consensus Guidelines 2016 for Flow Cytometric Immunophenotyping of Pediatric Acute Lymphoblastic Leukemia

Michael N. Dworzak,[1*] Barbara Buldini,[2] Giuseppe Gaipa,[3] Richard Ratei,[4] Ondrej Hrusak,[5] Drorit Luria,[6] Eti Rosenthal,[7] Jean-Pierre Bourquin,[8] Mary Sartor,[9] Angela Schumich,[1] Leonid Karawajew,[10] Ester Mejstrikova,[5] Oscar Maglia,[3] Georg Mann,[1] Wolf-Dieter Ludwig,[4] Andrea Biondi,[3] Martin Schrappe,[11] and Giuseppe Basso,[2] on behalf of the International-BFM-FLOW-network

Cytometry B Clin Cytometry, 2017

Immunophenotyping by flow cytometry (FCM) is a worldwide mainstay in leukemia diagnostics. For concordant multicentric application, however, a gap exists between available classification systems, technologic standardization, and clinical needs. The AIEOP-BFM consortium induced an extensive standardization and validation effort between its nine national reference laboratories collaborating in immunophenotyping of pediatric acute lymphoblastic leukemia (ALL). We elaborated common guidelines which take advantage of the possibilities of multi-color FCM: marker panel requirements, immunological blast gating, in-sample controls, tri-partite antigen expression rating (negative vs. weak or strong positive) with capturing of blast cell heterogeneities and subclone formation, refined ALL subclassification, and a dominant lineage assignment algorithm able to distinguish "simple" from bilineal/"complex" mixed phenotype acute leukemia (MPAL) cases, which is essential for choice of treatment. These guidelines are a first step toward necessary inter-laboratory standardization of pediatric leukemia immunophenotyping for a concordant multicentric application.

- multi-color (≥ 6)
- single panel recommended for all ALs
- immunological gate: „Bermude"-area of CD45 plus Lin marker
- in-sample cross-lineage negative controls
- semi-quantitative expression rating
- blast heterogeneities – subclone resolution
- dominant lineage assignement
- MPAL distinction
- refined ALL subclassification

Consensus antibody panel

- Extensive single-platform panel for acute leukemia in children

Mandatory and optional markers (each combined with CD45)	
Intracellular[@#]	iCD3, iCD22, iCD79a, iIgM (μ-chain), iLysozyme, iMPO
Surface[@]	CD2[§], CD3, CD5, CD7; CD10, CD19, CD20; CD11c, CD11b, CD13, CD14, CD15, CD33, CD64, CD65[&], CD117; CD34, (CD45), CD56, HLA-DR if T-ALL: CD1a, CD4, CD8, TCRαβ, TCRγδ if B-IV suspected: κ-chain, λ-chain (surface staining after pre-washing or intracellular)
Optional / Recommended	all cases: NG2[$], CD371[§%] if BCP-ALL: CD11a[§], CD22, CD24, CD38, CD44, CD58, CD66c, CD123[§], CRLF2[§*] if T-ALL: CD99, iTdT if BAL according to general panel: CD24, iTdT

@ mandatory markers for WHO, EGIL, ETP classifications
# prefix "i" stands for intracellular staining
§ phycoerythrin-conjugate (PE) recommended
& available only labelled with fluorescein isothiocyanate (FITC)
$ clone 7.1
% clone 50C1
* clone 1D3

Compatible with:

- WHO 2008/2016
- EGIL score
- „New" ALL subtypes
  - ETP
  - Switch ALL
  - CRLF2+ ALL

New entity: ETP

Early T-cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia

Elaine Coustan-Smith, Charles G Mullighan, Mihaela Onciu, Frederick G Behm, Susana C Raimondi, Deqing Pei, Cheng Cheng, Xiaoping Su, Jeffrey E Rubnitz, Giuseppe Basso, Andrea Biondi, Ching-Hon Pui, James R Downing, Dario Campana

Summary

Background About a fifth of children with acute T-lymphoblastic leukaemia (T-ALL) succumb to the disease, suggesting an unrecognised biological heterogeneity that might contribute to drug resistance. We postulated that T-ALL originating from early T-cell precursors (ETPs), a recently defined subset of thymocytes that retain stem-cell-like features, would respond poorly to lymphoid-cell-directed therapy. We studied leukaemic cells, collected at diagnosis, to identify cases with ETP features and determine their clinical outcome.

Findings 30 patients (12·6%) had leukaemic lymphoblasts with an ETP-related gene-expression signature or its associated distinctive immunophenotype ($CD1a^-$, $CD8^-$, $CD5^{weak}$ with stem-cell or myeloid markers). Cases of ETP-ALL showed increased genomic instability, in terms of number and size of gene lesions, compared with those with typical T-ALL. Patients with this form of leukaemia had high risk of remission failure or haematological relapse (72% [95% CI 40–100] at 10 years *vs* 10% [4–16] at 10 years for patients with typical T-ALL treated at St Jude Children's Research Hospital; and 57% [25–89] at 2 years *vs* 14% [6–22] at 2 years for patients treated in the AIEOP trial).

Interpretation ETP-ALL is a distinct, previously unrecognised, pathobiological entity that confers a poor prognosis with use of standard intensive chemotherapy. Its early recognition, by use of the gene expression and immunophenotypic criteria outlined here, is essential for the development of an effective clinical management strategy.

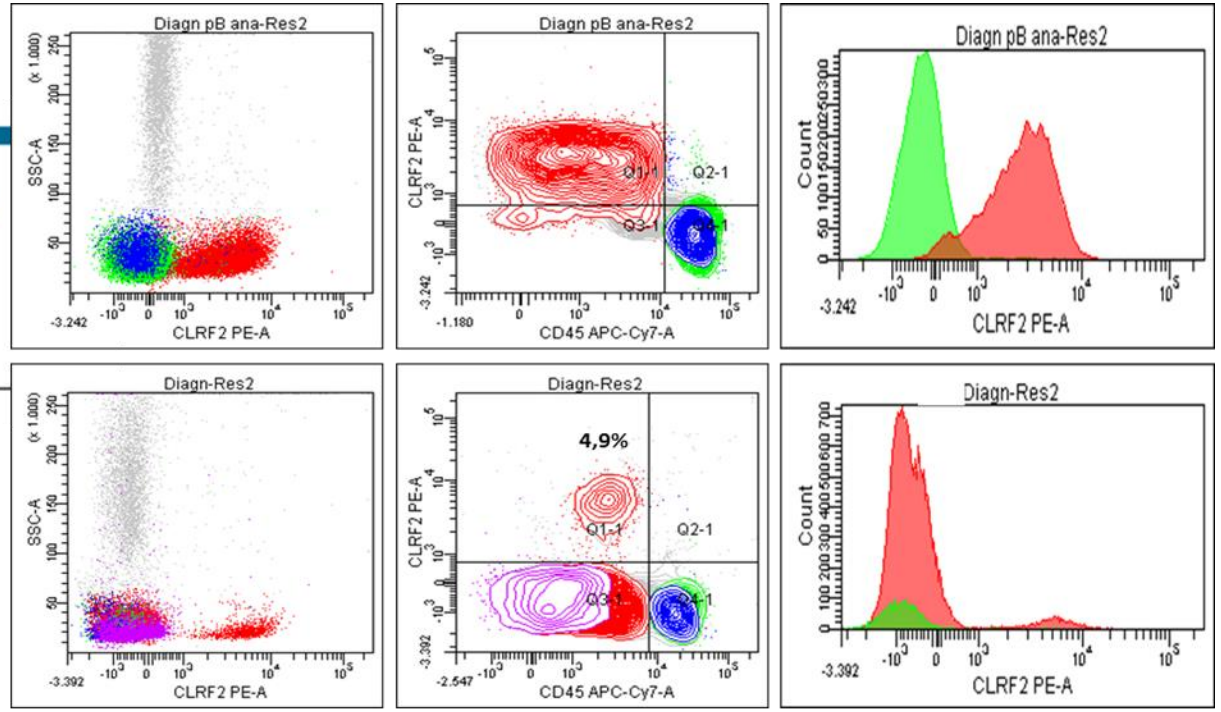
Lancet Oncol 2009; 10: 147–56

New entity: CRLF2r-ALL – CRLF2+

haematologica 2015; 100:e

Fine tuning of surface CRLF2 expression and its associated signaling profile in childhood B-cell precursor acute lymphoblastic leukemia

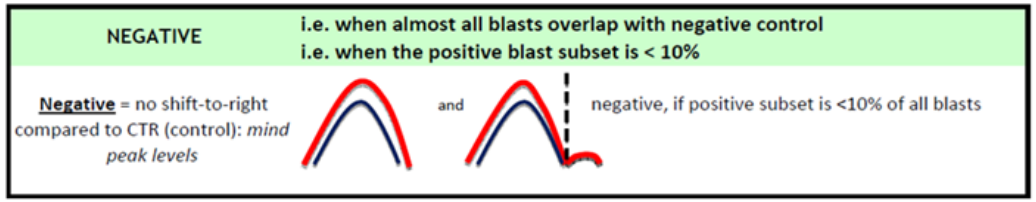
Cristina Bugarin,[1] Jolanda Sarno,[1] Chiara Palmi,[1] Angela Maria Savino,[1] Geertruy te Kronnie,[2] Michael Dworzak,[3] Angela Slumich,[3] Barbara Buldini,[2] Oscar Maglia,[1] Simona Sala,[1] Ilaria Bronzini,[2] Jean-Pierre Bourquin,[4] Ester Mejstrikova,[5] Ondrej Hrusak,[5] Drorit Luria,[6] Giuseppe Basso,[2] Shai Izraeli,[6] Andrea Biondi,[1,7] Giovanni Cazzaniga[1] and Giuseppe Gaipa;[1] on behalf of the I-BFM study group

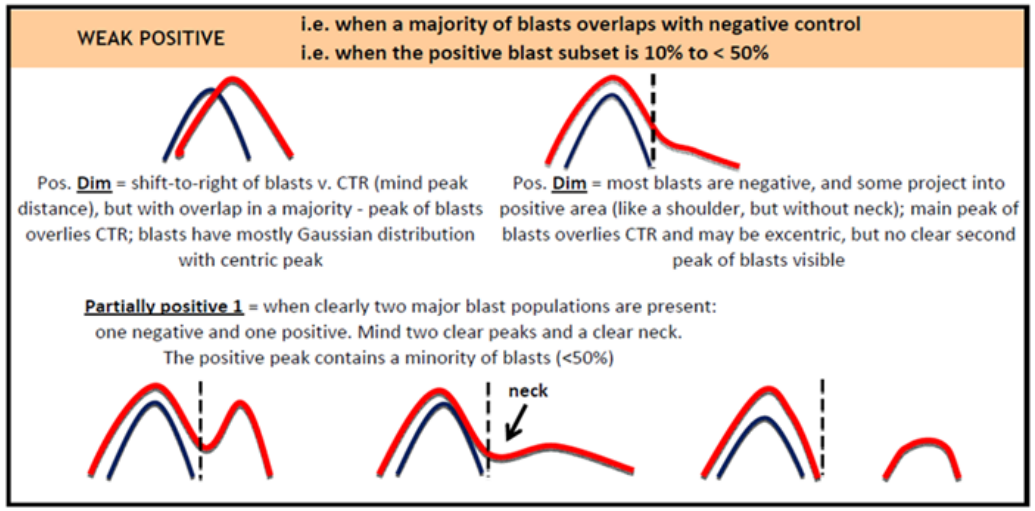


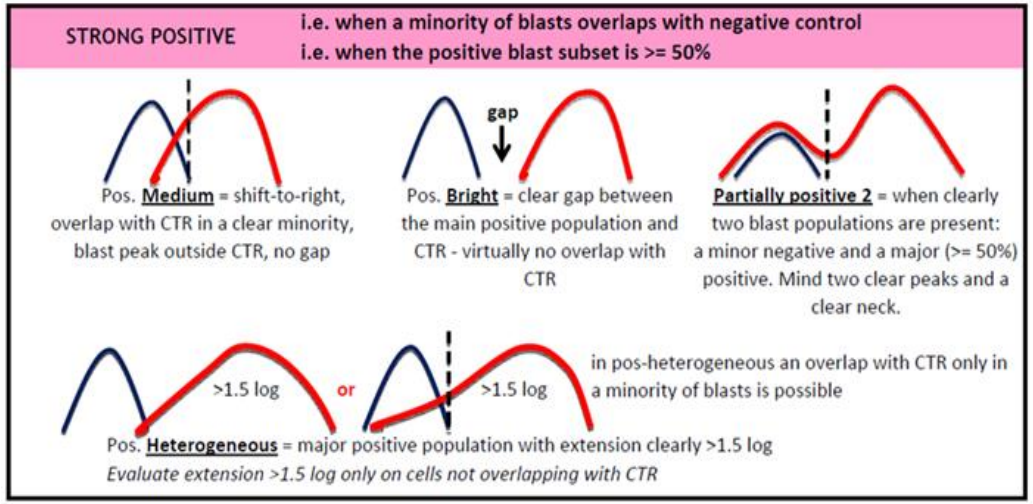
P2RY8-CRLF2+, *IGH@-CRLF2+*)

Expression rating: neg / weak / strong

- In-sample cross-lineage negative control populations







Cytometry Part B (Clinical Cytometry) 72B:S14–S22 (2007)

2006 Bethesda International Consensus Recommendations on the Immunophenotypic Analysis of Hematolymphoid Neoplasia by Flow Cytometry: Optimal Reagents and Reporting for the Flow Cytometric Diagnosis of Hematopoietic Neoplasia

Brent L. Wood,[1*] Maria Arroz,[2] David Barnett,[3] Joseph DiGiuseppe,[4] Bruce Greig,[5] Steven J. Kussick,[6] Teri Oldaker,[7] Mark Shenkin,[8] Elizabeth Stone,[9] and Paul Wallace[10]

[1]Departme ...shington

[3]Department of Haema ...tal, Sheffield, England

[4]Depar ...cticut

[5]De ...ee

[6]Flow Cytomet ...of Washington

[7]I

[10]Roswell Park C ...atory Medicine, Buffalo, New York

The group strongly affirmed the conclusion from the 1997 consensus conference that the reporting of numerical values for each antibody in a simple tabular form is generally unsatisfactory to indicate the presence of abnormal cells, cannot describe their phenotype in sufficient detail, and limits the ability of the recipient of the report to interpret results. Reporting of results in this manner is to be strongly discouraged.

WHO 2008 Criteria for MPAL Definition

Myeloid lineage:
Myeloperoxidase (flow cytometry, immunohistochemistry, or cytochemistry)
or
Monocytic differentiation (NSE, CD11c, CD14, CD64, or lysozyme)

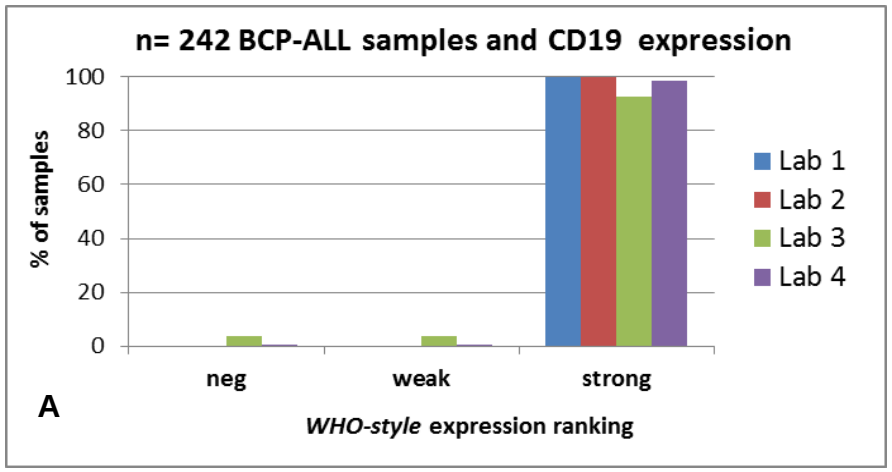
T-lineage:
Cytoplasmic CD3 (flow cytometry with antibodies to CD3 epsilon chain; immunohistochemistry using polyclonal anti- CD3 antibody may detect CD3 zeta chain, which is not T-cell specific)
or
Surface CD3 (rare in mixed phenotype leukaemias)

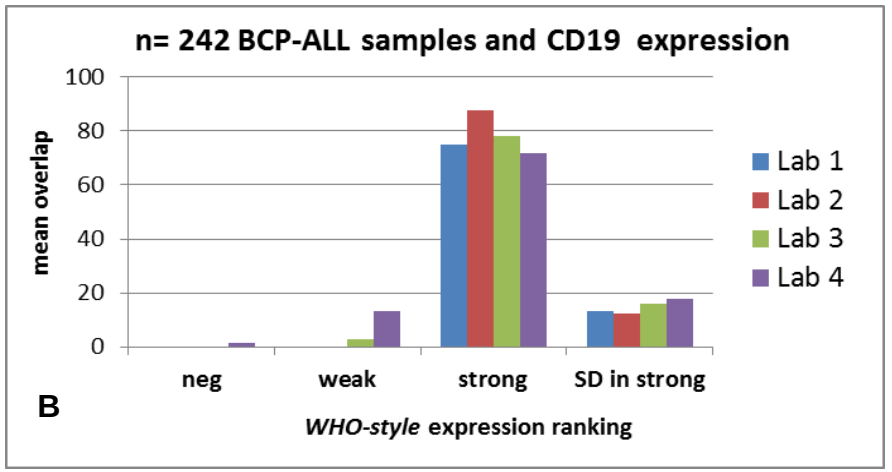
B-lineage (multiple antigens required):
Strong CD19 with at least 1 of the following strongly expressed: CD79a, cytoplasmic CD22, CD10
or
Weak CD19 with at least 2 of the following strongly expressed: CD79a, cytoplasmic CD22, CD10

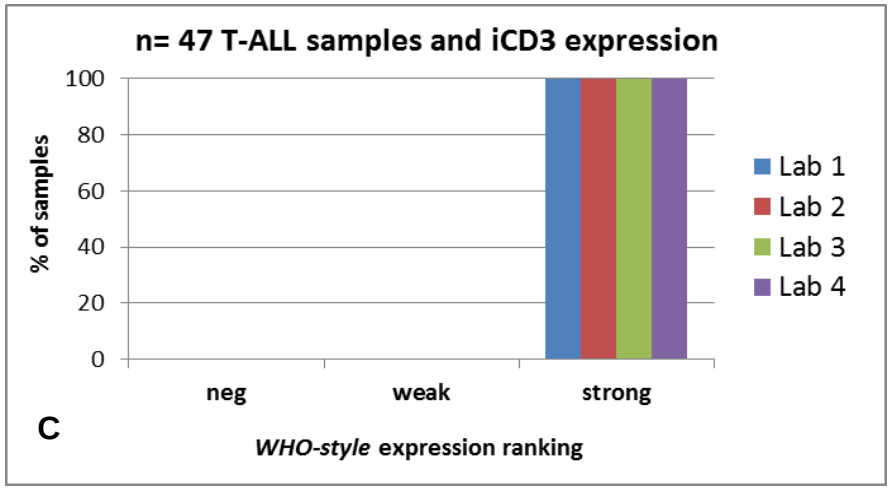
Note: **Monocytic differentiation** requires **positivity of ≥2** of these antigens;

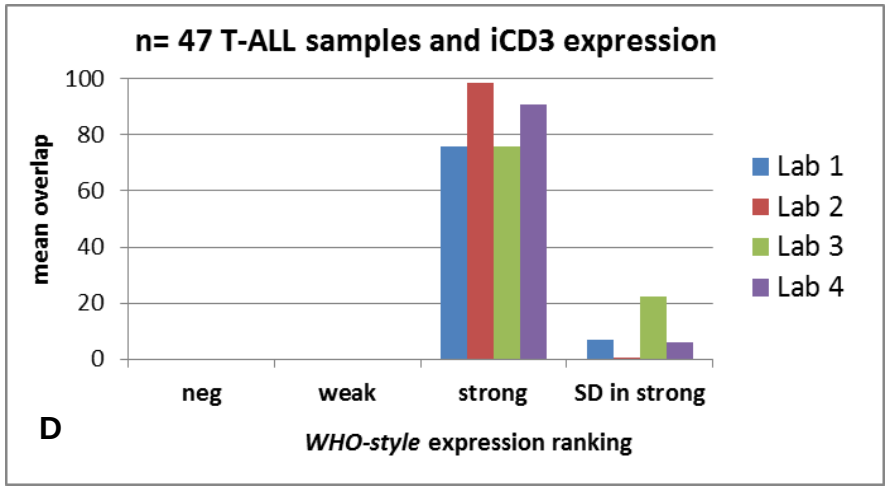
The **T-cell component** is recognized by **bright expression of iCD3**, either on the entire blast population **or on a separate subpopulation of leukemic cells** … should be as bright or nearly as bright as that of normal residual T cells present in the sample.

WHO-style expression ranking









Process	Output
Sample **preparation** and **acquisition**	
Gating on blast population (CD45/LIN/SSC)	**Identify** blast population(s) and determine **blast percentage** among total NC
Complete immunophenotype assessment: **Antigen expression, distribution and intensity** according to *WHO & adapted Bethesda style*	**Inter-lineage** blast clone heterogeneity
Dominant lineage assignement according to *AIEOP-BFM criteria* (negative/weak/strong): based on iMPO, (i)CD3, CD7; CD10, CD19, (i)CD22, iCD79a	**ALL or not**
Further ALL blast characterization	**Intra-lineage** blast clone heterogeneity Immunological **subtype** (BI-BIV; TI-TIV; ETP) **MPAL status** (WHO); BAL status (EGIL)
Immunophenotyping report	**Indication for inclusion into AIEOP-BFM ALL treatment trial**

Dominant lineage concept

TABLE 2. THE AIEOP-BFM DOMINANT LINEAGE ASSIGNMENT [@]

Lineage	Criteria	Antigens
BCP-ALL	≥2 positive of:	[§] CD19; CD10, (i)CD22, iCD79a
T-ALL	all 3 of:	[#] (i)CD3^{pos}, CD7^{pos}; iMPO$^{negative\ or\ weak}$
AML	≥2 positive of: and:	iMPO, CD13, CD33, CD64, CD65, CD117 BCP-/T-ALL criteria not met

[@] Of note, these markers are relevant for dominant lineage assignment, but are insufficient for a thorough description of leukemic immunophenotypes.

[§] BCP-ALL needs strong positivity in ≥2 of the four antigens – in the rare case of CD19-negativity, specifically CD10 must be strong positive. Mind that rare cases of MLL-rearranged BCP-ALL may drop out of this scheme due to biology-inherent lack of CD10, as well as weak (i)CD22 and iCD79a expression (CD19 is then usually strong positive).

[#] For T-ALL, iCD3 positivity must be either strong, or if rated weak, CD2 and/or CD5 should be any positive in addition. Surface CD3 expression needs to be tested in addition.

$P = 0{\cdot}027$). Our data suggest that an intensive therapy regimen including stem cell transplantation may be favourable for bilineal or lineage switch cases, whereas patients with *ETV6/RUNX1* fusion, lymphoid morphology and patients with expression of cyCD22 and cyCD79a should be treated with an ALL-directed therapy.

Gerr et al.,
BJH 2010

Therapy: BFM

MPAL in pediatrics

primarily shows that cytochemical MPO expression in childhood acute leukemia revealing typical lymphoblastic morphology and phenotype does rarely exist. Although a small number of patients studied, cytochemical MPO expression in acute leukemia does not seem to require myeloid leukemia treatment in case of otherwise lymphoblastic cytomorphology and phenotype.

Steiner et al.,
JPHO 2010

Therapy: BFM

Simple immunophenotypic criteria are useful for therapy decisions in MPAL. In B lineage leukemia, MPAL confers poorer prognosis. However, our data do not justify a preferential use of current acute myeloid leukemia-based therapy in MPAL.

Mejstrikova et al.,
Haematologica 2010

Therapy: BFM

An acute lymphocytic leukemia type of induction therapy, using agents that are active against lymphoid and myeloid leukemias, appears to be more effective in achieving and maintaining complete remissions regardless of whether the patients are classified according to EGIL criteria or the new WHO criteria. Hematopoietic stem cell transplantation may not be necessary for all patients in first complete remission.

Al-Seraihy et al.,
Haematologica 2009

Therapy: TTX 13

MPAL – trial inclusion & treatment

Dominant Lineage Assignment

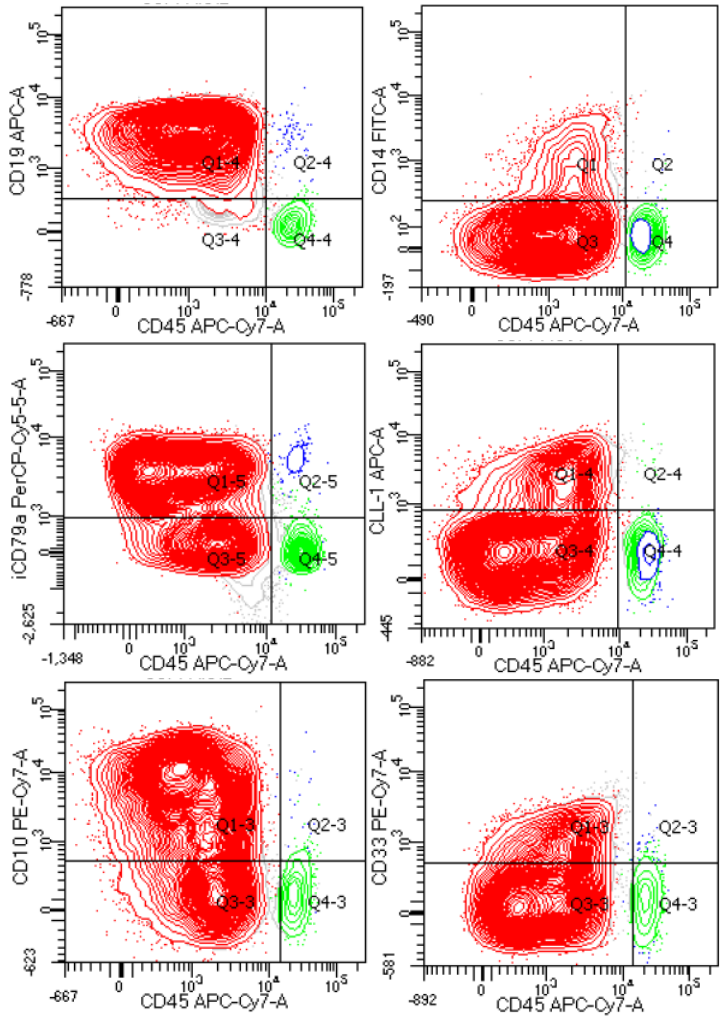
Inclusion into AIEOP-BFM ALL treatment trials is based on morphologically and genetically defined lymphoblastic leukemia with a dominant ALL-immunophenotype according to the AIEOP-BFM lineage assignment criteria (Table 2) adapted from Mejstrikova et al. (20). This is irrespective of whether or not a case fulfils also criteria of MPAL (WHO 2008/2016, Refs. 3,4, and 16; Supporting Information Table 1) or BAL (EGIL, Refs. 9 and 10; Supporting Information Table 2)—even in case of MPO positivity—in a single blast population.

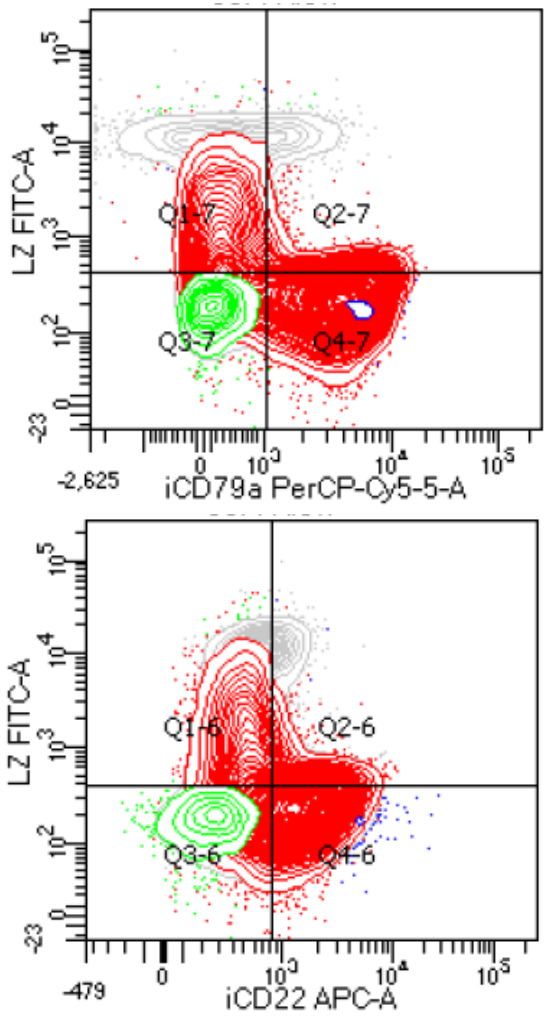
Nevertheless, apart from assigning the dominant lineage of a single or simply branched leukemic clone (e.g., by partial positivity with MPO), a finding of fulfilled MPAL/BAL-criteria needs to be reported as secondary detail. Thus, dominant lineage assignment overrules French-American-British (FAB)-classification as well as MPAL/BAL designations for clinical decision making in AIEOP-BFM with the—important—exception of cases with more than one (separated or complex) blast population including a non-lymphoblastic component (see Supporting Information Fig. 6). In such case, dominant lineage assignment is not applicable as far as both separate components comprise each ≥10% of cells of the sample. In

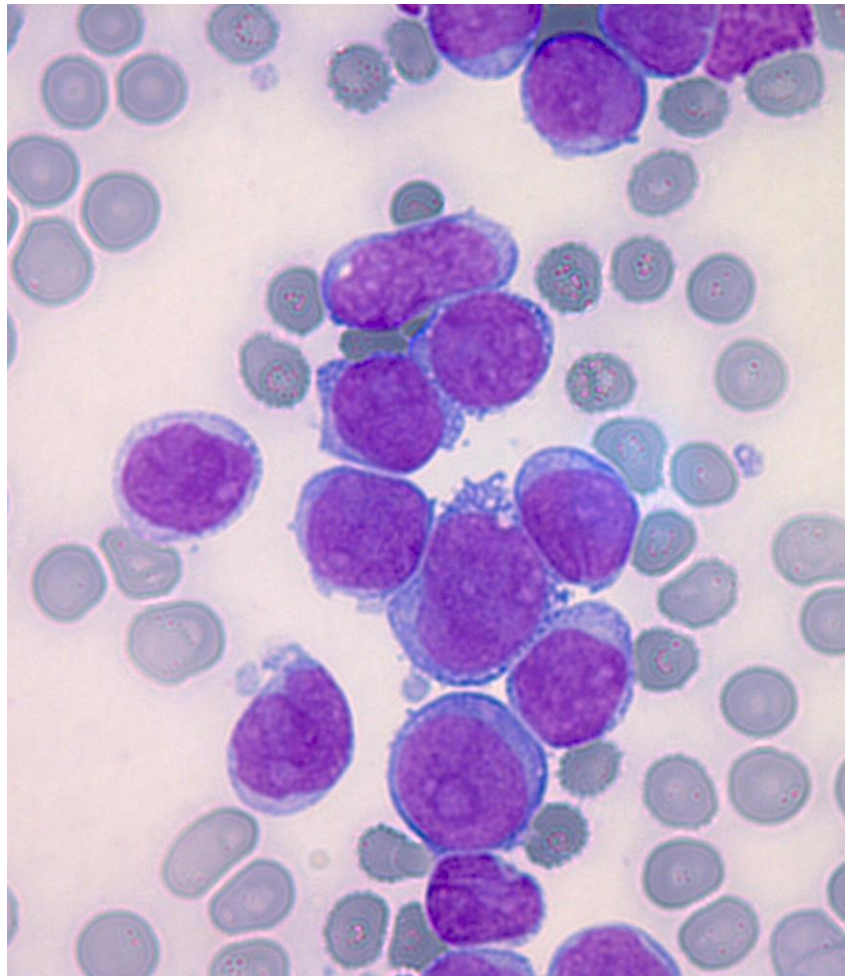
AIEOP-BFM Consensus Guidelines 2016 for Flow Cytometric Immunophenotyping of Pediatric Acute Lymphoblastic Leukemia

Cytometry Part B (Clinical Cytometry)

Dominant lineage: ALL or MPAL?







Dominant lineage: ALL or MPAL?

Phänotyp:

myeloisch		T-lymphoid		B-lymphoid		non-lineage	
MPO	part pos 1	iCD3	neg	CD19	pos med	CD34	part pos 2
Lysozym	part pos 1	CD7	neg	iCD22	pos med	CD38	pos med
CD117	neg	CD2	neg	iCD79a	part pos 2	CD45	part pos 1
CD13	neg	CD5	neg	CD10	part pos 2	HLADR	pos het
CD33	part pos 1	CD1a	n.d.	CD20	part pos 1	CD11a	pos med
CD15	neg	sCD3	neg	CD22	pos med	CD11b	neg
CD65	neg	CD4	n.d.	CD24	pos dim	CD36	n.d.
CD14	part pos 1	CD8	n.d.	CRLF2	neg	CD44	pos bright
CD11c	neg	TCR a/b	n.d.	iIgM	neg	CD56	neg
CD64	neg	TCR g/d	n.d.	iKappa	neg	CD99	pos bright
CD41	n.d.			iLambda	neg	CD123	pos med
CD61	n.d.					CD184	n.d.
CD71	pos med					NG2	neg
CD133	n.d.					CLL-1	part pos 1

Diagnose: BCP-ALL B-II mit B-I Subklon von 26%; ebenfalls subklonal monozytäre Differenzierung - Verdacht auf switch - ALL; CRLF2-Expression auf 1% der Blasten

Lineage: B Subtype (EGIL): BI/II Blastzellanteil: 95%

BAL (EGIL): yes MPAL (WHO): yes multiclonal: yes

Cytometry Part B (Clinical Cytometry) 86B:152–153 (2014)

Editorial

Mixed Phenotype Acute Leukemia

Michael J. Borowitz*
Professor of Pathology and Oncology,
Johns Hopkins Medical Institutions,
Baltimore, Maryland

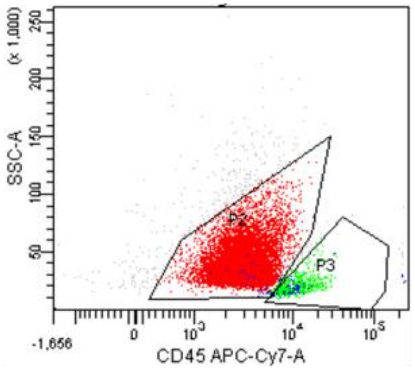
criteria for T/Myeloid MPAL (mixed phenotype acute leukemia) can be met in one of two ways. The criterion most are familiar with requires the expression of the most specific markers for each lineage—in this case cytoplasmic CD3 and myeloperoxidase.

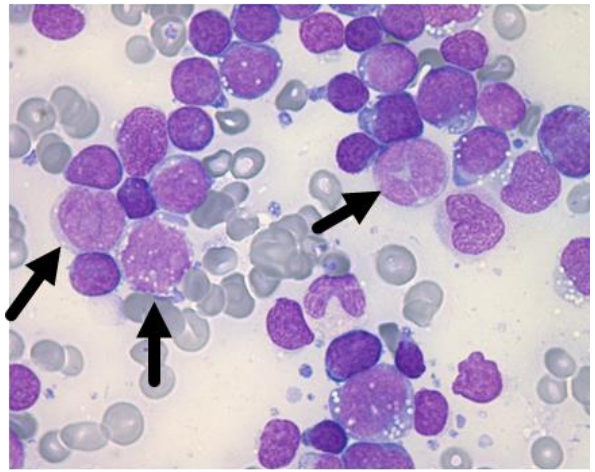
However, less frequently recognized is the fact that expression of these specific markers only applies to the situation in which there is a single population of blasts; criteria for identifying a myeloid component are also met "...when there are two or more distinct populations of leukaemic cells, one of which would meet immunophenotypic criteria for acute myeloid leukaemia (with the exception that this population need not comprise 20% of all nucleated cells)..."

Simple co-expressing MPAL

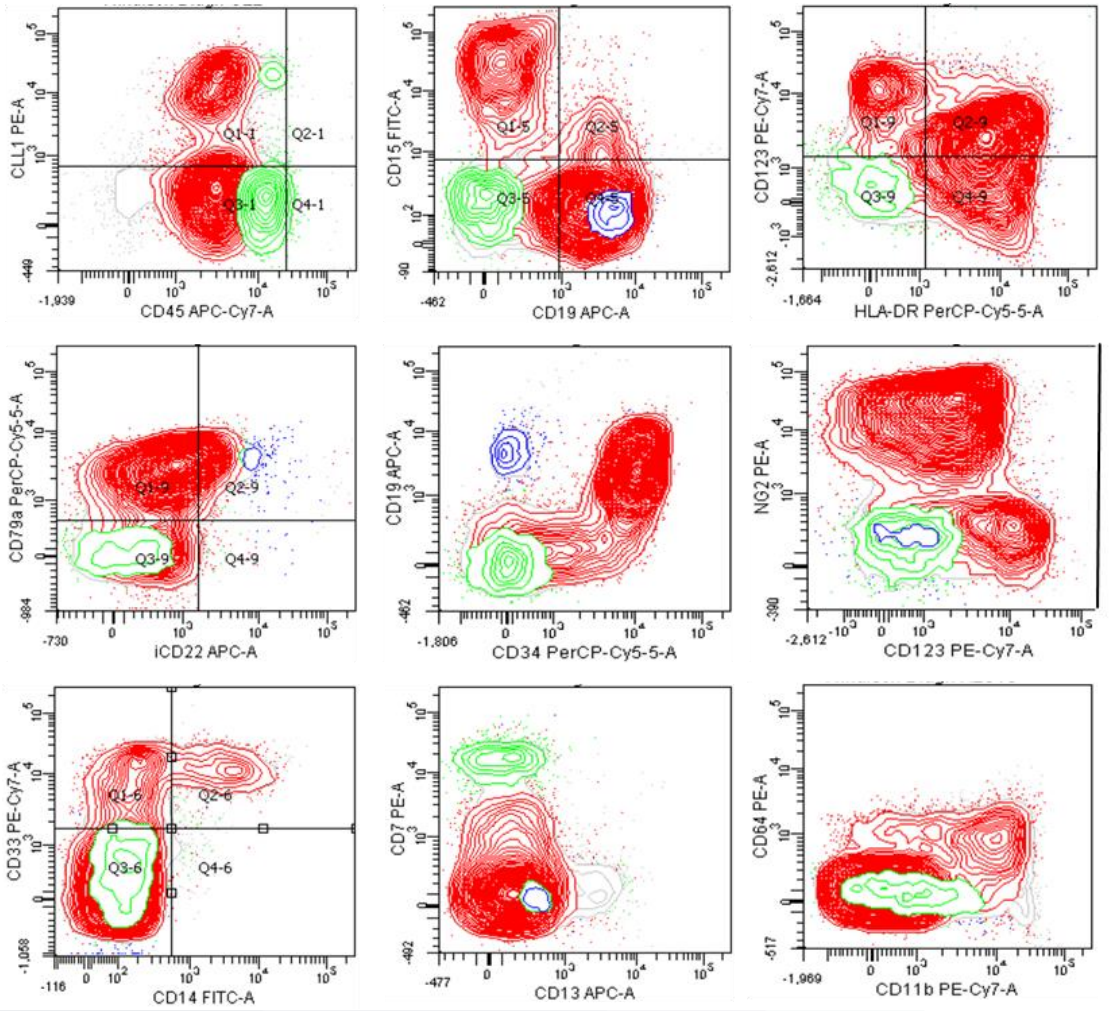
Bi-lineal MPAL

Heterogeneous blasts: MPAL - bilineal



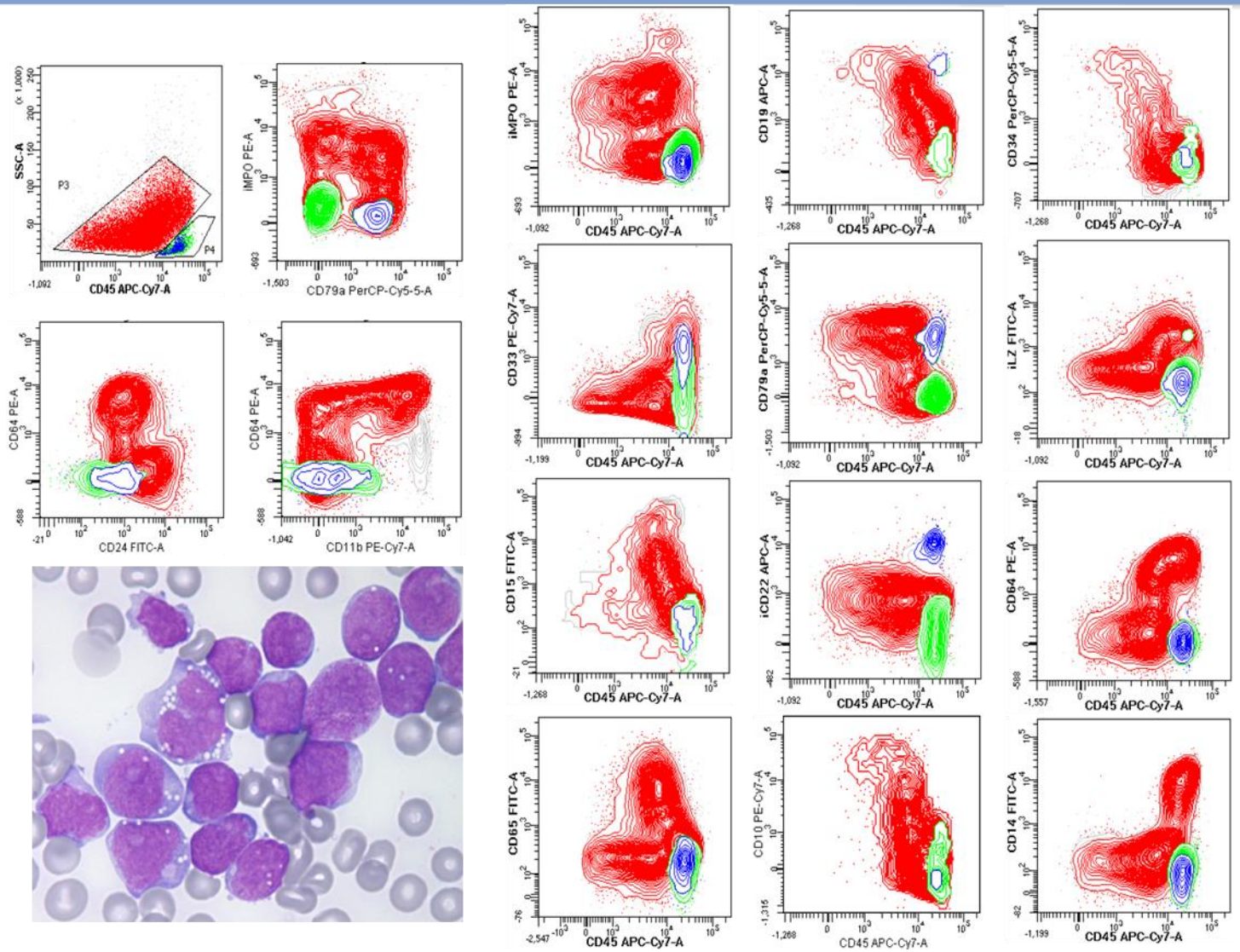


Genetics: 46XY; t(4;11)



- Diagnosis: MPAL B/M
- Complex immunophenotype
- Separate blast subsets with differentiation drift into opposing lineage directions
- No common antigenic denominator of lineage
- Dominant lineage cannot be determined
- No inclusion into AIEOP-BFM trial

Heterogeneous blasts: MPAL - complex



- Diagnosis: MPAL B/M
- Complex immunophenotype
- Branched, interconnected blast subsets with differentiation drift into opposing lineage directions
- No common antigenic denominator of lineage
- Dominant lineage cannot be determined
- No inclusion into AIEOP-BFM trial

Genetics: MLLr & BCR/ABL negative

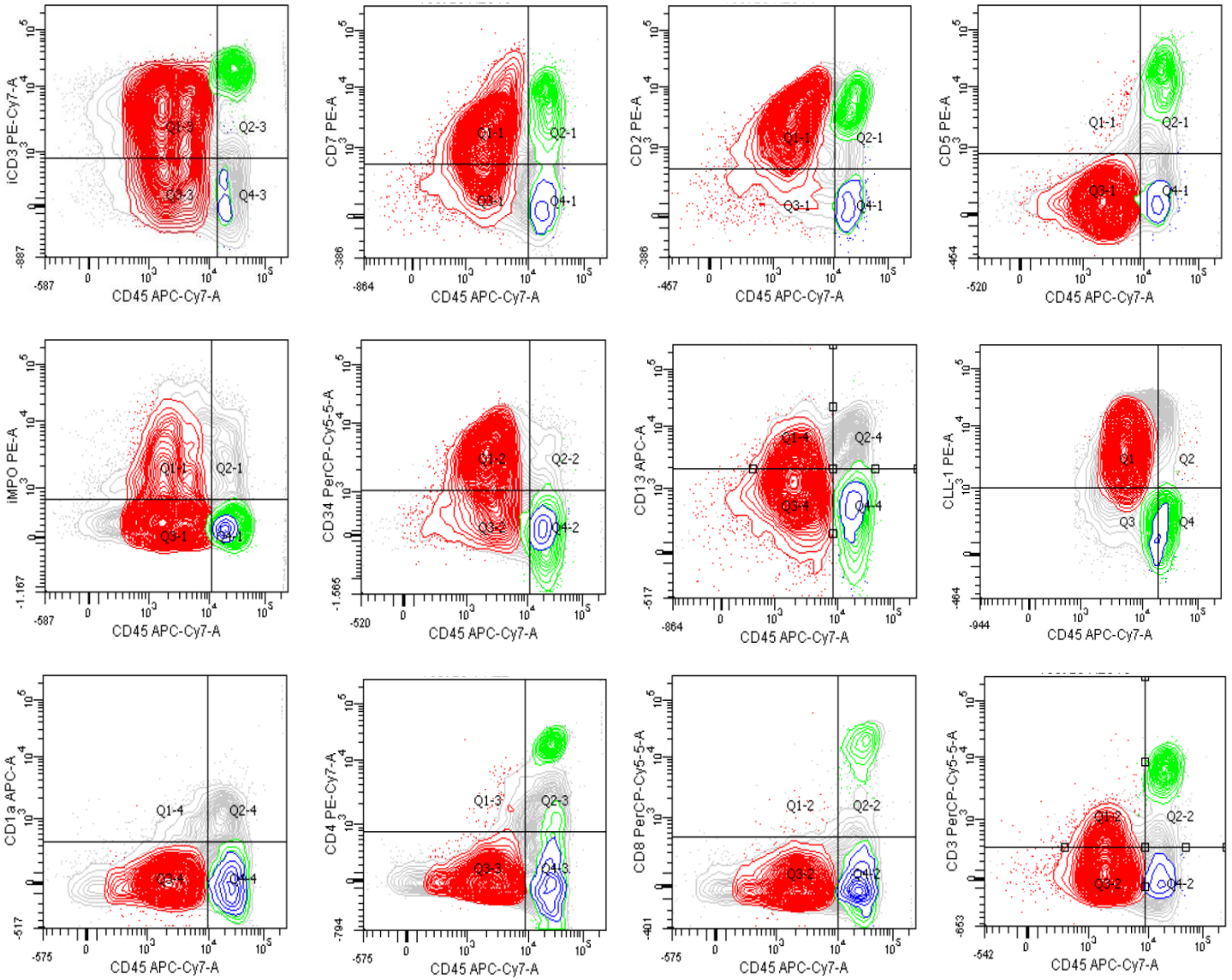
New entity: ETP

Thus, in the broadest sense, ETP ALL is a kind of "T/myeloid" leukemia. From a definitional perspective, however, MPO expression excludes ETP ALL, while the great majority of cases of MPAL are MPO positive. In addition, the T cell component of T/myeloid leukemia frequently would meet criteria for ETP ALL. Thus, these two leukemias appear more alike than different, although because of the central importance of MPO to labeling something as myeloid, and the way leukemia treatment protocols are structured, they are typically treated differently. Unfortunately, this may make it difficult ever to understand whether these do in fact constitute different leukemic entities. It will be interesting to see how this situation will be treated in the next iteration of the WHO classification.

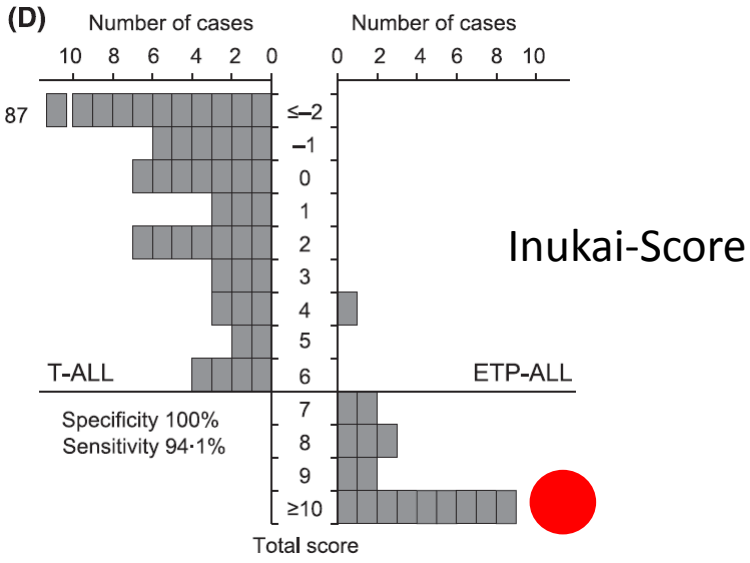
Cytometry Part B (Clinical Cytometry) 86B:152–153 (2014)

Michael J. Borowitz*
Professor of Pathology and Oncology,
Johns Hopkins Medical Institutions,
Baltimore, Maryland

Subtype	Discriminators	Remarks
ETP (only additive to T-I or T-II)	CD1a^{neg}, CD8^{neg} usually CD5$^{neg\ or\ weak\ pos}$ and ≥1^{pos} of HLADR, CD11b,13,33,34,65,117	if CD5$^{strong\ pos}$: ≥2^{pos} of HLADR, CD11b,13,33,34,65,117; sCD3$^{weak\ pos}$ may occur*



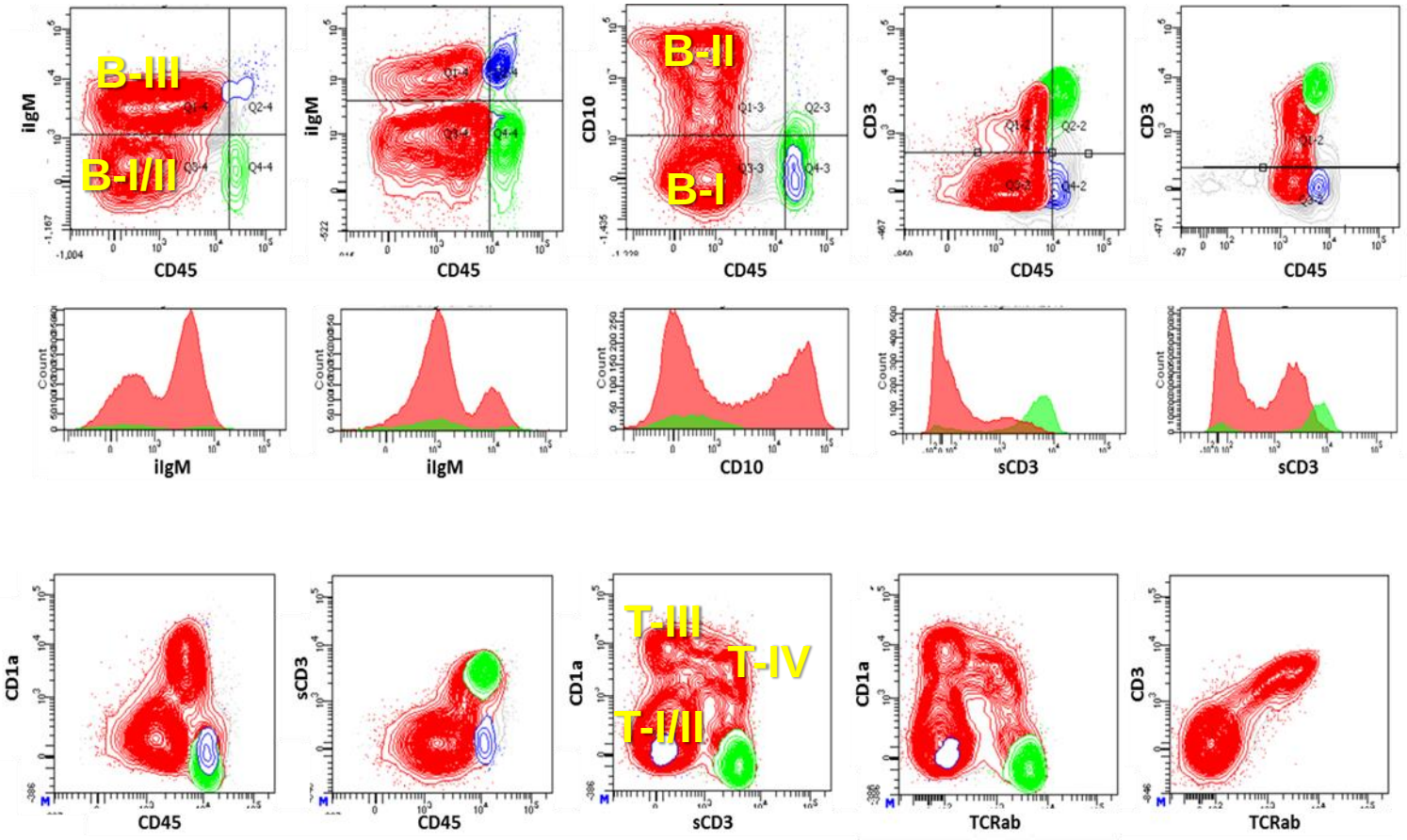
Dgn.: T-ALL
EGIL: **T-II**
ETP: yes
MPAL: **yes**
BAL: **yes**
Blast clone heterogeneity: **yes**



Inukai-Score

Process	Output
Sample **preparation** and **acquisition**	
Gating on blast population (CD45/LIN/SSC)	**Identify** blast population(s) and determine **blast percentage** among total NC
Complete immunophenotype assessment: **Antigen expression, distribution and intensity** according to *WHO & adapted Bethesda style*	**Inter-lineage** blast clone heterogeneity
Dominant lineage assignement according to *AIEOP-BFM criteria* (negative/weak/strong): based on iMPO, (i)CD3, CD7; CD10, CD19, (i)CD22, iCD79a	**ALL or not**
Further ALL blast characterization	**Intra-lineage** blast clone heterogeneity Immunological **subtype** (BI-BIV; TI-TIV; ETP) **MPAL status** (WHO); BAL status (EGIL)
Immunophenotyping report	**Indication for inclusion into AIEOP-BFM ALL treatment trial**

Heterogeneous blasts: ALL subtype drift



"heterogeneous blast populations"

- immunophenotypic **subclone formation**
- **may lead to divergent interpretation and reporting**
- characterized by partial expression of certain markers:
  - B-ALL: iIgM (in 40% of cases), CD10, K/λ-chain
  - T-ALL: sCD3 (in 50% of cases), CD1a, TCR
- differentiation may even drift in a single case from
  - B-I till B-IV
  - T-I/II till T-IV
- cumulative designation adapted from EGIL: e.g. TII/III/IV

TABLE 4: THE AIEOP-BFM SUBCLASSIFICATION OF ALL#

Subtype	Discriminators	Remarks
B-I (pro-B)	$CD10^{neg}$	BCP-ALL lineage criteria fulfilled
B-II (common)	$CD10^{pos}$	/
B-III (pre-B)	$iIgM^{pos}$	$CD10^{neg\ or\ weak\ pos}$ may occur§
B-IV (mature B)	κ- or λ-$chain^{pos}$	may occur with FAB L1/L2 morphology&
T-I (pro-T)$^{\$}$	only $iCD3^{pos}$ and $CD7^{pos}$	T-ALL lineage criteria fulfilled
T-II (pre-T)	≥1 of $CD2^{pos}$, $CD5^{pos}$, $CD8^{pos}$	surface (s) $CD3^{weak\ pos}$ allowed*
T-III (cortical T)	$CD1a^{pos}$	$sCD3^{weak}$ may occur*
T-IV (mature T)	$CD1a^{neg}$ and $sCD3^{pos}$*	$sCD3^{strong}$, or $sCD3^{weak\ pos}$ with TCR^{pos}
ETP (only additive to T-I or T-II)	$CD1a^{neg}$, $CD8^{neg}$ usually $CD5^{neg\ or\ weak\ pos}$ and ≥1^{pos} of HLADR, CD11b,13,33,34,65,117	if $CD5^{strong\ pos}$: ≥2^{pos} of HLADR, CD11b,13,33,34,65,117; $sCD3^{weak\ pos}$ may occur*

adapted from refs. 8 & 9.

§ $CD10^{neg/weak}$ B-III is frequently associated with MLL-rearrangements (12).

& light-$chain^{pos}$ cases without FAB L3-morphology and without *MYC*-translocation are eligible for conventional ALL treatment, and thus must be separated from Burkitt-type mature B-ALL (40-43).

$ T-I is very rare and can be reported together with T-II (as T-I/II)

* Dim or even more frequently partial surface positivity with CD3 (e.g. in a minor blast subpopulation) occurs when sensitive methodology is used and should not mislead to diagnose mature T-ALL in the absence of TCR expression.





Mixed Lineage Leukemia **– Rearranged Childhood Pro-B and CD10-Negative Pre-B Acute Lymphoblastic Leukemia Constitute a Distinct Clinical Entity**

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Refined subclassification: prec-B-IV (non-Burkitt)

Precursor B Lymphoblastic Leukemia With Surface Light Chain Immunoglobulin Restriction

A Report of 15 Patients

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Key Words: Precursor B cell; Acute lymphoblastic leukemia; Surface immunoglobulin–positive acute leukemia; Flow cytometry; WHO classification; Immunophenotyping

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Abstract

We describe 15 patients (9 children) with precursor B-cell (pB) acute lymphoblastic leukemia (ALL) with surface immunoglobulin (sIg) light chain restriction revealed by flow cytometric immunophenotyping (FCI). The same sIg+ immunophenotype was present at diagnosis and in 3 relapses in 1 patient. In 15 patients, blasts were CD19+CD10+ (bright coexpression) in 14, CD34+ in 12, surface κ+ in 12, surface λ+ in 3; in 8 of 8, terminal deoxyribonucleotidyl transferase (TdT)+; and in 4, surface IgD+ in 2 and surface IgM+ in 1. The 3 CD34– cases included 1 TdT+ case, 1 with t(1;19)(q23;p13), and 1 infant with 70% marrow blasts. One adult had CD10–CD19+CD20–CD22+CD34+ TdT+sIg+ blasts with t(2;11)(p21;q23). Blasts were L1 or L2 in all cases (French-American-British classification). Karyotypic analysis in 12 of 12 analyzable cases was negative for 8q24 (myc) *translocation. Karyotypic abnormalities, confirmed by fluorescence in situ hybridization in 6 cases, included hyperdiploidy, t(1;19)(q23;p13), t(12;21)(p13;q22), t(9;22)(q34;q11), t(2;11)(p21;q23), and trisomy 12. The sIg light chain restriction in pB ALL might be present in neoplasms arising from the early, intermediate, and late stages of precursor B-cell maturation; sIg light chain restriction revealed by FCI does not necessarily indicate a mature B-cell phenotype, further emphasizing the importance of a multidisciplinary approach to diagnosing B-lymphoid neoplasms.*

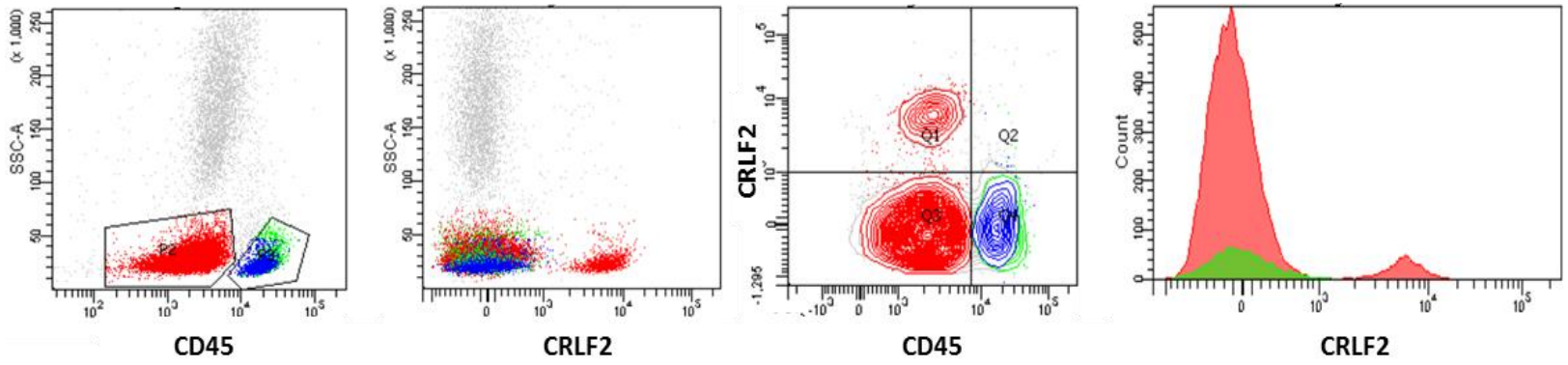
Reporting

TABLE 4: CONTENTS OF IMMUNOPHENOTYPING REPORTS IN AIEOP-BFM TRIALS

Item	Contents
Identification	Name, code number, date of birth, question/suspicion, type of sample, dates of sampling, processing/analysis, reporting, name/signature(s) of technical analyst and medical reporter
Antigen expression	"*WHO-style*" rating: *negative*, *positive weak*, *positive strong* "*Bethesda-style*" rating: as add-on, at least *partially positive*
Dominant lineage[#]	B-, T-, Myeloid-lineage independently of MPAL/BAL-status; not applicable if bilineal leukemia with non-lymphoblastic component, AUL, NK/myeloid precursor- or DC-leukemia
Subtype[§]	B-I to B-IV, T-I to T-IV; report ETP as additive result
Heterogeneities	WHO 2008 MPAL, adapted EGIL BAL criteria (also B/T and B/T/My) (Intra-lineage) blast clone heterogeneity
Quantities	Blast percentage among NC (if applicable, separate by subclones)
Conclusions	Diagnosis as relevant for treatment choice; additional information of potential clinical interest (e.g. towards underlying genetic lesions)

[#] Bilineal leukemia with a lymphoblastic and a separate non-lymphoblastic blast subset, as well as AUL, NK/myeloid precursor- and DC-leukemia are excluded from AIEOP-BFM ALL trials.
[§] Combinations of subtype labels are appropriate in case of blast subset formation at different maturation levels; B-IV with FAB-L3 morphology and *MYC*-rearrangements ("Burkitt-type") are excluded from AIEOP-BFM ALL trials, whereas B-IV cases with L1/L2-morphology and other genetic lesions are included.

Heterogeneous blasts: clones <10%



Blast cell heterogeneities in the sense of **immunophenotypic subclone formation** should not be overlooked even if discriminative antigen-positivity occurs in less than 10% **of blasts**, i.e. the usual threshold to claim an antigen as being positive on a relevant population of cells. Of note, this **threshold is artificial and derives from the necessity to separate unambiguous expression from background** which is **influenced by a plenitude of factors** like sample quality, cell viability, autofluorescence, antigen of interest, fluorochrome, antibody combination, compensation matrix, negative control strategy etc. Hence, as shown in this BCP-ALL case with a minor CRLF2-positive blast sub-clone of 5%, the case result for CRLF2 expression is "negative" (for the major blast population). However, the **existence of the small** CRLF2-positive blast subclone must be captured in the item "heterogeneous blast population" and described in the report conclusion.

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SUPPL. TABLE 1: WHO 2008/2016 LINEAGE CRITERIA FOR MPAL DEFINITION[#]

Lineage	Antigens
Myeloid	MPO or ≥ 2 monocytic antigens (NSE, CD11c, CD14, CD64, iLysozyme)
T-lymphoid	Strong[§] expression of CD3 (ε-chain; usually cytoplasmic)
B-lymphoid	Strong expression of ≥ 2 antigens of CD19, CD79a, iCD22, CD10

[#] adapted from refs. 3,4 &16

[§] The T-cell component is recognized by strong expression of iCD3 … on the entire blast population, or on a separate subpopulation of leukemic cells … should be as bright or nearly as bright as that of normal residual T cells present in the sample (see ref. 16).

SUPPL. TABLE 2: EGIL SCORING SYSTEM FOR BIPHENOTYPIC ACUTE LEUKEMIA[#]

Points	B-lineage	T-lineage	Myeloid lineage
2	iCD22, iCD79a, iIgM	CD3, TCR	iMPO, iLysozyme
1	CD10, CD19, CD20	CD2, CD5, CD8, CD10	CD13, CD33, CD65, CD117
0.5	CD24, TdT	CD1a, CD7, TdT	CD14, CD15, CD64

[#] adapted from refs. 9 & 10

Note: **BAL is defined** when the **scores from two separate lineages are both > 2 points**.
Specific rules for cases of B/T or tri-lineage differentiation – see *chapter 7 in the Guidelines*.