Dr. Mattias Hofmans
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UZ Gent

EuroFlow standardization for FCM MRD applications in hemato-oncology

Towards Next Generation Flow cytometry

09/02/2024
Workshop Molecular Biology and Cytometry Course 2024

UZ GENT EuroFlow

UZ GENT

UNIVERSITEIT GENT

Introduction

EuroFlow consortium

Measurable Residual Disease (MRD)

From PCR methods to next generation flow (NGF)

UZ GENT

EuroFlow

European networks for laboratory diagnostics

Since 1996
BMH4-CT98-3936 CA
FP4 BIOMED-2 program

Since 2001
BMH-CMT94-1675 CA
FP2 BIOMED-1 program
+ I-BFM-SG and Pre-BMT-SG

Since 2006
LSHB-CT-2006-018708
FP6 STREP LSH-2004

Participants: Based on experience and participation in (inter)national clinical trails

EuroFlow

EuroFlow: independent scientific consortium, aiming at innovation in flow cytometry for improvement of diagnostic patient care

- Initiated in 2004 (FP6 STREP LSH-2004)
- Formal project duration: April 2006-Oct 2009 (LSHB-CT-2006-018708)
- Sustained based on collective IP and patents and collective revenues

Chairmen:
J.J.M. van Dongen & A. Orfao

20 institutes (23 laboratories)
in 11 countries

www.EuroFlow.org

January 2024

Aims of the EuroFlow consortium

1. Research & Innovation of diagnostic patient care
   - Development and standardization of fast, accurate, and highly sensitive flow cytometric tests for diagnosis and prognostic (sub)classification as well as for evaluation of treatment effectiveness during follow-up.
     - Hematological malignancies
     - Immune disorders, including immunodeficiencies
     - Immune monitoring in different medical conditions, including immunotherapies
     - Solid tumors (STOT tube)

2. Standardization of laboratory diagnostics
   - All protocols/standard operating procedures (SOPs) that are developed and standardized by EuroFlow are freely available (https://euroflow.org/)

3. Quality Assessment, including QA evaluation meetings
   - external Quality Assessment program
   - Current aim to get ISO 17043 Accreditation for organisation of EQA programs

4. Continuous education: Seminars, Workshops, and Trainings

Diagnostics for hematological malignancies

1. Making the diagnosis

   Normal ↔ reactive/regenerating ↔ malignant

   Annually > 300,000 new patients with a hematological malignancy in developed countries. Consequently at least 4-fold more patients are annually being checked for exclusion of such malignancy!

Screening tubes

2. Classification of hematopoietic malignancies

   Relation with prognosis: relevance of risk-group definition

   Based on differentiation characteristics and chromosome aberrations, resulting in fusion gene transcripts or aberrantly (over) expressed genes

Classification tubes

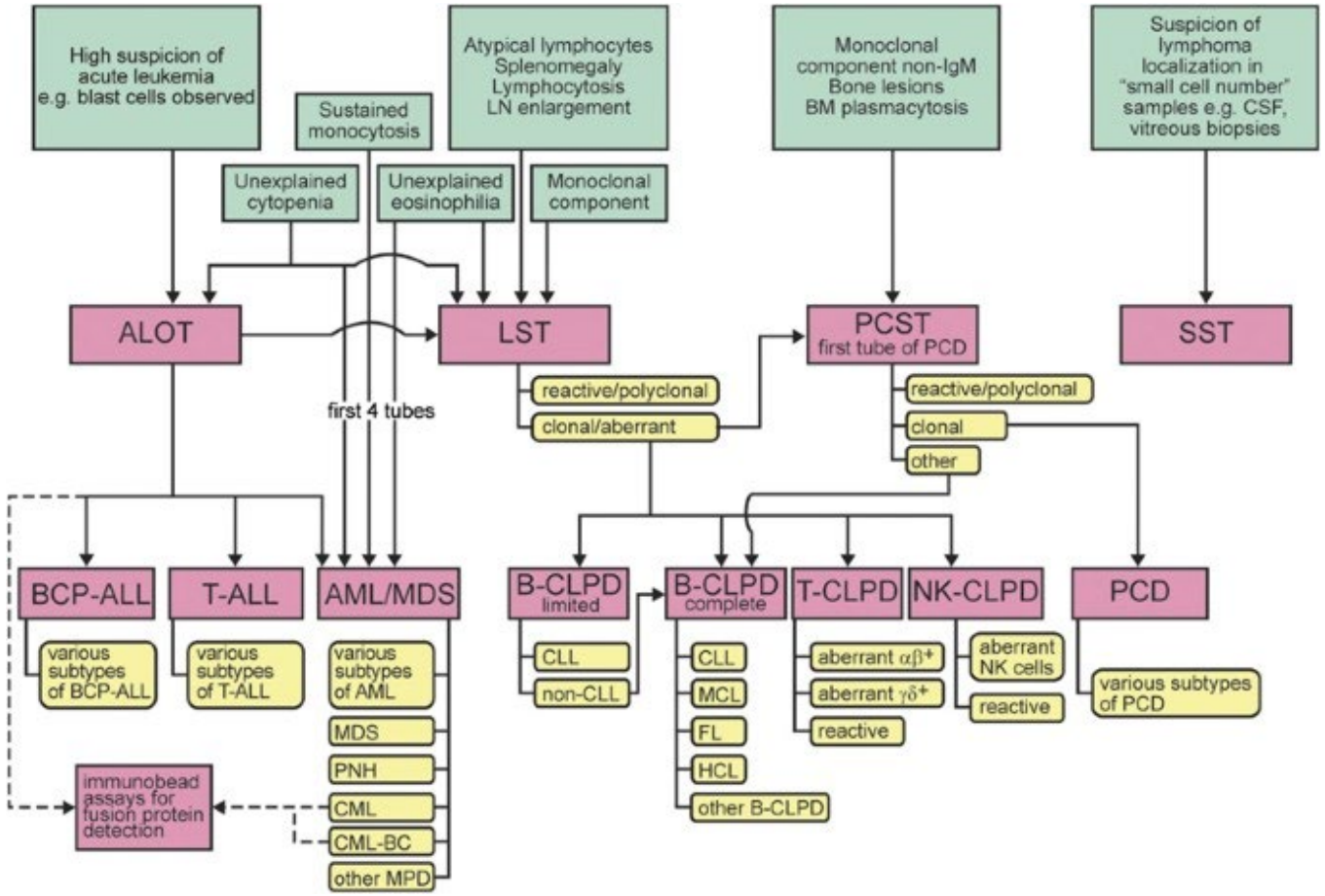
3. Evaluation of treatment effectiveness (MRD):

   MRD-based risk-group stratification (treatment reduction or escalation)

   Annually > 400,000 follow-up samples in protocol-based leukemia patients (ALL, AML, CML); patients with CLL and NHL will follow soon (> 1,000,000 follow-up samples)

MRD tubes

EuroFlow antibody panels for standardized *n*-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes



Screening tubes

Classification tubes

van Dongen JJM et al. EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. Leukemia 2021

New therapies warrant extensive patient monitoring in many different diseases

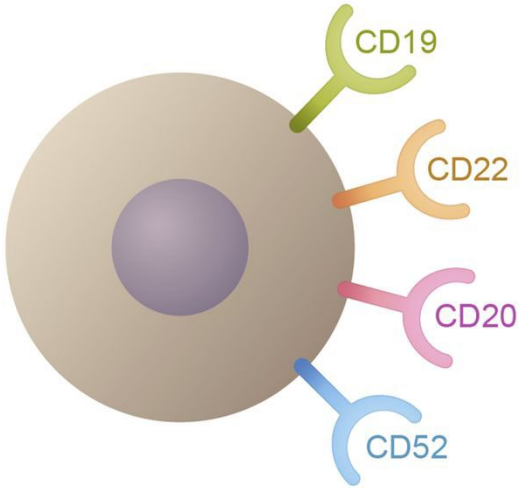
auto-immune diseases, infectious diseases, cardiovascular diseases, oncology

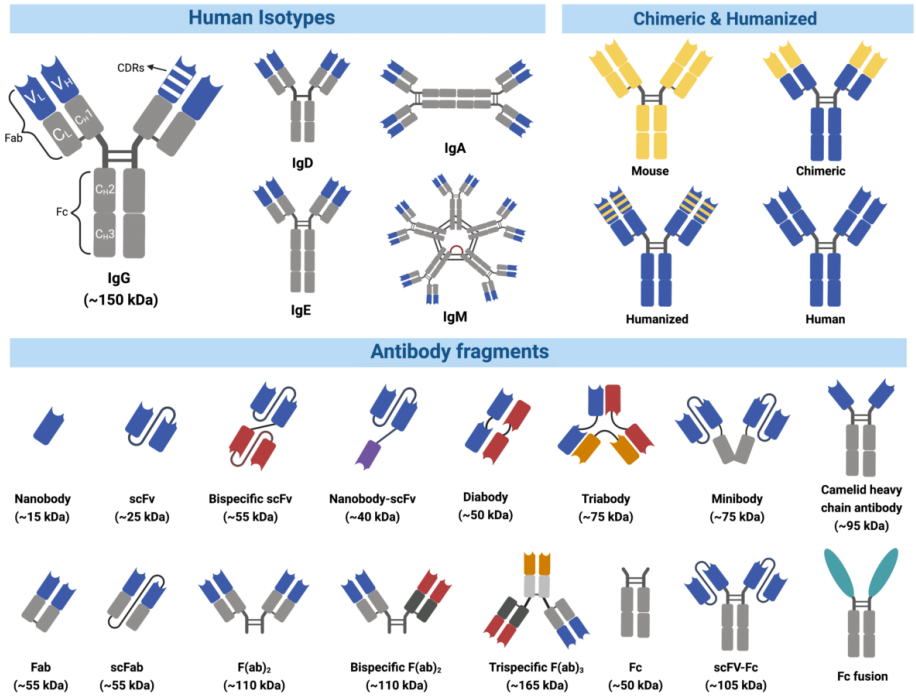
EuroFlow

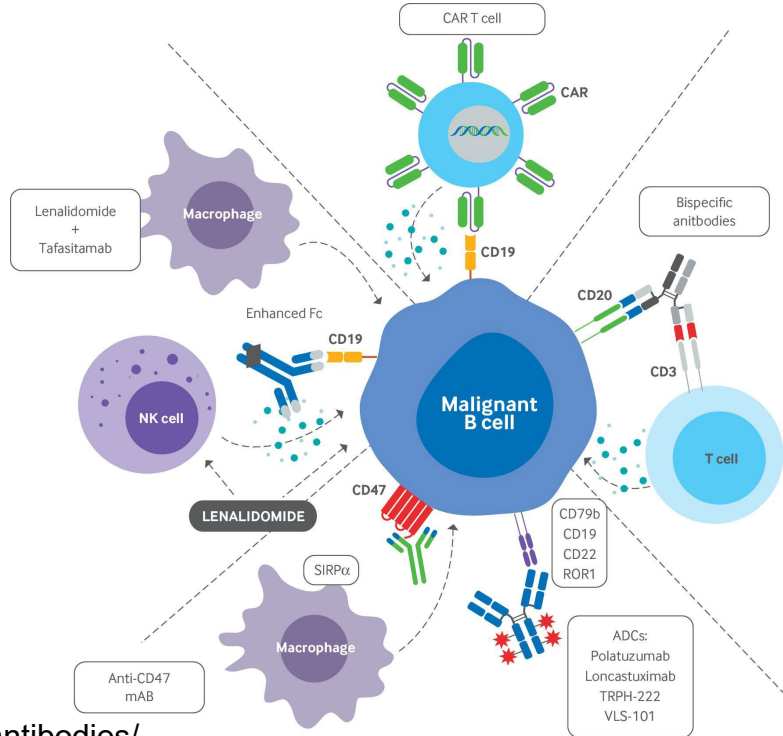
Changes in diagnostic strategies

- Many new therapeutics for targeted (immune) therapies are being developed

Many B-cell targets CD19, CD20, CD22, CD38, CD47, BCMA, …







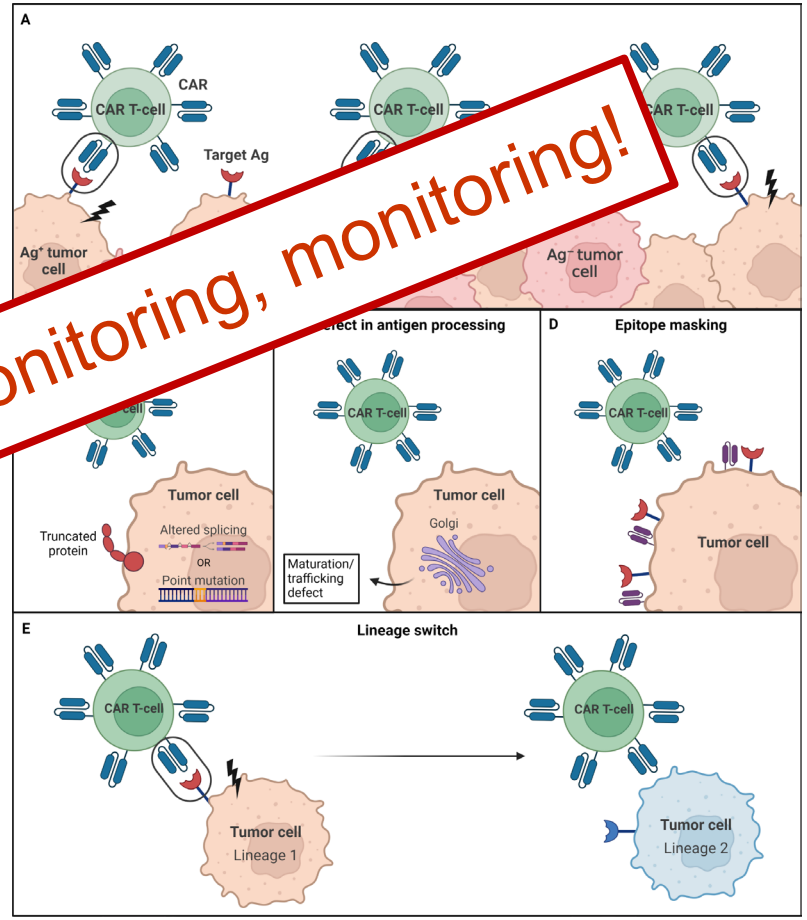
Jabbour et al. Blood 2015; Sawalha Y and Maddocks K. BMJ 2022; https://www.rapidnovor.com/what-are-monoclonal-antibodies/

New therapies warrant extensive patient monitoring in many different diseases

EuroFlow

- Most new immune-therapeutics block or manipulate specific molecules.
  - Frequently this leads to disease control with significant increase of QOF (not necessarily cure).
- The more targeted the therapy, the more chance of mutation-induced resistance
  - Early detection of th… will become incre… (for new therapy…

CONSEQUENCE: Monitoring, monitoring, monitoring!

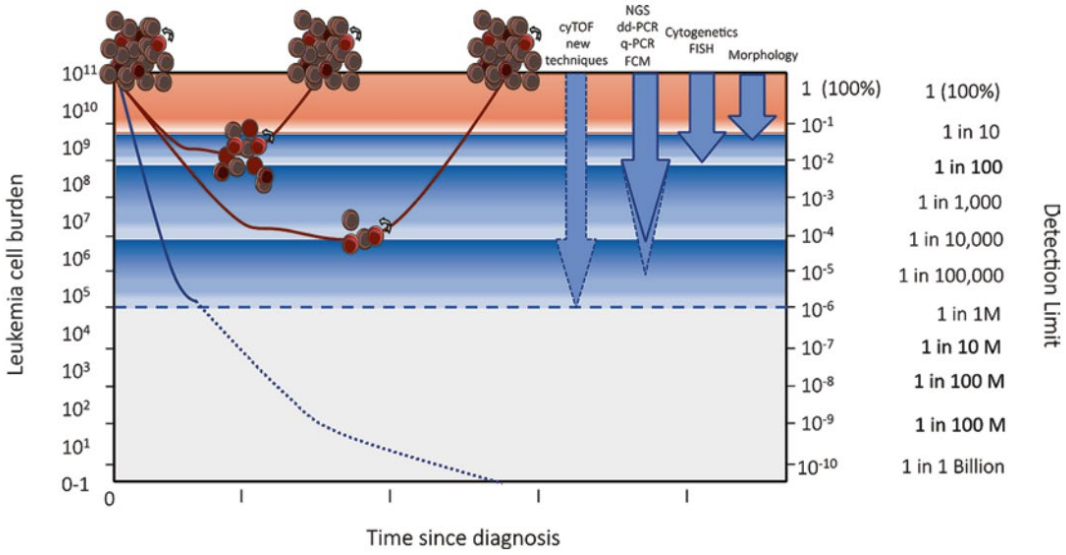


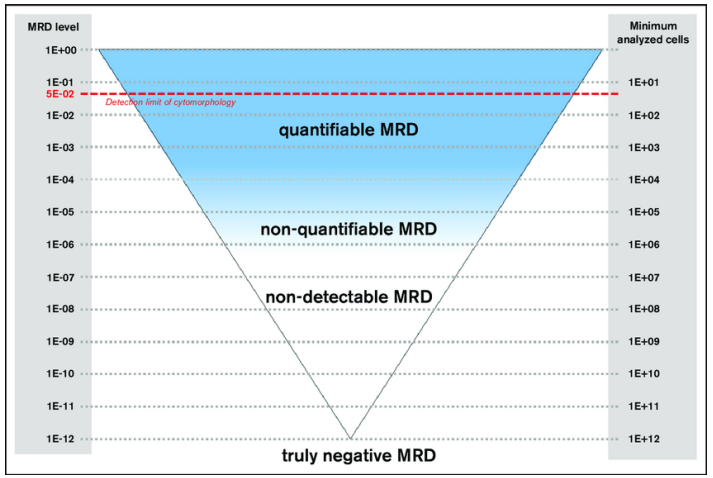
https://lymphomahub.com/medical-information/educational-theme-or-tumor-intrinsic-resistance-mechanisms-to-car-t-cell-therapy-in-lymphomas

Detection of minimal/measurable residual disease (MRD)

- Complete remission (CR): less than 5% of residual blast in BM and normalization of CBC

- Measurable residual disease (MRD) is the number of leukemic cells that remain after the treatment.
  - Dependent on the sensitivity of the technique used
  - A good MRD approach should be
    - Sensitive
    - Highly specific
    - Reproducible
    - Standardized procedure with ideally a wide interinstitutional validation.
  - In theory, if the sensitivity increases to a maximum, then there will be higher chances of achieving a longer relapse free survival (RFS) and potentially achieve a cure.





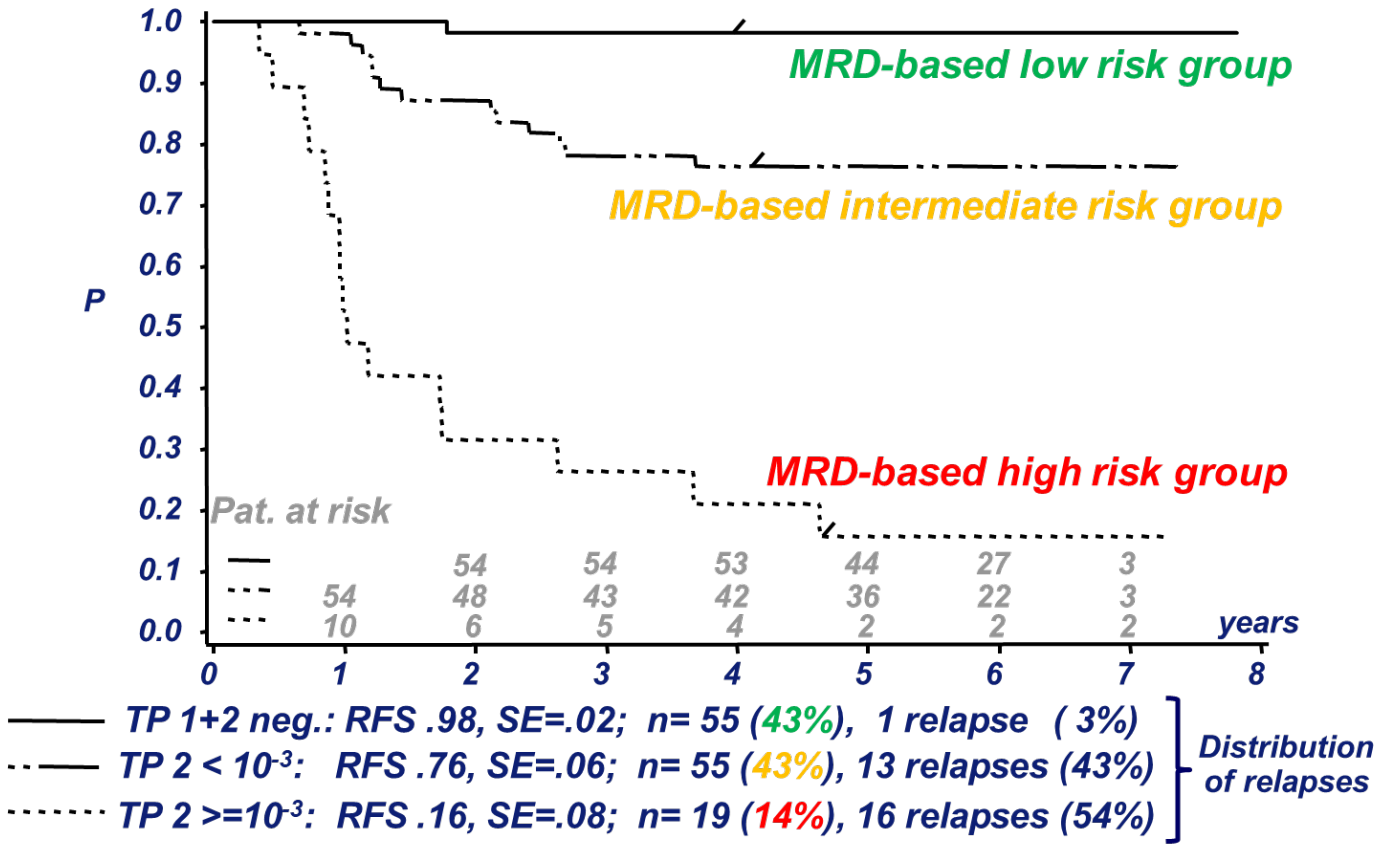
Short N et al. Am. J. Hematology 2018; Letetsu R et al Leukemia 2020; JJM van Dongen et al. The Lancet 1998

Clinical VALUE of MRD detection

- Kinetics:
  - tumor load reduction during and after induction treatment provides crucial information about the response to treatment
- Prognosis:
  - Predictor (independent prognosticator) of outcome of patients with leukemia
  - Prediction of relapse
  - Prognostic relevance of patients undergoing stem cell transplantation
- Treatment:
  - Surrogate endpoint to define response to treatment and to define treatment allocation.
  - MRD-based stratification: identification of low-risk (therapy reduction), intermediate and high-risk (therapy intensification) patients

MRD as prognostic marker

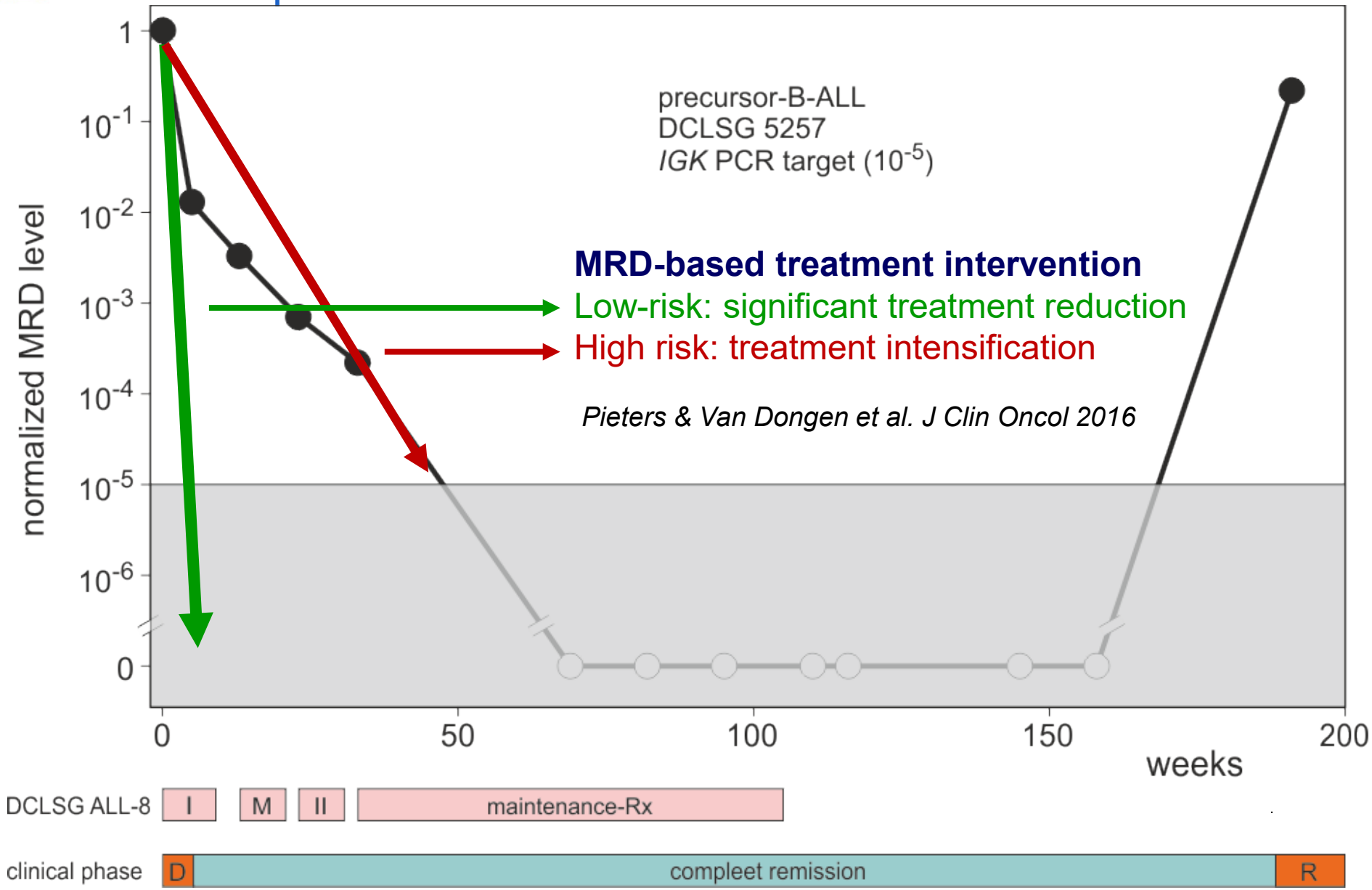
Relapse-free survival of the three MRD-based risk groups in childhood ALL



** Data update, see JJM van Dongen et al., Lancet 352 (1998): 1731*

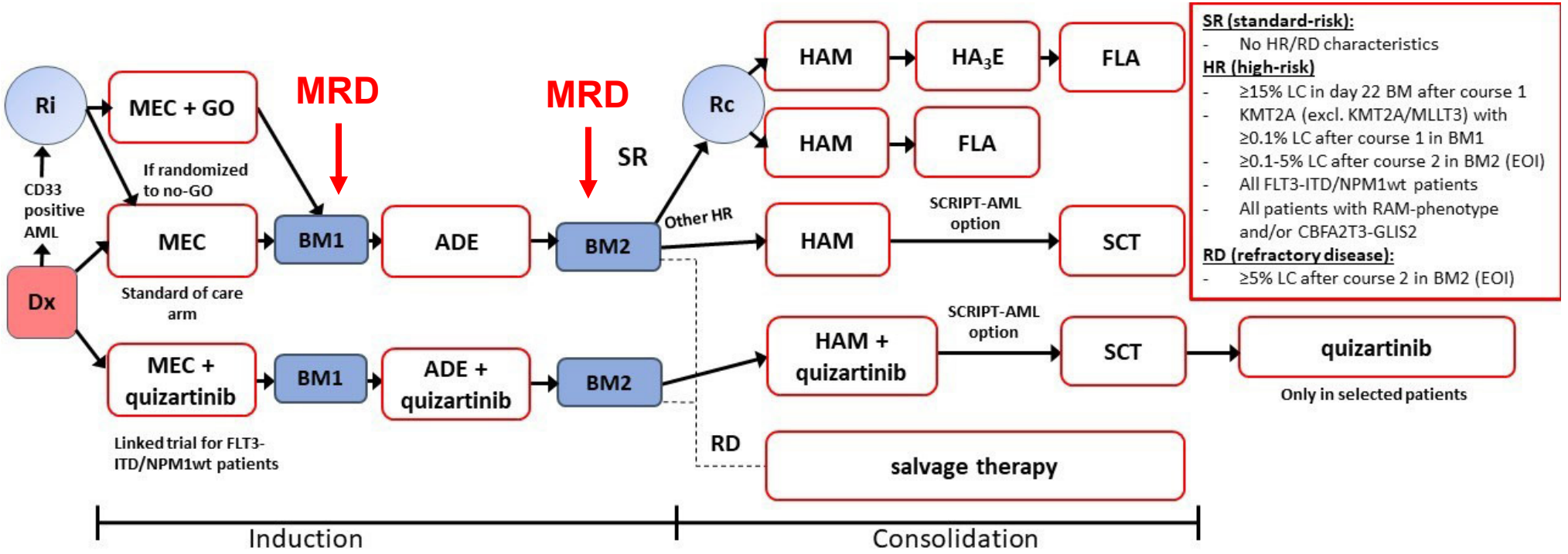
Short N et al. Am. J. Hematology 2018; Letetsu R et al Leukemia 2020; JJM van Dongen et al. The Lancet 1998

MRD as surrogate endpoint to define treatment response and treatment allocation



Van Dongen et al, Lancet 1998; Van der Velden et al. Leukemia 2007; Van der Velden et al., Leukemia 2009

MRD incorporation in clinical trials



Chip-AML22 Master Protocol: An Open-Label Clinical Trial in Newly Diagnosed Pediatric De Novo Acute Myeloid Leukemia (AML) Patients Including a Linked Phase II Trial with Quizartinib in *FLT3*-ITD/ *NPM1*wt Patients - a Study By the NOPHO-DB-SHIP Consortium

Kaspers GJL, ASH abstract 2023

Comparison of different MRD techniques

- ALL as example

	Multi-color flow cytometry	qPCR for fusion genes	ASO-qPCR for IG/TR genes	High-throughput NGS
Sensitivity	10^{-4}	10^{-4} to 10^{-5}	10^{-4} to 10^{-5}	10^{-6}
Applicability	>90%	40-50%	90-95%	>90%
Advantages	- Rapid - Relatively inexpensive - DfN method does not require access to diagnostic specimen	- Sensitive - Standard primers used for specific fusions	- Sensitive - Applicable to most patients - Standardized guidelines in Europe	- Very sensitive - Applicable to almost all patients - Clone-unbiased (can track multiple clones and evolution) - Only US FDA-approved assay (ClonoSEQ) - Data for MRD use in peripheral blood
Limitations	- Variable sensitivity - Requires technical expertise - Fresh cells required - Less standardized - Immunophenotypic shifts can lead to false negative results	- Not applicable to all patients	- Time-consuming - Expensive - Relies on pre-treatment sample - Requires extensive experience and labor	- Expensive - Longer turn-around time than MFC - Requires diagnostic pre-treatment sample

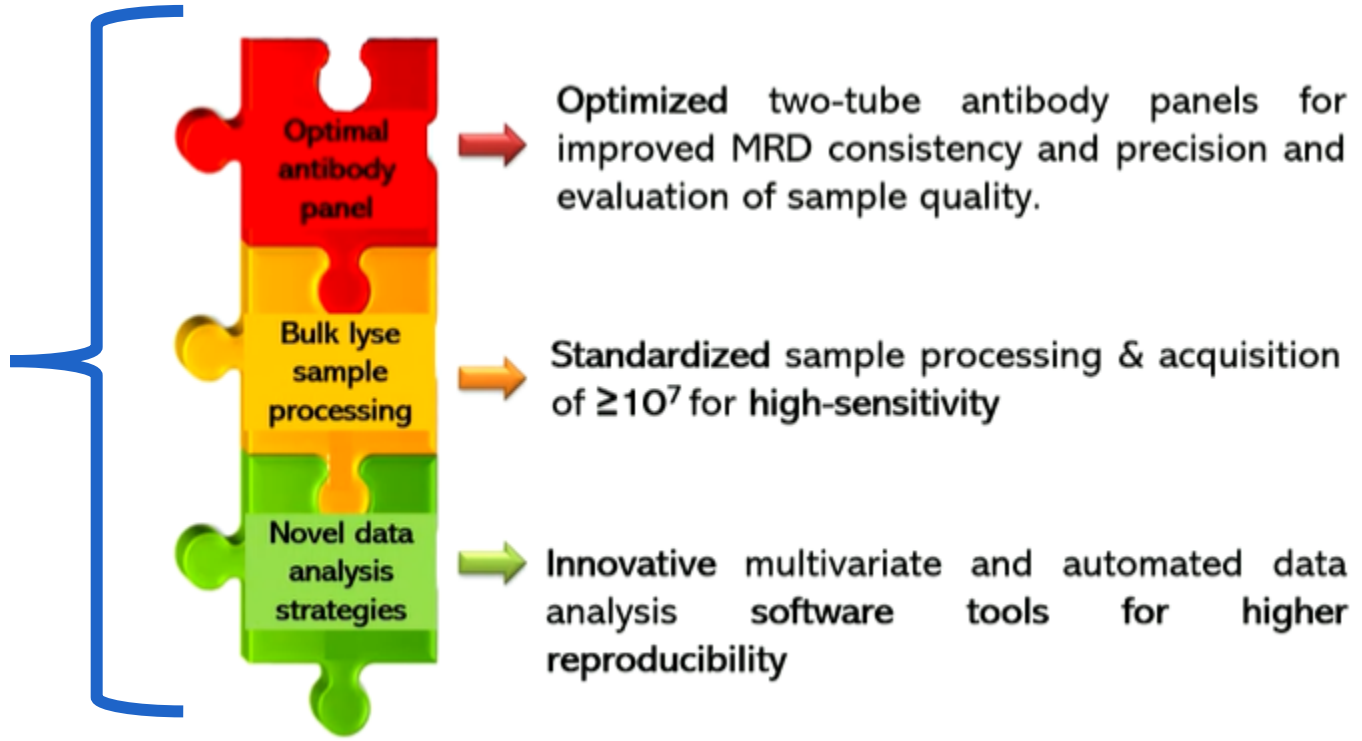
ALL: acute lymphoblastic leukemia; ASO: allele-specific oligonucleotide; DfN: different-from-normal; FDA: Food and Drug Administration; IG: immunoglobulin; MFC. multicolor flow cytometry; NGS, next-generation sequencing; qPCR, quantitative polymerase chain reaction; TCR, T-cell receptor.

Saygin C *et al* Measurable residual disease in acute lymphoblastic leukemia: methods and clinical context in adult patients. Haematologica, 2022

Major limitations of MRD by flow cytometry

- Heterogeneity of blast cells, especially in AML (# subpopulations in ~75% of AML patients)
- Sensitivity and specificity depend on the discriminatory level of the LAIPs
  - Expertise and knowledge required for LAIP recognition
  - LAIP not always present at diagnosis
  - Low frequency of LAIP expression on normal regenerative BM (reduced sensitivity)
- sensitivity depends on the numbers of cells analyzed (sample volume and cell concentration)
- Difficult to standardize > many laboratories/groups use their own MFC-MRD assay
- Analysis and interpretation of data require relevant expertise
- How to quantify residual MRD?
  - percentage per MNC
  - Percentage per leukocytes; including erythroid precursors?
  - Log reduction of blasts?
  - Correction of % LAIP at diagnosis?

Solution:
EuroFlow Next Generation Flow cytometry (NGF)



Aimed at higher sensitivity ($<10^{-5}$-10^{-6})

Theunissen P, Standardized flow cytometry for highly sensitive MRD measurements in B-cell acute lymphoblastic leukemia. Blood. 2017
Flores-Montero J et al. Next Generation Flow for highly sensitive and standardized detection of minimal residual disease in multiple myeloma. Leukemia 2017

Classical Flow Cytometry versus Next Generation FCM

	Classical flow cytometry	Next Generation Flow
Sample handling	No standardization (not needed?)	**Fully standardized**
Immunostaining procedures	No standardization (we will harmonize later?)	**Fully standardized (SOP)**
Instrument settings	No standardization (we use internal calibrator?)	**Fully standardized (SOP)**
Multi-color staining	Custom 8-10 colors tubes	**Standardized 8-14 color tubes**
Cell acquisition	50,000 to 100,000 cells	**5 to 10 million cells**
Data analysis: gating procedures	Subjective eye-balling ("flow cytometry is art")	**Objective** (automated gating, based on reference data bases)
Speed	4 to 5 hours, including ~1 hour for data analysis	**2 to 3 hours**, including 10-15 minutes for data analysis

Technical aspects of FCM MRD

From bone marrow samples to bulk lysis procedure and data analysis

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EuroFlow

Technical aspects of FCM MRD

- Sample preparation
  - Bone marrow sampling
  - Sample transport
  - Sample processing
- Flow cytometry
  - Monoclonal antibody panels
  - Cytometer settings
  - Sample running
  - Selection of control samples
- Data analysis
  - Gating strategy
  - Data analysis and interpretation
  - Report

HemaSphere

EHA EUROPEAN HEMATOLOGY ASSOCIATION

Guideline Article - Evidence based

Open Access

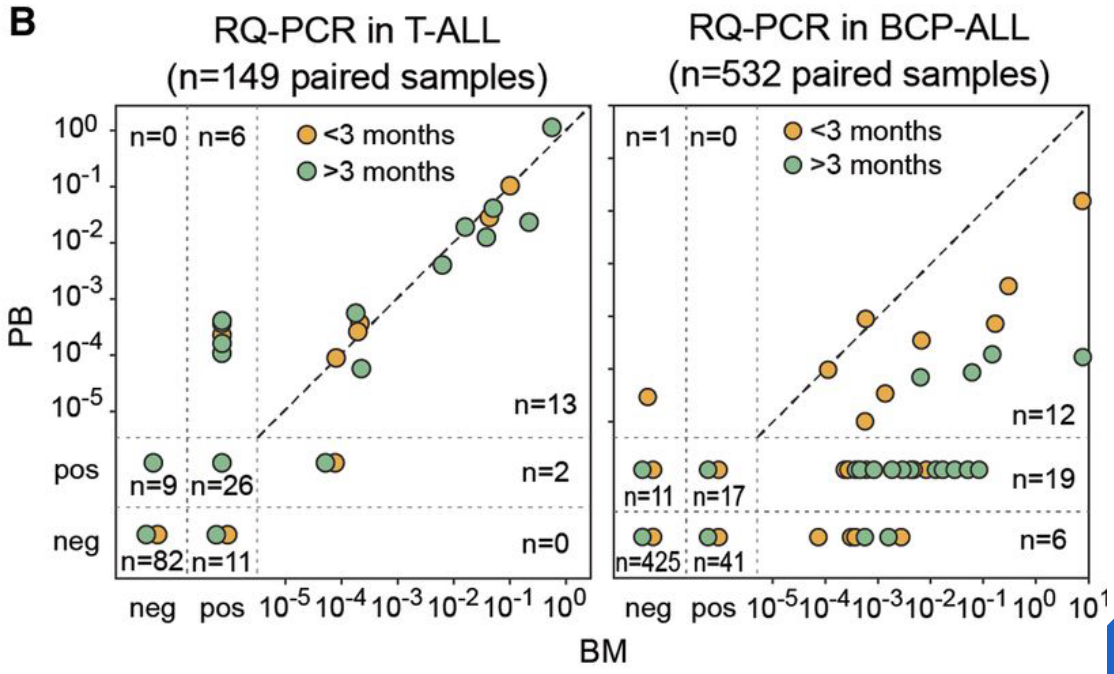
Technical Aspects of Flow Cytometry-based Measurable Residual Disease Quantification in Acute Myeloid Leukemia: Experience of the European LeukemiaNet MRD Working Party

Jesse M. Tettero[1], Sylvie Freeman[2], Veit Buecklein[3], Adriano Venditti[4], Luca Maurillo[4], Wolfgang Kern[5], Roland B. Walter[6], Brent L. Wood[7], Christophe Roumier[8], Jan Philippé[9], Barbara Denys[9], Jeffrey L. Jorgensen[10], Marie C. Bene[11], Francis Lacombe[12], Adriana Plesa[13], Monica L. Guzman[14], Agnieszka Wierzbowska[15], Anna Czyz[16], Lok Lam Ngai[1], Adrian Schwarzer[17], Costa Bachas[1], Jacqueline Cloos[1], Marion Subklewe[3], Michaela Fuering-Buske[18], Francesco Buccisano[4]

Standardization

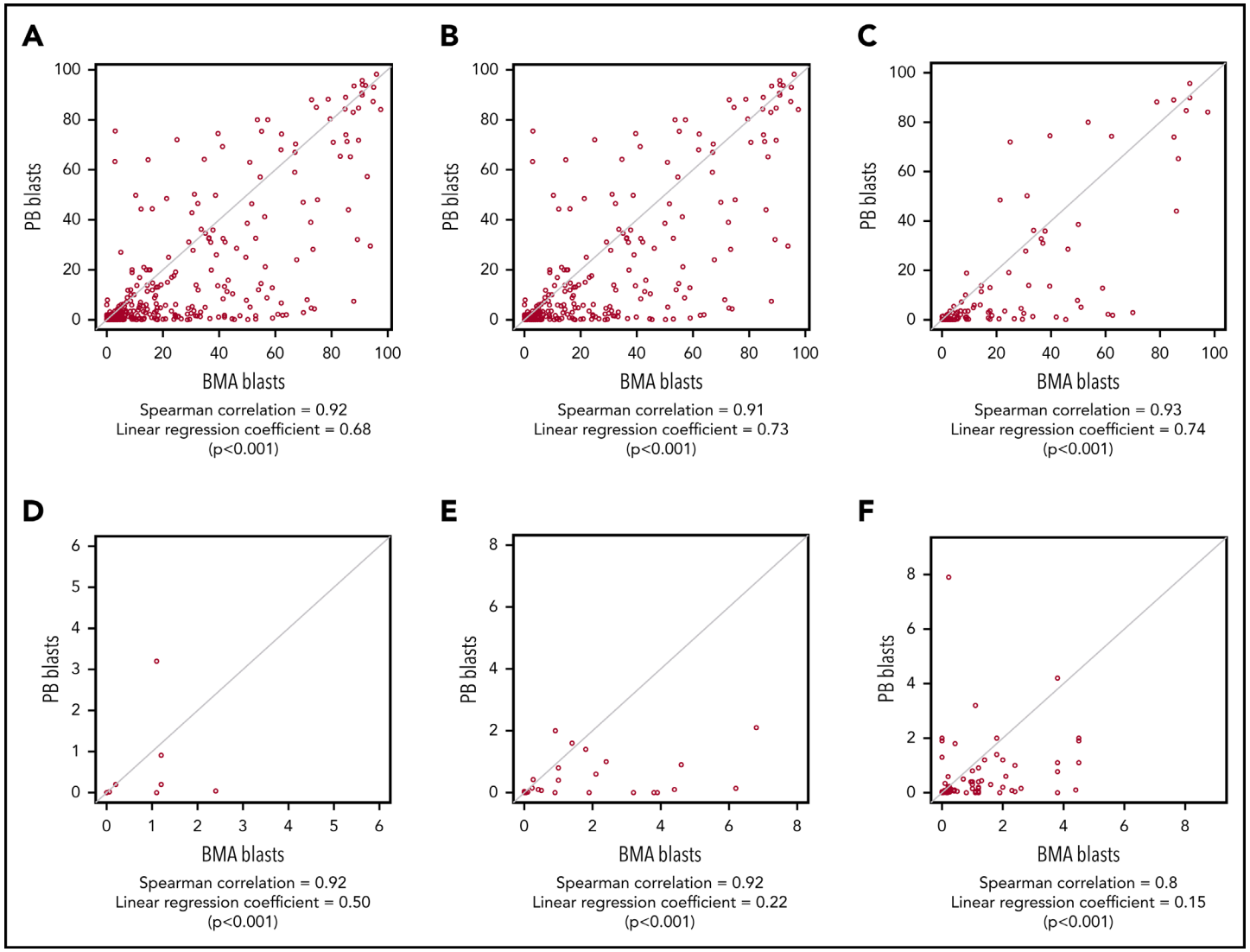
Bone marrow vs peripheral blood sampling

- Early microscopic MRD studies in **T-ALL** suggested that blood samples might be used instead of more invasive and traumatic BM samples samples in both **BCP-ALL and T-ALL.**
  - Studies in T-ALL confirmed that blood MRD levels in T-ALL patients were comparable or up to 1 log lower than in BM.
  - In BCP-ALL patients, blood MRD levels were 1 to 3 logs lower than in BM, making MRD studies via blood sampling impossible in BCP-ALL patients.
- Consequently, for both BCP-ALL and T-ALL patients, **BM sampling** is a prerequisite



Van Dongen JJM et al. Minimal residual disease diagnostics in acute lymphoblastic leukemia: need for sensitive, fast, and standardized technologies, Blood 2015

Bone marrow vs peripheral blood sampling (2)



AML

PB MFC testing could facilitate serial monitoring, thereby providing AML patients and providers with additional information to guide discussions of prognosis and treatment

Godwin CD et al. Acute myeloid leukemia measurable residual disease detection by flow cytometry in peripheral blood vs bone marrow, Blood, 2021

Analytical sensitivity

- **Rare event analysis = sensitivity**
- Limit of detection (LoD)
- Limit of quantitation (LoQ)

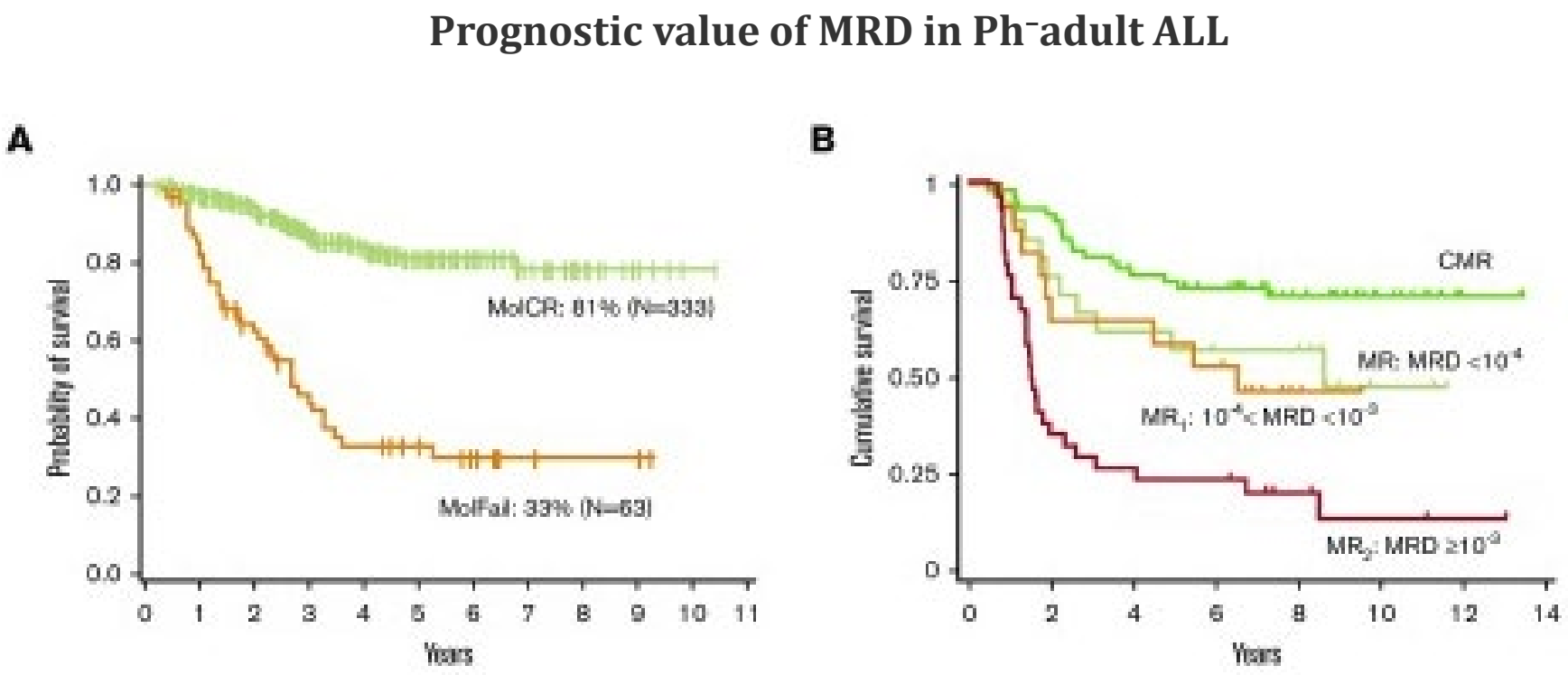
TABLE 1 | Total number of cells to collect in detection of rare events.

Frequency of Rare Events (1/x)	% of total	Desired coefficient of variation % (rare events required)			
		30 (11)	10 (100)	5 (400)	3 (1,111)
20	5	222	2,000	8,000	22,222
50	2	556	5,000	20,000	55,556
100	1	1,111	10,000	40,000	111,111
1,000	0.1	11,111	100,000	400,000	1,111,111
10,000	0.01	111,111	1,000,000	4,000,000	11,111,111
100,000	0.001	1,111,111	10,000,000	40,000,000	111,111,111
1,000,000	0.0001	11,111,111	100,000,000	400,000,000	1,111,111,111

For very rare cell populations, number of cells to be analyzed increases substantially.

- Calculation LOD and LOQ depends on the total number of cells analyzed
- Smallest homogeneous population than can be detected is 20 events (10 events more liberal?)
  - 19 events = maximum # of events < LOD
  - 95% confidence interval for a count of 19 events = 11 - 30 events.
  - Thus, the LOD can be estimated as **(30/total number of cells analyzed) × 100%**
  - Similarly, it is also widely accepted that more than 50 (40?) events is a standard threshold for reproducible enumeration. Consequently, the **LOQ = (50/total number of cells analyzed) × 100%**

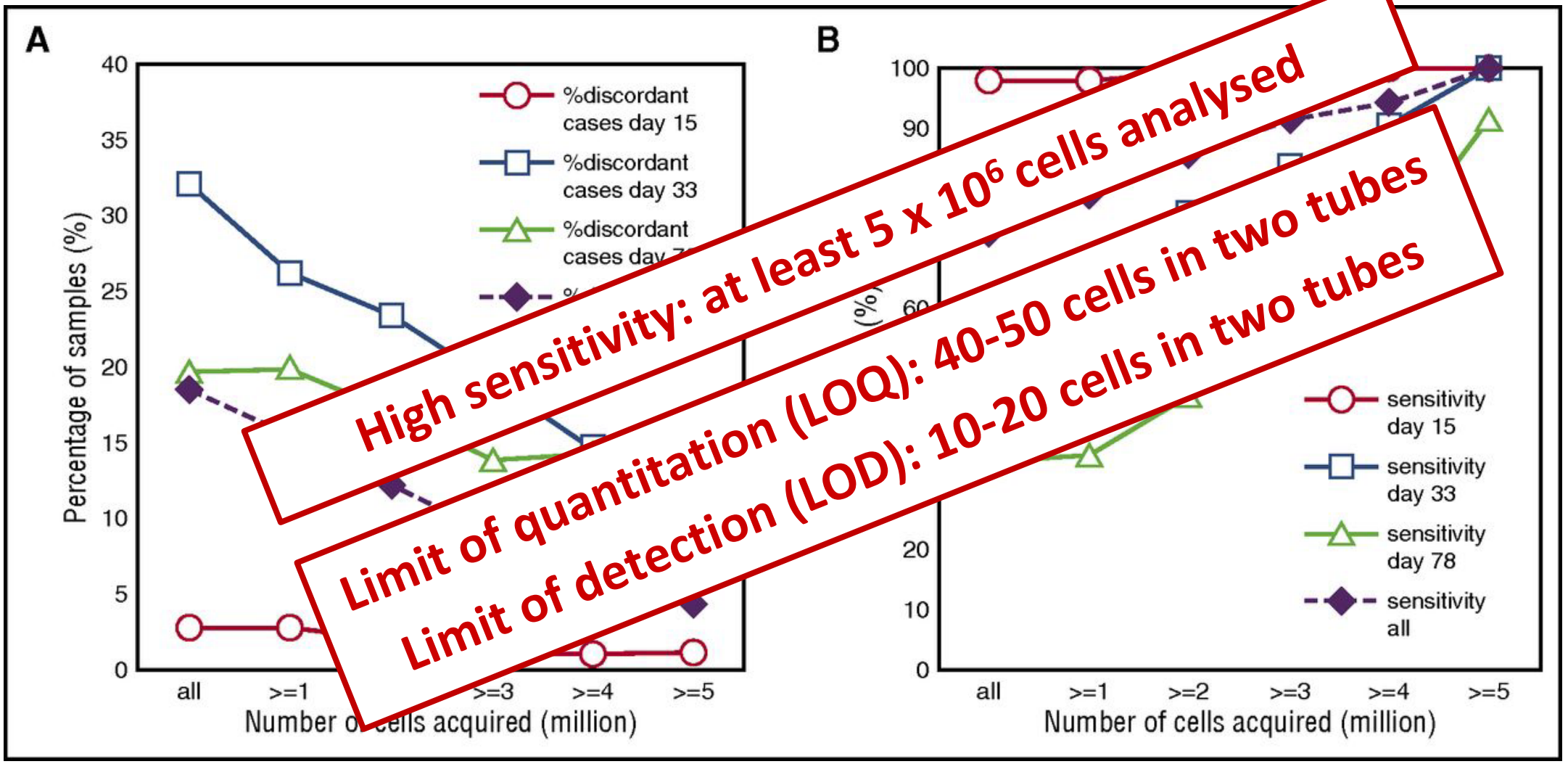
The sensitivity of the assay is important



- Complete molecular remission (MolCR) is defined as MRD negativity with an assay sensitivity of at least 10^{-4}
- High sensitivity needed for optimal risk stratification

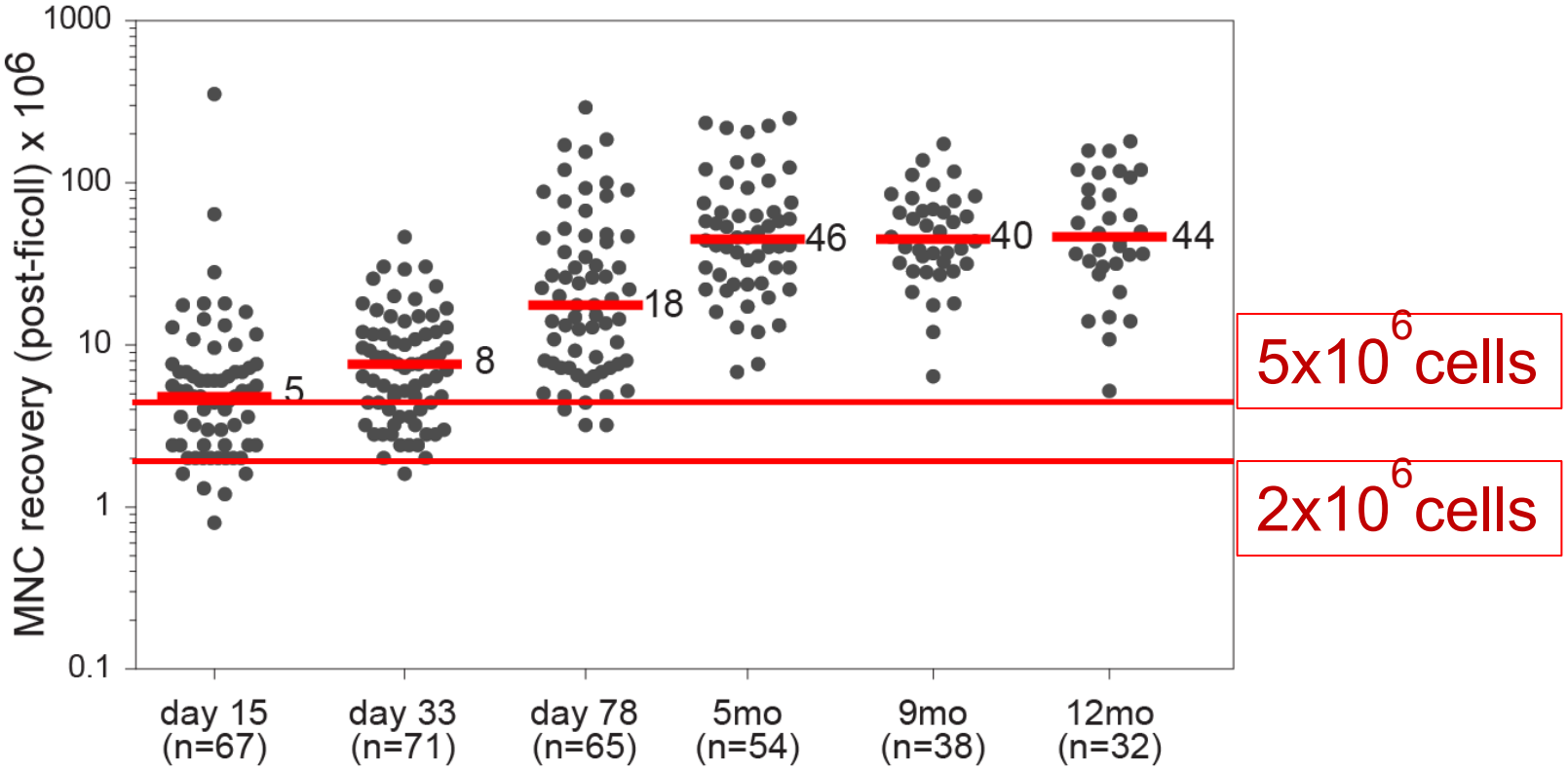
Brüggeman M and Kotrova M. Minimal residual disease in adult ALL: Technical aspects and implications for correct clinical interpretation, Blood Adv. 2017

Performance of FCM-MRD vs PCR-based MRD is dependent on the number of acquired cells.



Theunissen P, Standardized flow cytometry for highly sensitive MRD measurements in B-cell acute lymphoblastic leukemia. Blood. 2017

Bone marrow sampling



Number of cells for MRD diagnostics during follow-up:

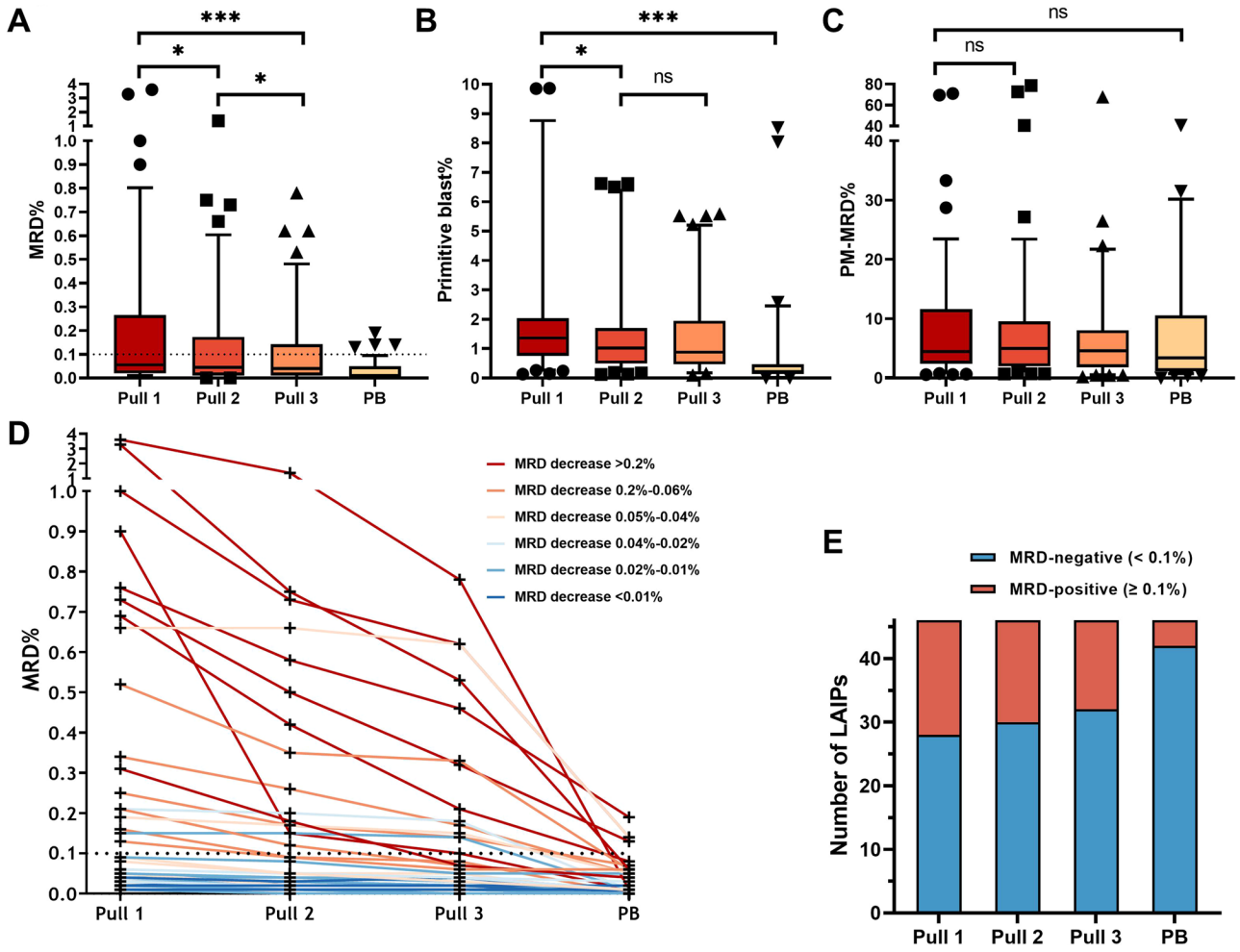
- **RQ-PCR**: 2x10^6 cells for ≥6µg of DNA (attention: recovery is 50% of 13µg)
- **NGS**: 1x10^6 cells for ≥3µg of DNA
- **EuroFlow-based FCM MRD**: 5 to 10 x10^6 cells for below 10^{-5} sensitivity (40 cells)

Advise: Only use the **first aspirate** to have high cellularity (without hemodilution)
Collect ≥ 2½ ml, but **not more than 5 ml** of first aspirate (avoid hemodilution)
Include clear descriptions and **guidelines in diagnostic protocols**.

Brüggeman M and Kotrova M. Minimal residual disease in adult ALL: Technical aspects and implications for correct clinical interpretation, Blood Adv. 2017

Impact of hemodilution on FCM based MRD assessment in acute myeloid leukemia

- Hemodilution can yield false-negative MRD results in AML



Tettero J M et al. Impact of hemodilution on flow cytometry based measurable residual disease assessment in acute myeloid leukemia, Leukemia 2024

How to assess BM hemodilution

- Different formula tested
- Best from the test:
  - The gra > 9(
  - CD < 0,

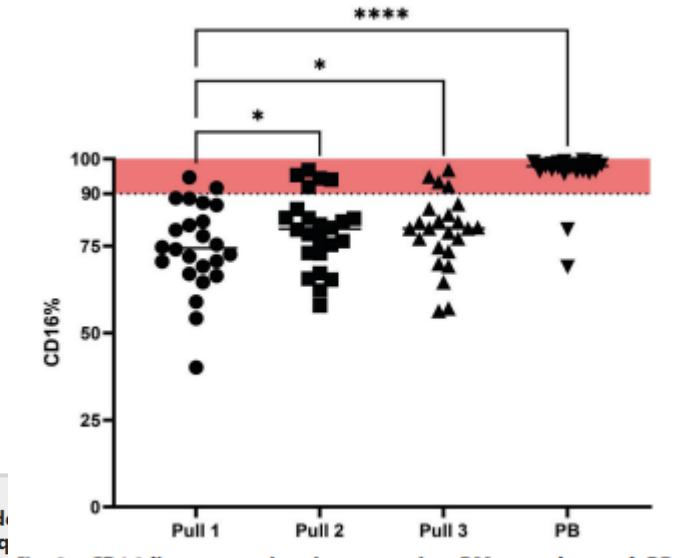
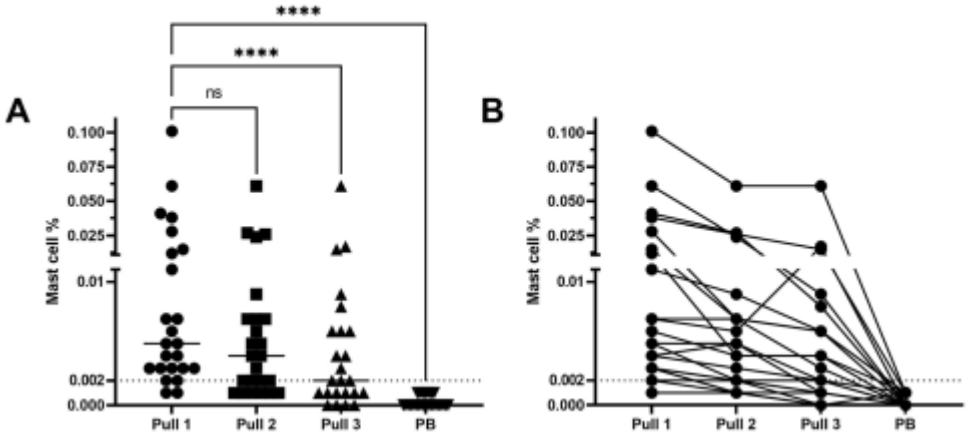


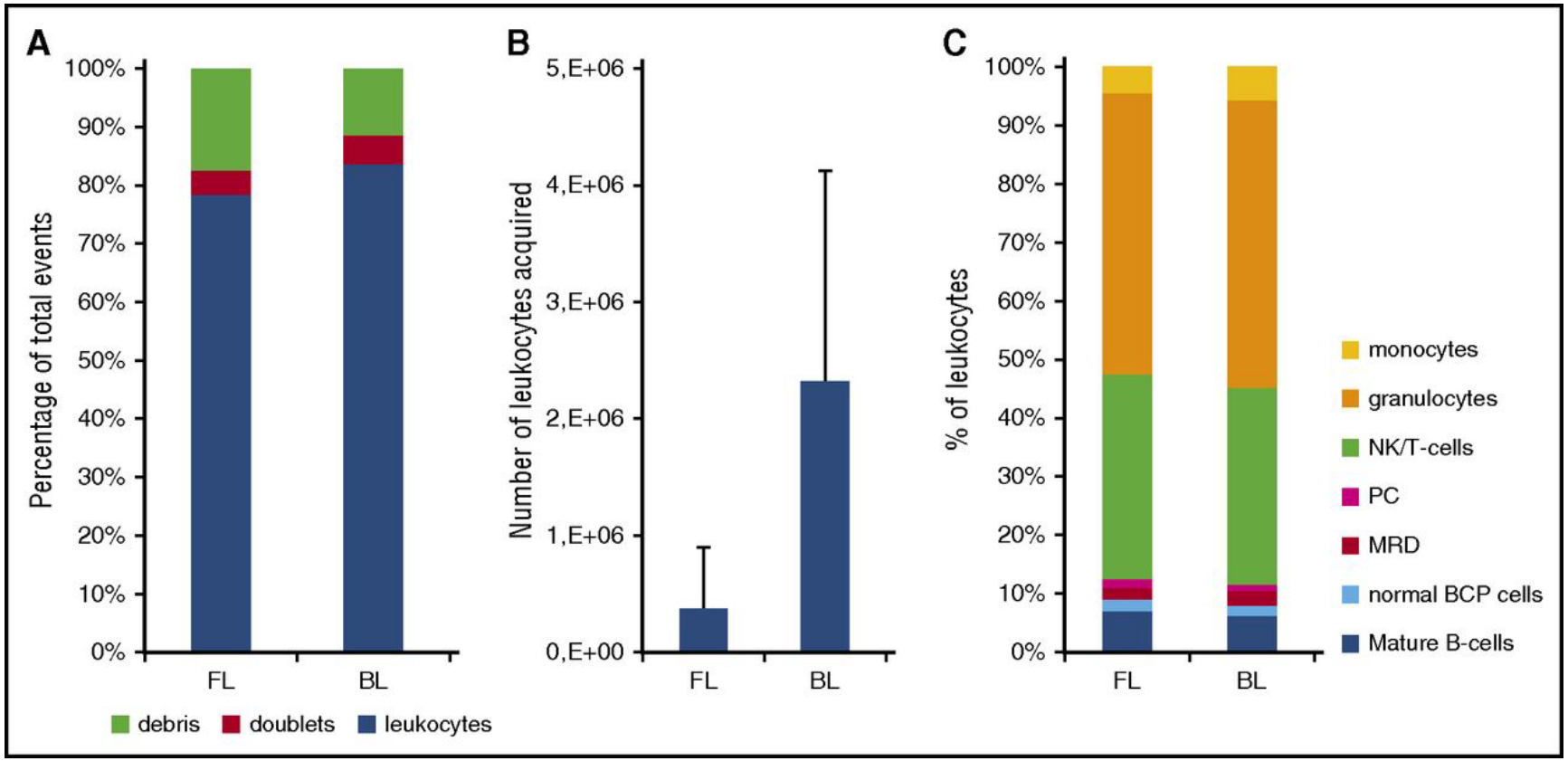
Fig. 3 CD16dim expression in successive BM samples and PB.

Reference	Formula for detecting hemodilution	Ad req		
Holdrinet et al. [16]	Bone marrow purity = [1-(erythrocytes BM/erythrocytes PB) × (leukocytes PB/leukocytes BM)] × 100%	Matched PB	No	No
Delgado et al. [23]	PB contamination index = −3.052 + 0.065 × (%CD10+ neutrophils of granulocytes) −0.609 × (%CD34 +) − 2.008 × (%plasma cells)	CD10 marker	Yes	Yes
Aldawood et al. [24]	Predicted bone marrow purity = [1 − (Lymphocytes FCM / Lymphocytes PB / Leukocytes FCM)] × 100%			
Loken et al. [25]	Normalized blast count = (80%/% dim CD16) × blast count			
Schuurhuis et al. [8]	>90% mature neutrophils			
Flores-Montero et al. [26]	Suggested blood contamination if mast cell population ($CD117^{high}$) ≤ 0			



Tettero JM et al. Impact of hemodilution on flow cytometry based measurable residual disease assessment in acute myeloid leukemia, Leukemia 2024

Sample processing: Bulk lysis



Each of the BM samples (day 15: n = 15; day 33: n = 15; day 78: n = 12) was processed according to the standard EuroFlow protocol (FL) and in parallel according to the EuroFlow bulk-lysis protocol (BL)

Using Bulk-lysis, on average 12-fold more leukocytes could be acquired ($P < .0001$)

Bulk-lysis resulted in

- Less debris ($P = .032$)
- More leukocytes ($P = .03$)

Theunissen P, Standardized flow cytometry for highly sensitive MRD measurements in B-cell acute lymphoblastic leukemia. Blood. 2017

Data analysis

Dependent on the tubes

- LAIP
  - Identification of Leukemia Associated Immuno-Phenotype or LAIPs, based on immunophenotypic aberrancies
- Different from normal approach (DfN)

The quality of a LAIP

1) **Specificity**

   depends on the percentage of LAIP expression on normal cells

2) **Sensitivity**

   depends on the percentage of LAIP expression on the leukemic blast population at diagnosis (minimal 10%) and the number of cells analyzed

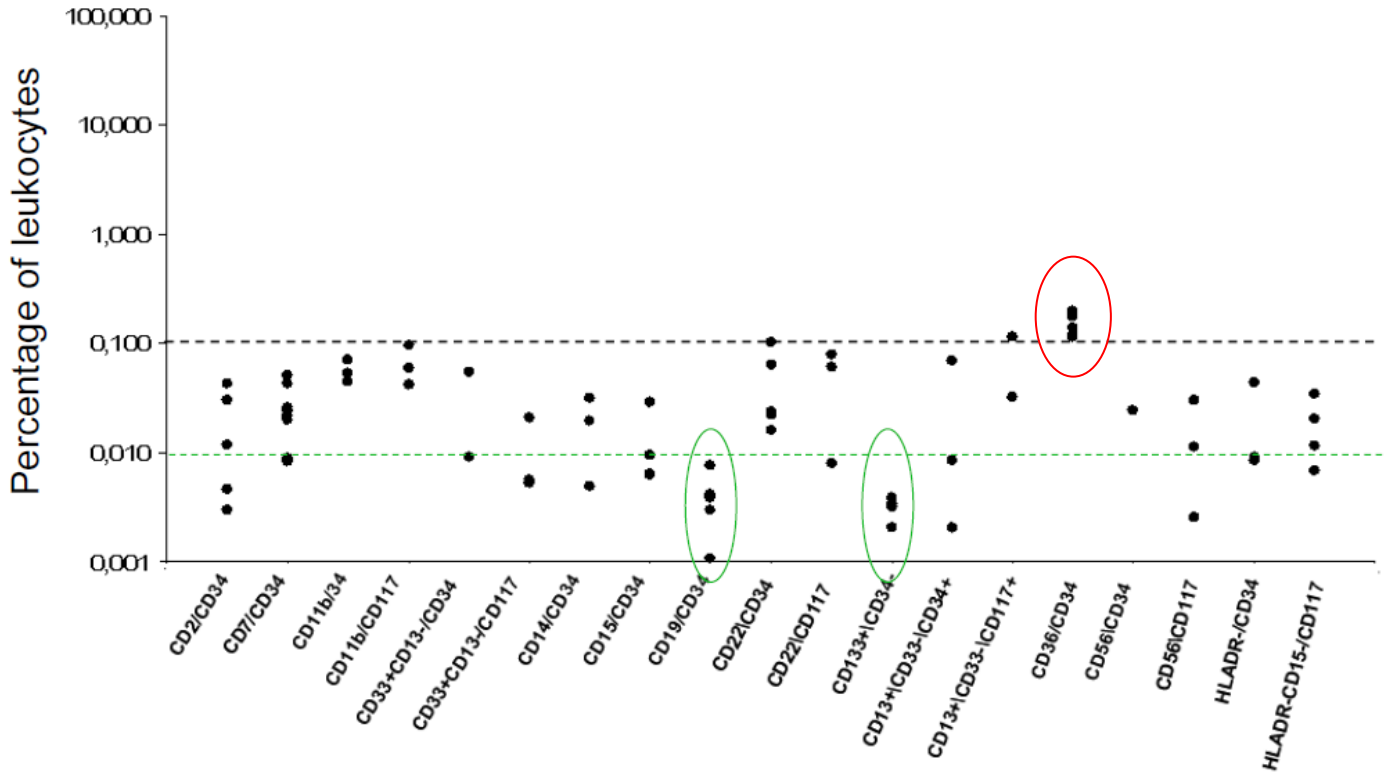
3) **Stability**

   phenotypic shifts can results in fals-negativity (especially dim expression of markers is susceptible)

Choice of LAIP is important

- Background of LAIP in normal and regenerating BM
  - ➡ NOT every LAIP = leukemic specific
  - ➡ Sensitivity and specificity is variable

Aberant marker expression on normal BM: % of primitive marker compartment (CD34 or CD117)



Feller et al, Blood Cancer Journal 2013

Flow cytometry: Improvement of data analysis

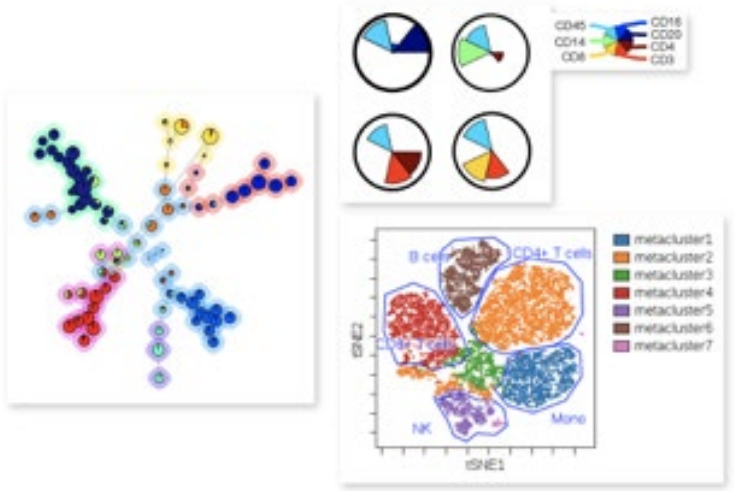
Conventional methods of manual data analysis

- Based on visualization of multiple bi-dimensional plots
- Operator's selection of population of interest (subjective)
- Depending on the expertise of the operator

Infinicyt CYTOGNOS

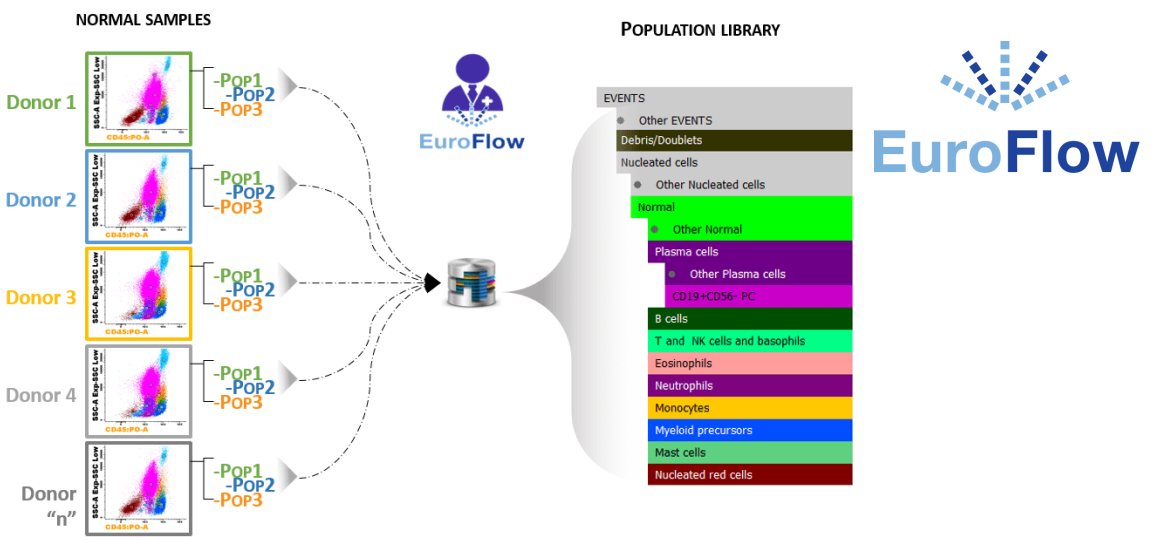
FlowSom

- Enormous increase in number of data by merging and calculations
- Automated method for analysis of flow cytometry immunophenotypic data
- Reference picture will facilitate MRD analysis
- Reducing expert-based data-analysis

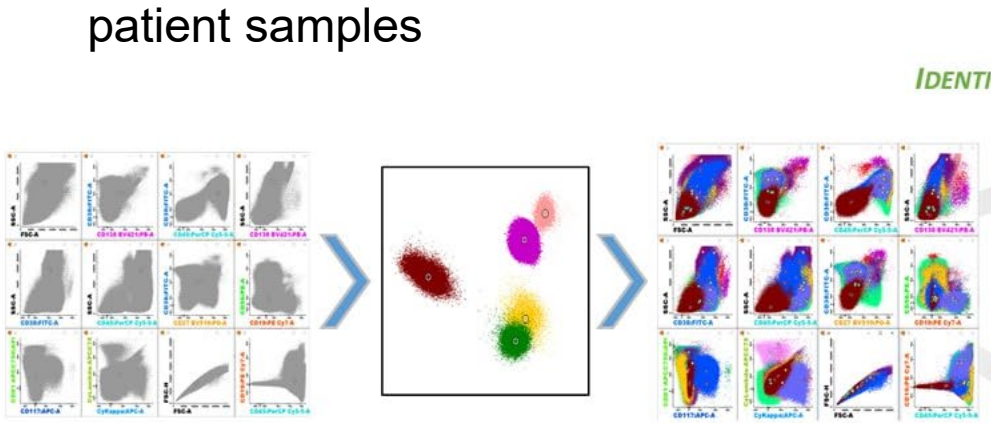


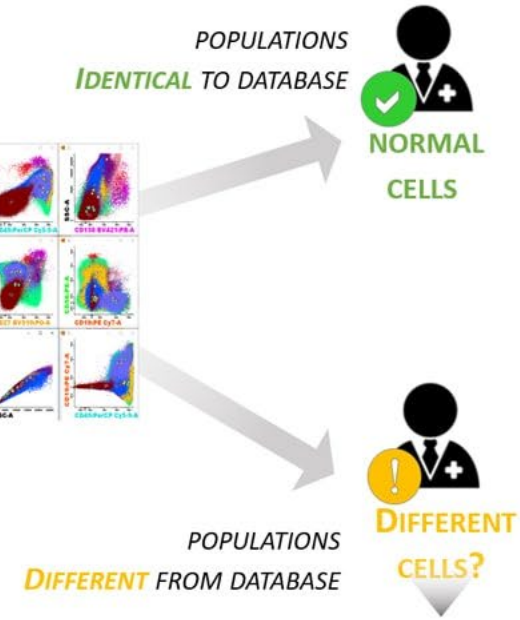
AG&I tool Infinicyt

1. Database construction



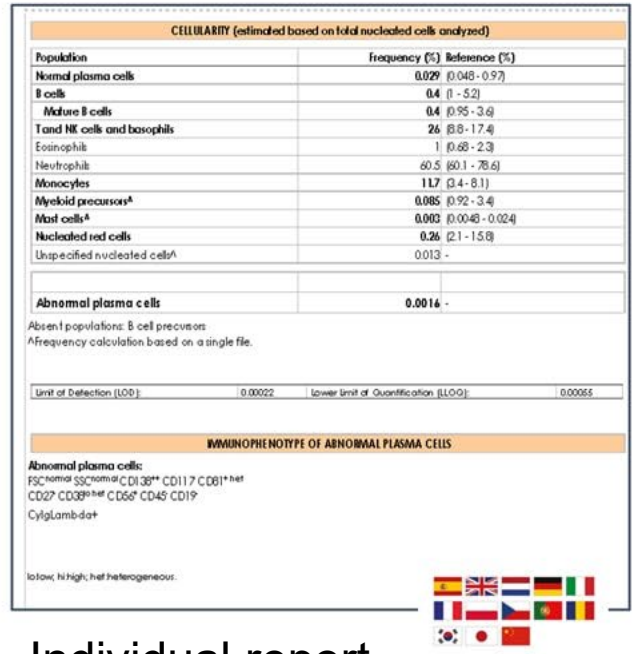
2. Analysis of individual patient samples





3. Analyze checks

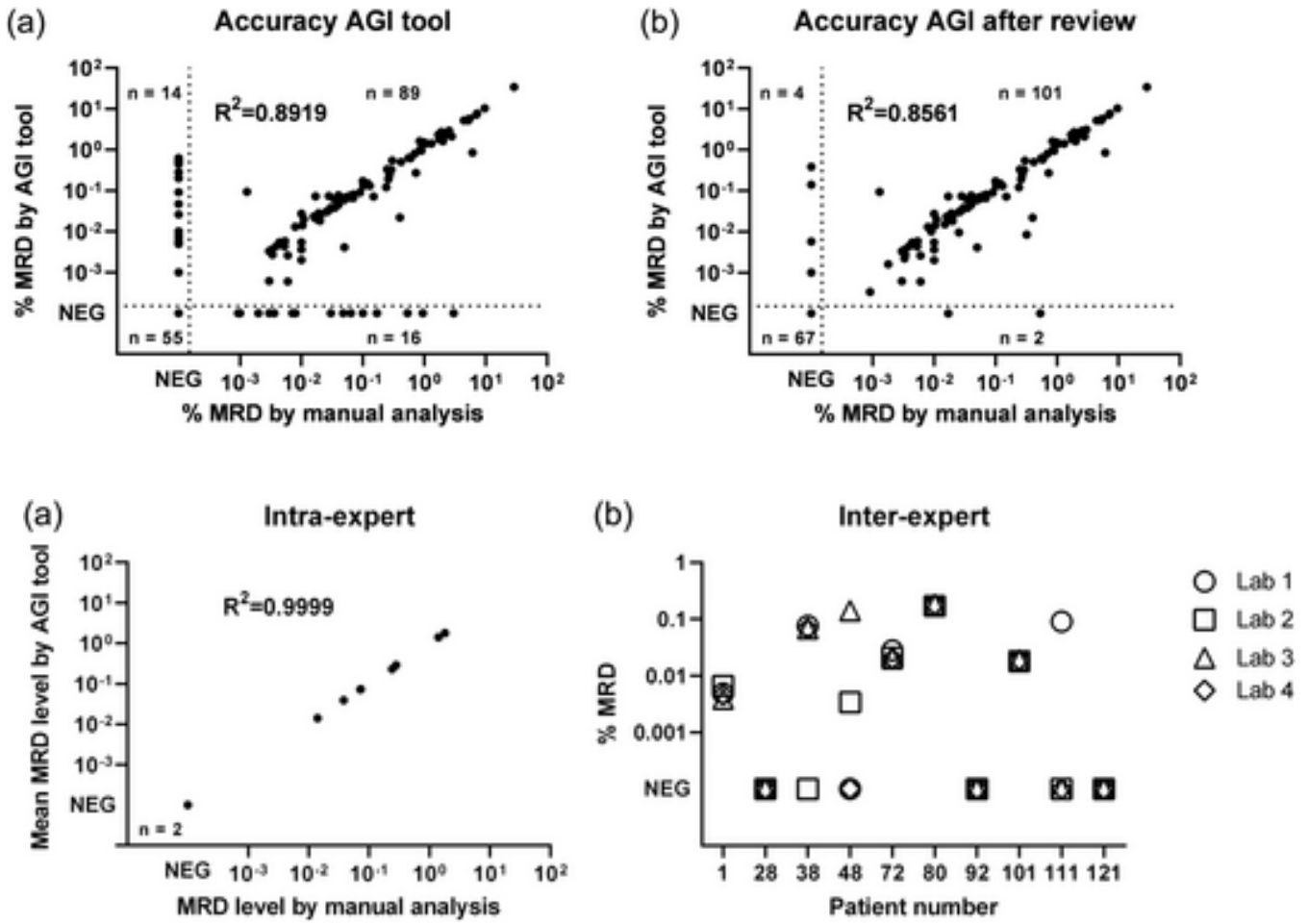
NEEDS SUPERVISION: CHECK POPULATIONS



4. Individual report

1 - - - - - - - 2 - - - - - - - - - - - 3 - - - - - - - - - - - - - - - - 4

Performance of the AG&I tool in BCP-ALL



Comparison of MRD levels between manual and AG&I showed a concordance rate of 83%. After review of discordant cases by additional experts, the concordance increased to 97%.

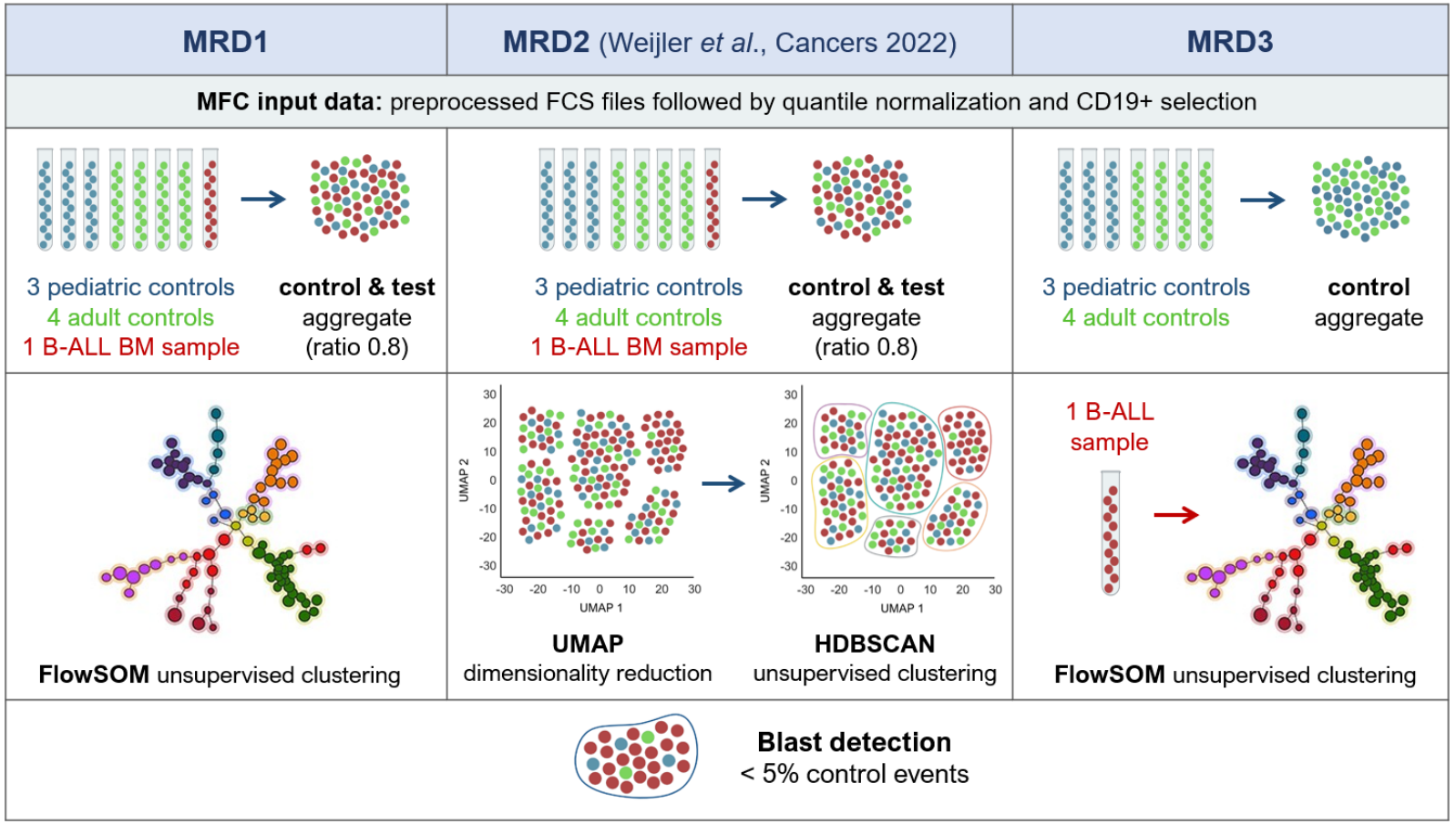
Furthermore, the AGI tool showed excellent intra-expert concordance (100%) and good inter-expert concordance (90%).

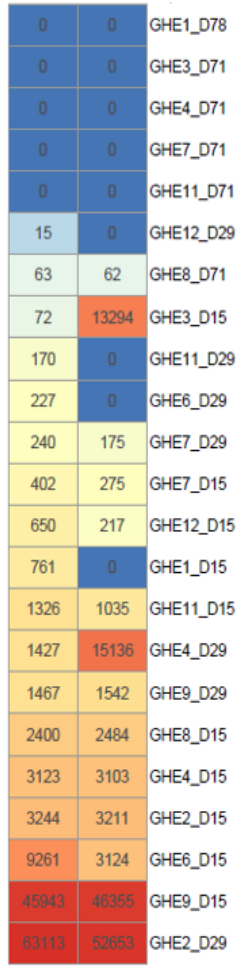
In addition to MRD levels, also percentages of normal cell populations showed excellent concordance between manual and AGI tool analysis.

Verbeek M et al. Minimal residual disease assessment in B-cell precursor acute lymphoblastic leukemia by semi-automated identification of normal hematopoietic cells: A EuroFlow study. Clinical Cytometry 2023

Automated detection of measurable residual disease in acute lymphoblastic leukemia

- Three ML models to tackle MRD detection in B-ALL





Masterthesis Sofie Ellegiers

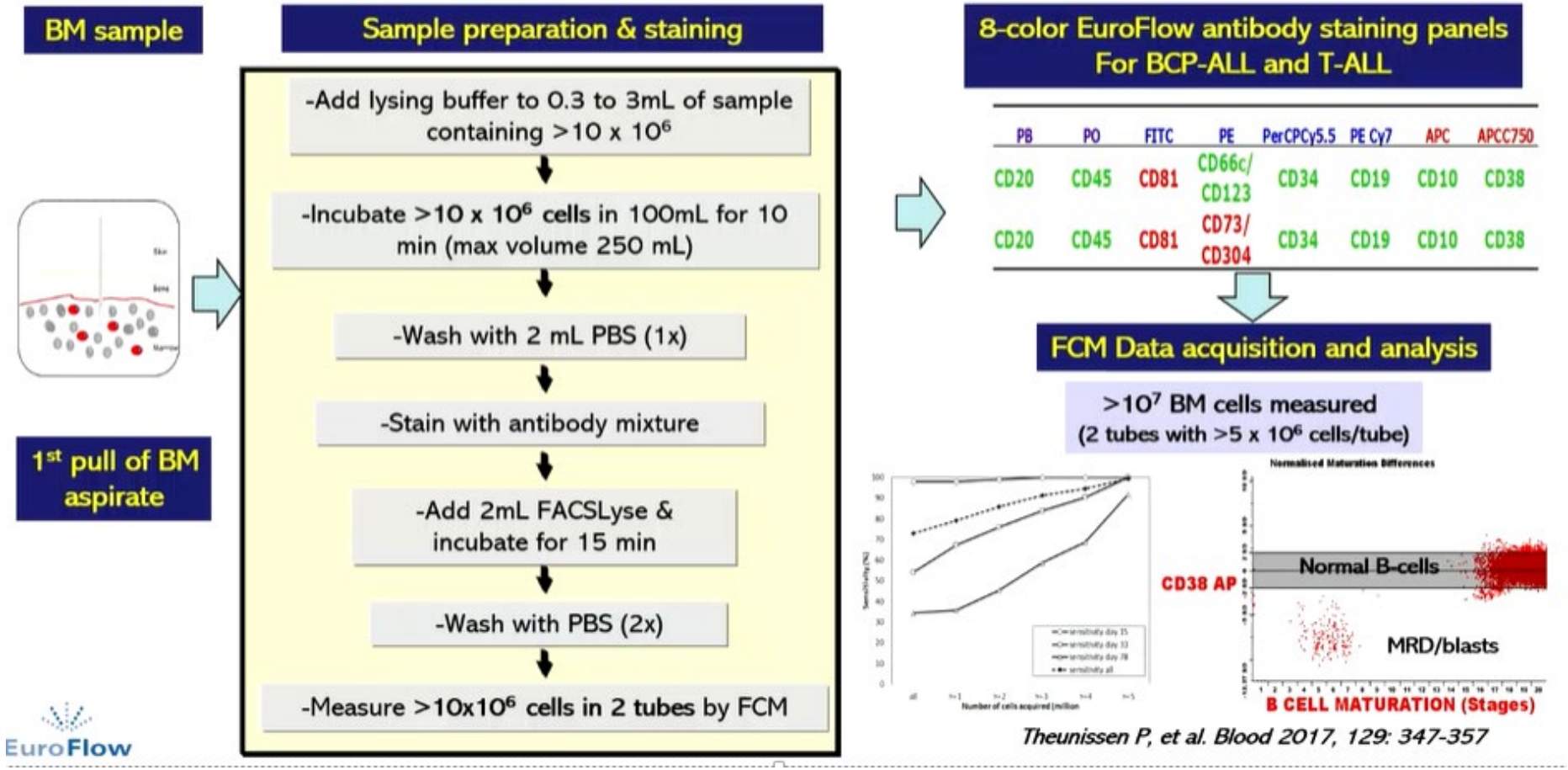
Conclusion

1. MRD technique requirements:
   - Broad availability, easy implementation and affordable
   - Applicability in vast majority of patients (preferably ≥95%)
   - Sufficient sensitivity (Quantitative range ≤10^{-4}, preferably ≤10^{-5})
   - Fast (short turn-around time: 1-2 days)
   - Standardization (≠ harmonization) with international QA programs
2. Flow-based MRD is stepwise being introduc
3. Sample requirements should be b
   1. MRD should be assessed from a small v … Hemodilution
   2. Store at ambient condition an… and processed using b…
   3. For clinical d… …ied assay, including adequate LOB, LOD, and LLOQ with a harm…
4. EuroFlow-b…
   - Broad availab… …n and affordable
   - Applicability in … patients (≥95%)
   - High sensitivity (… ≤10^{-5})
   - Fast: 3-4 hours (MRD report on the same day or next day)
   - Fully standardization with EQA program
5. Attention: many cells required for reaching high sensitivity (5-10 x10^{6})!

Fully standardized processes from pre-analytical phase, to sample acquisition and data interpretation

ALL

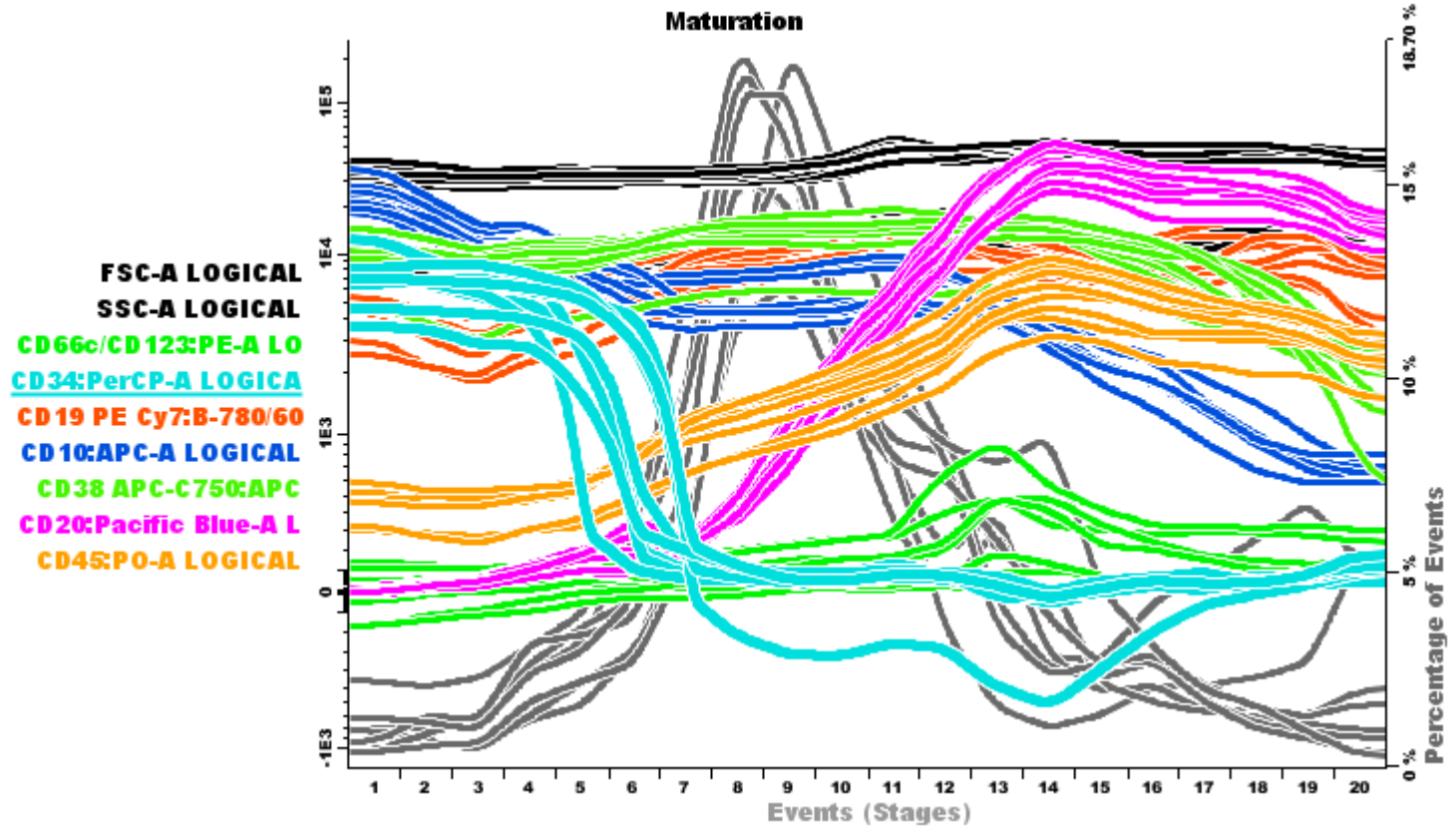
MRD detection in BCP-ALL by NGF



Theunissen P, Standardized flow cytometry for highly sensitive MRD measurements in B-cell acute lymphoblastic leukemia. Blood. 2017

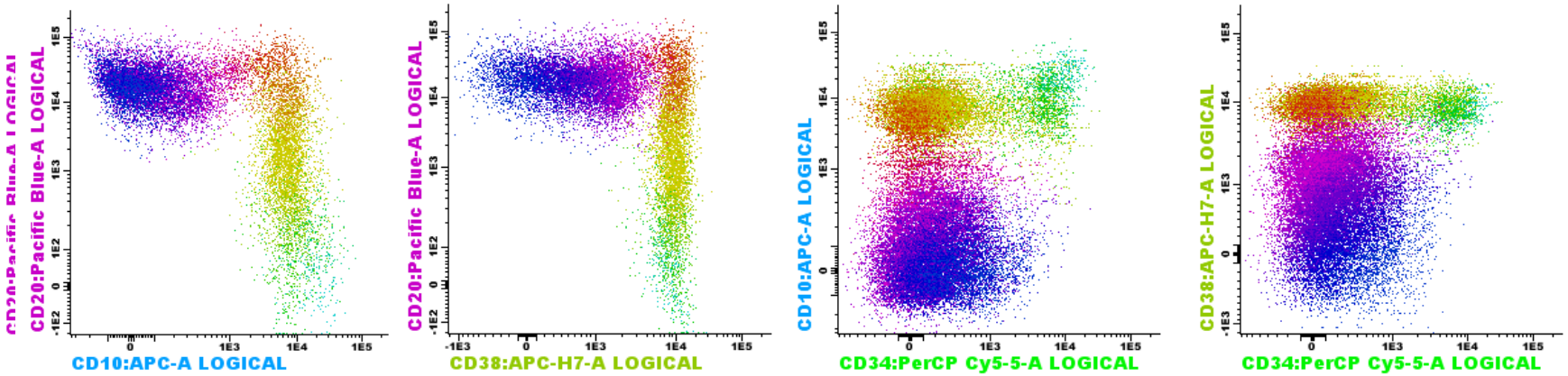
MRD based on maturation – new software tools

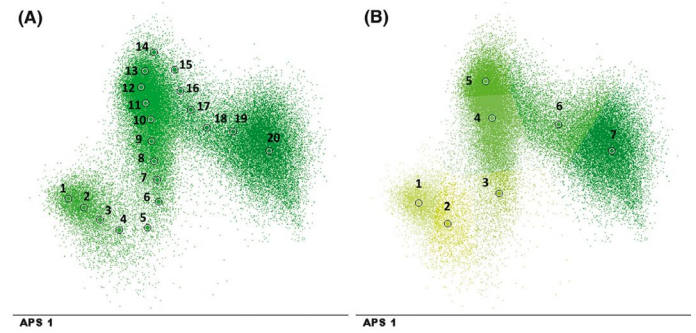
- Normal B-cell differentiation pathways

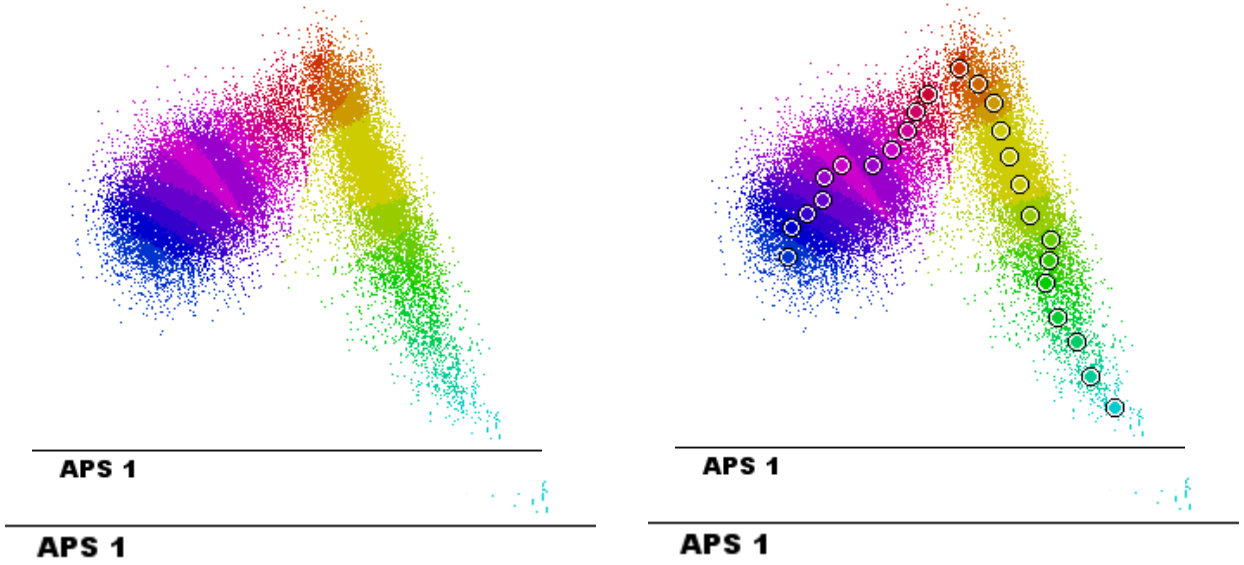


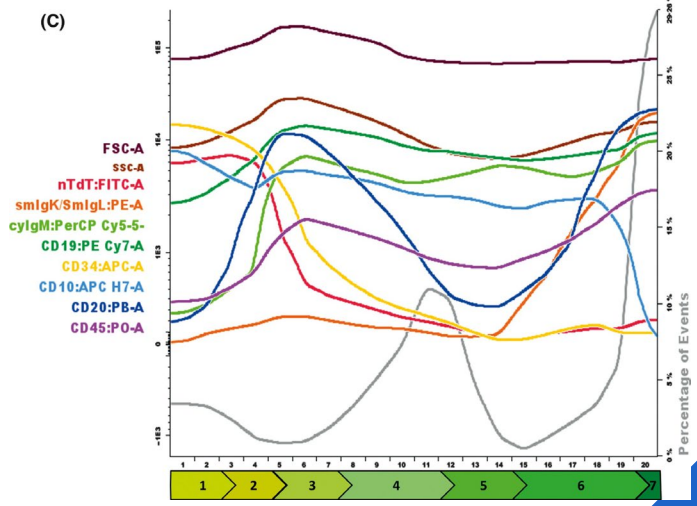
Theunissen PMJ et al. Detailed immunophenotyping of B-cell precursors in regenerating bone marrow of acute lymphoblastic leukaemia patients: implications for minimal residual disease detection BJH 2017

Dissection of normal precursor-B-cell differentiation implications for MRD monitoring





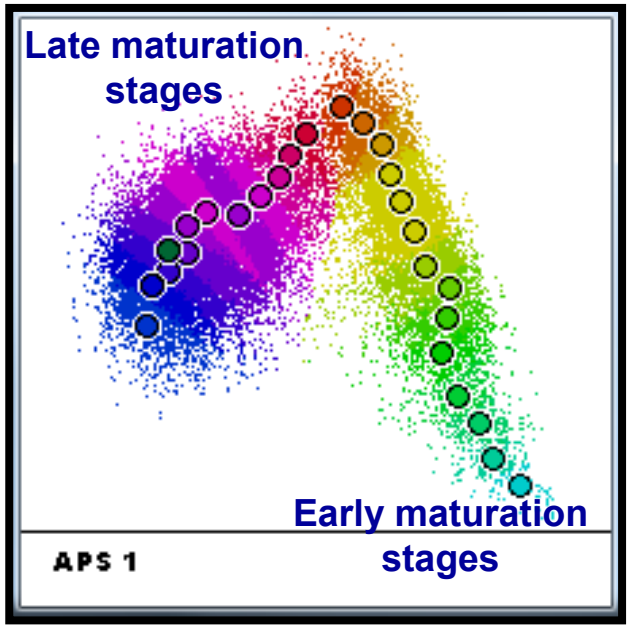




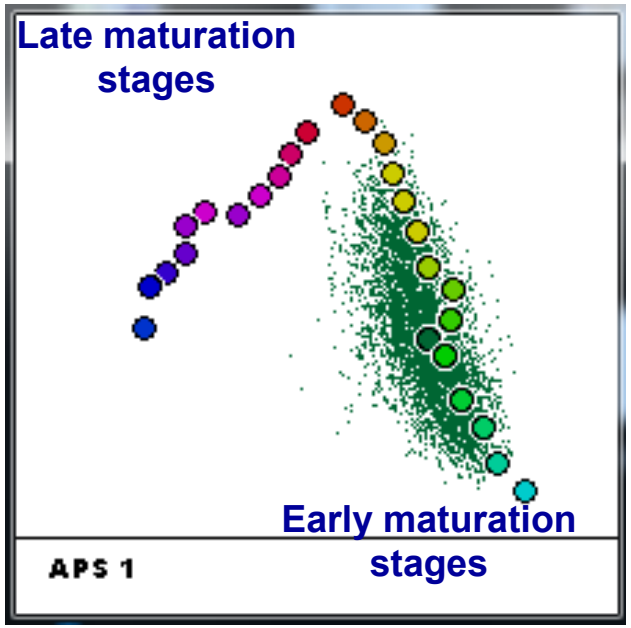
Theunissen PMJ et al. Detailed immunophenotyping of B-cell precursors in regenerating bone marrow of acute lymphoblastic leukaemia patients: implications for minimal residual disease detection BJH 2017

New concept for differentiation pathway analysis

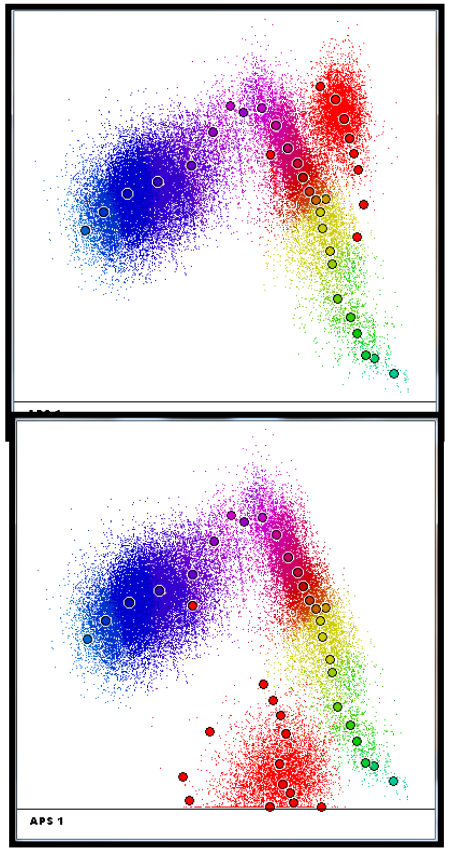
Dissection of normal BM



Regenerating BCP cells



ALL blast cells

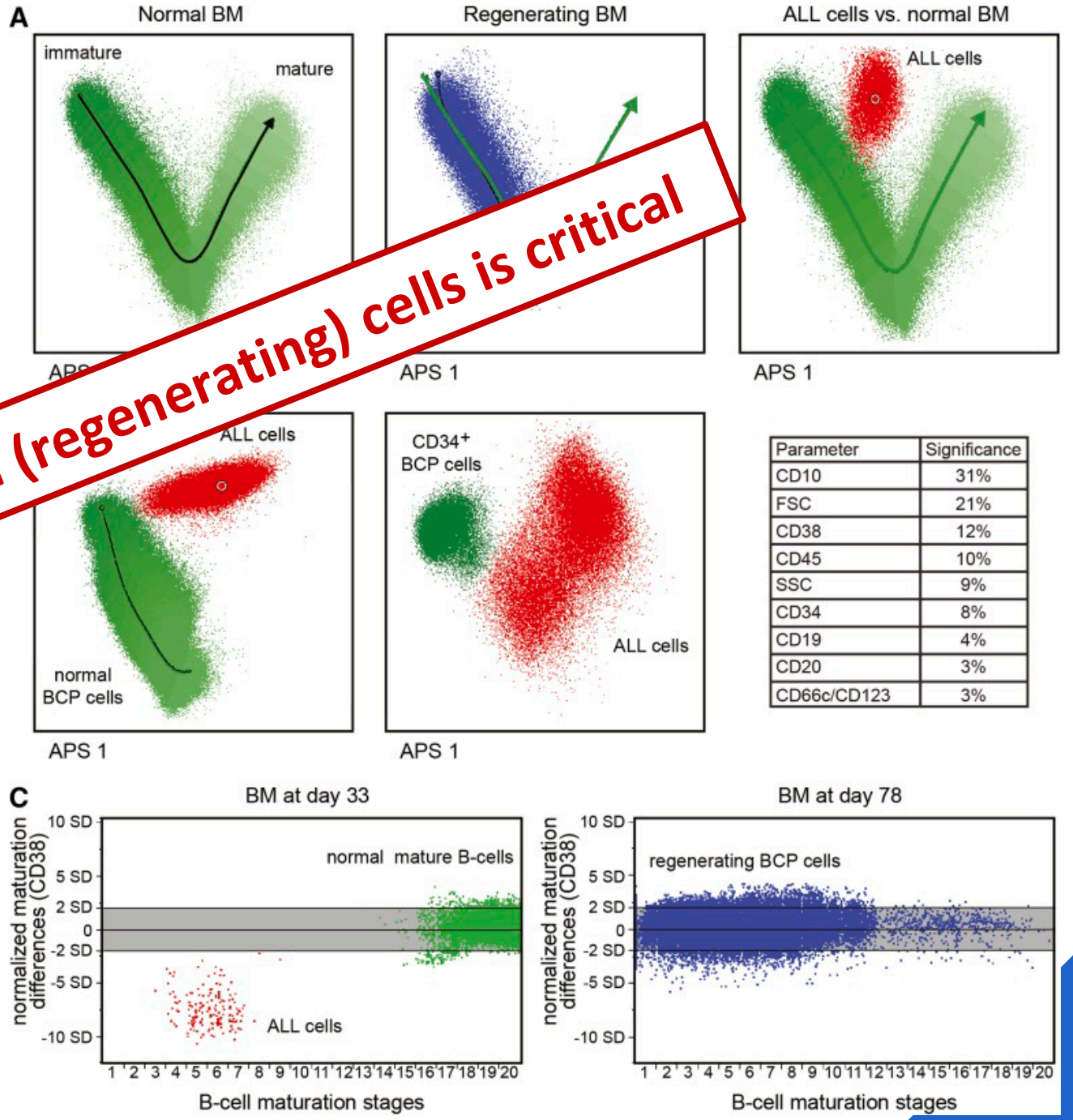


The immunophenotypic maturation of regenerating BCPs differs from that of normal BCPs and from ALL blasts

NGF-MRD

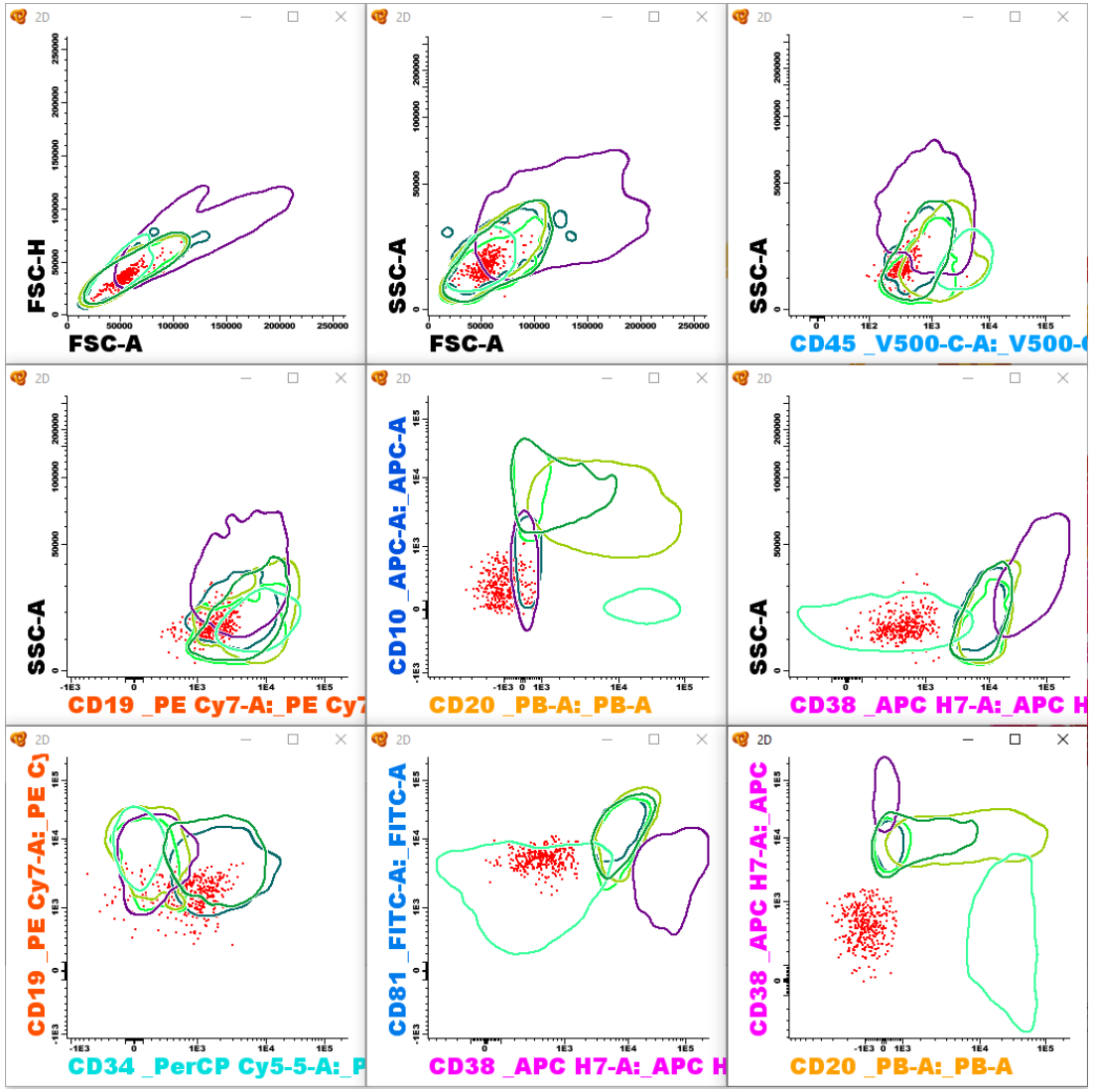
- Insights in the full normal BCP pathway in BM
- Better define the degree of IF deviation of BCP-ALL cells fr… normal BCP, also in re… BM
- Visualiz… …ivariate an…

Accurate recognition of normal (regenerating) cells is critical



Parameter	Significance
CD10	31%
FSC	21%
CD38	12%
CD45	10%
SSC	9%
CD34	8%
CD19	4%
CD20	3%
CD66c/CD123	3%

Reference profile of normal and regenerating BM samples



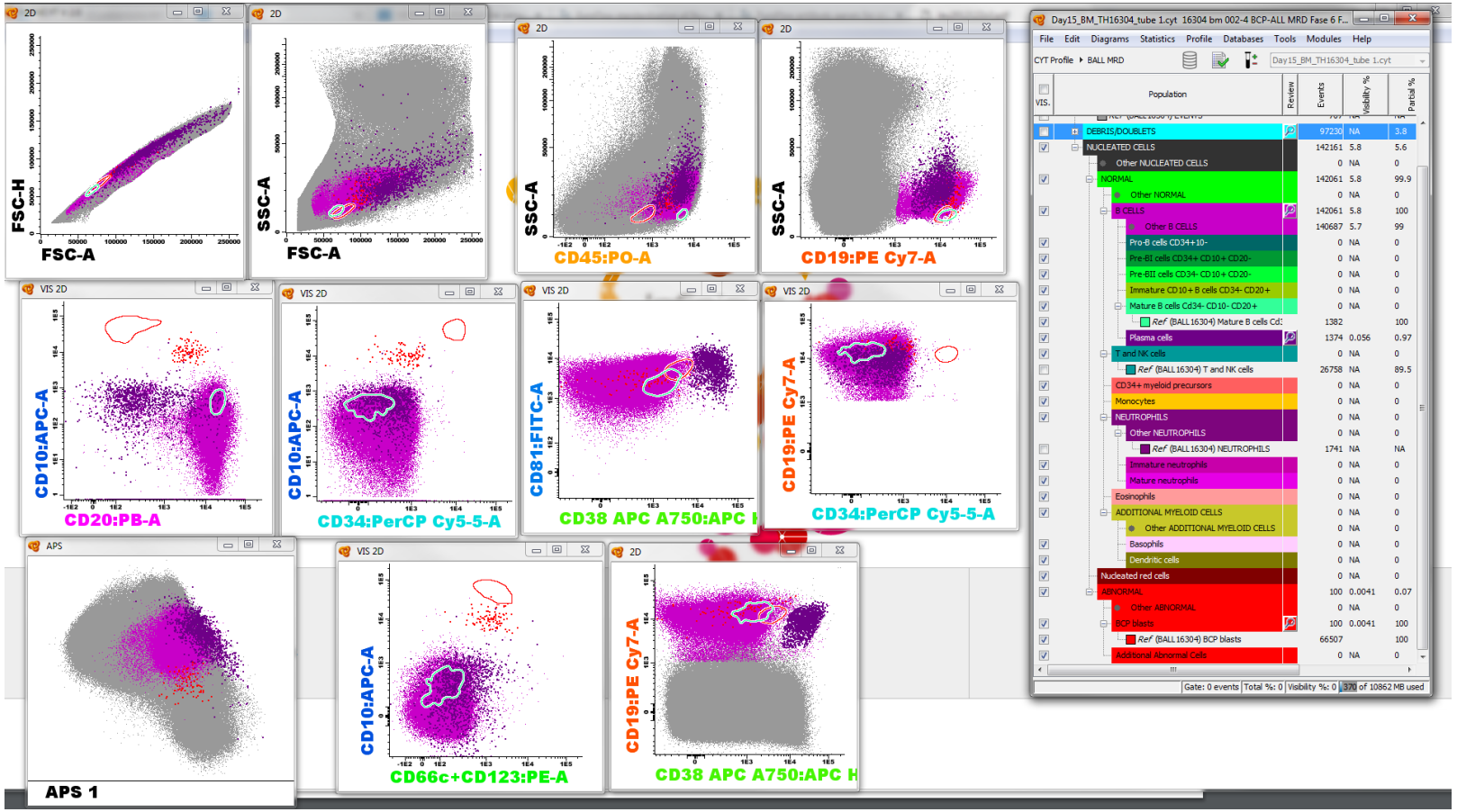
Gating strategy for MRD in ALL

- Use correct and standard profile
- General principes:
  - Dismiss debris (FSC-A/SSC-A) and doublettes (FSC-A/FSC-H)
- Define normal precursor B-cells
  - proB: CD19-/CD34+/CD10-/CD20- ;
  - pre BI:CD19+/CD34+/CD10+/CD20- ;
  - Pre BII: CD19+/CD34-/CD10+/CD20- ;
  - immature transitional B CD10+: CD19+/CD34-/CD10+/CD20+
  - mature B: CD19+/CD34-/CD10-/CD20+ (CD38w/CD81w).

All CD38/CD81 STRONG positive

- Important to look back at CD45/SSC-A plot; during maturation CD45 expressing gradually increases

Early MRD timepoints: beware of phenotypic shifts



- Analyze an early timepoint (D15)
- During treatment:
  - CD45 ↑
  - CD10 ↓
  - CD20 ↑
  - CD34 ↓
  - Correlate with aberrant markers
- APS view

Example 1 en 2

EuroFlow NGF MRD ALL approach for detection of CD19- leukemic cells

Recommendation: make a reference image of the different cell populations in the normal bone marrow, including regenerating B-cell precursors, mature B cells, myeloid progenitors, basophils and erythroid precursors as a reference for normal expression patterns

Generate dot plot diagrams:
FSC-A/FSC-H
FSC-A/ SSC-A
CD45/SSC-A
CD10/CD20
CD34/CD20
CD38/CD45
plot every marker vs. CD10
clustering: e.g. APS or CA

Eliminate Doublets by FSC-A /FSC-H

In FSC-A/SSC-A gate on nucleated cells by roughly excluding debris (to be refined after identification of blasts)

Remove aggregates

Visually check for CD10 expression in CD10/SSC-A

Gate CD10+/++ cells with low / int SSC

Check for aberrant antigen expression / clustering of events in comparison to normal B-cell precursors and refine BCP-ALL population

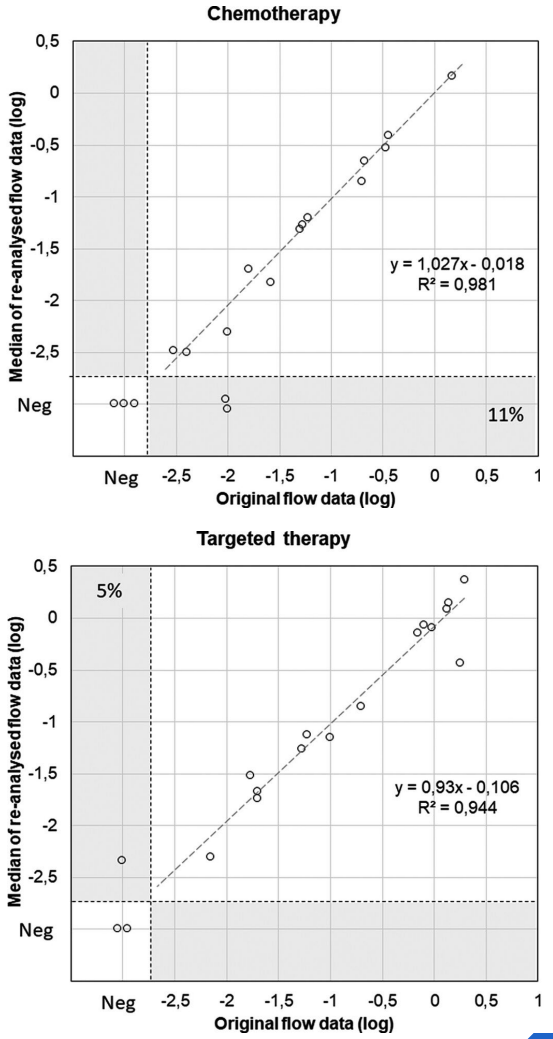
- Distribution of CD10/CD20 in combination with expected expression levels of CD34 and CD45
- Aberrant positivity of CD66c/CD123 or CD73/CD304
- Under-/overexpression of CD38 or CD81
- Increased SSC

Gate CD10- cells with low SSC Check both CD34+ and CD34- populations

Check for clustering to discriminate from myeloid progenitors and refine BCP-ALL population

- Low SSC
- Aberrant positivity of CD66c/CD123 or CD73/CD304
- Under/-overexpression of CD38 or CD81

Back gate on CD45/SSC-A and FSC-A/SSC-A for uniform clustering of the BCP-ALL population



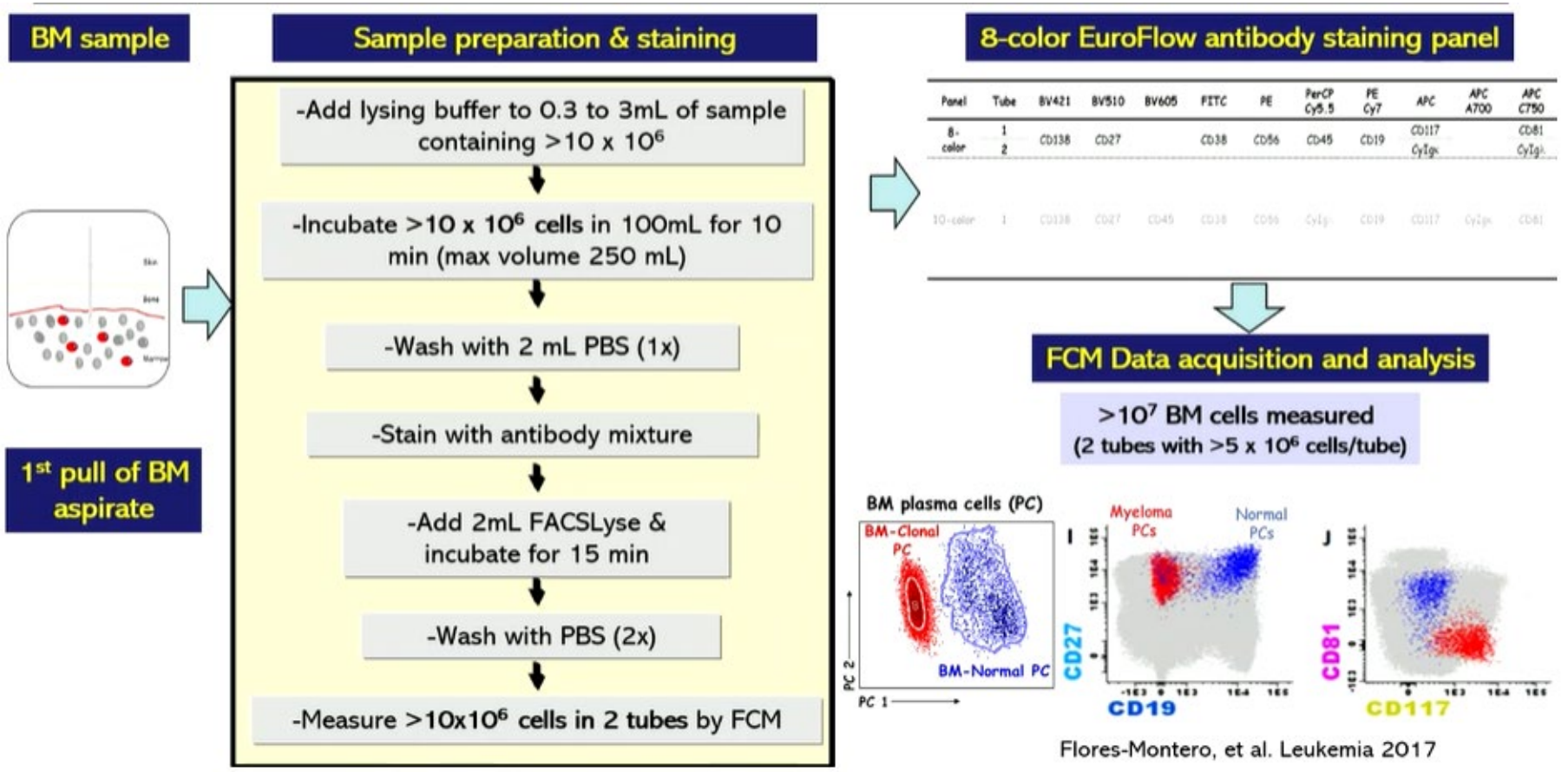
Verbeek MWC. Flow cytometric minimal residual disease assessment in B-cell precursor acute lymphoblastic leukaemia patients treated with CD19-targeted therapies —a EuroFlow study. BJH, 2021

EuroFlow NGF MRD ALL approach for detection of CD19- leukemic cells

- Example 3

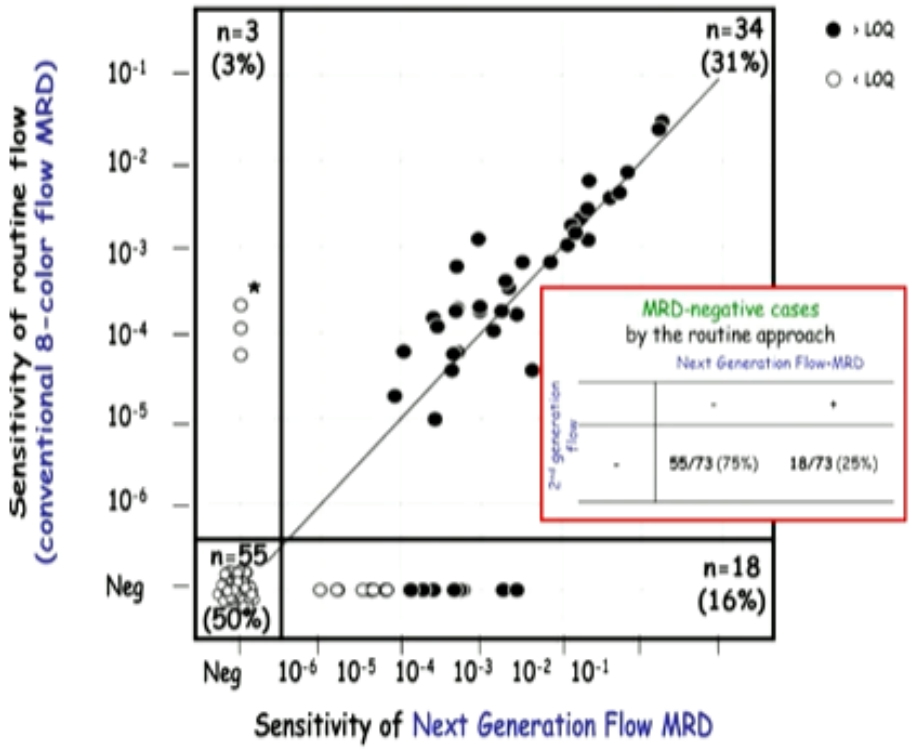
MM MRD

MRD detection in Multiple Myeloma by NGF



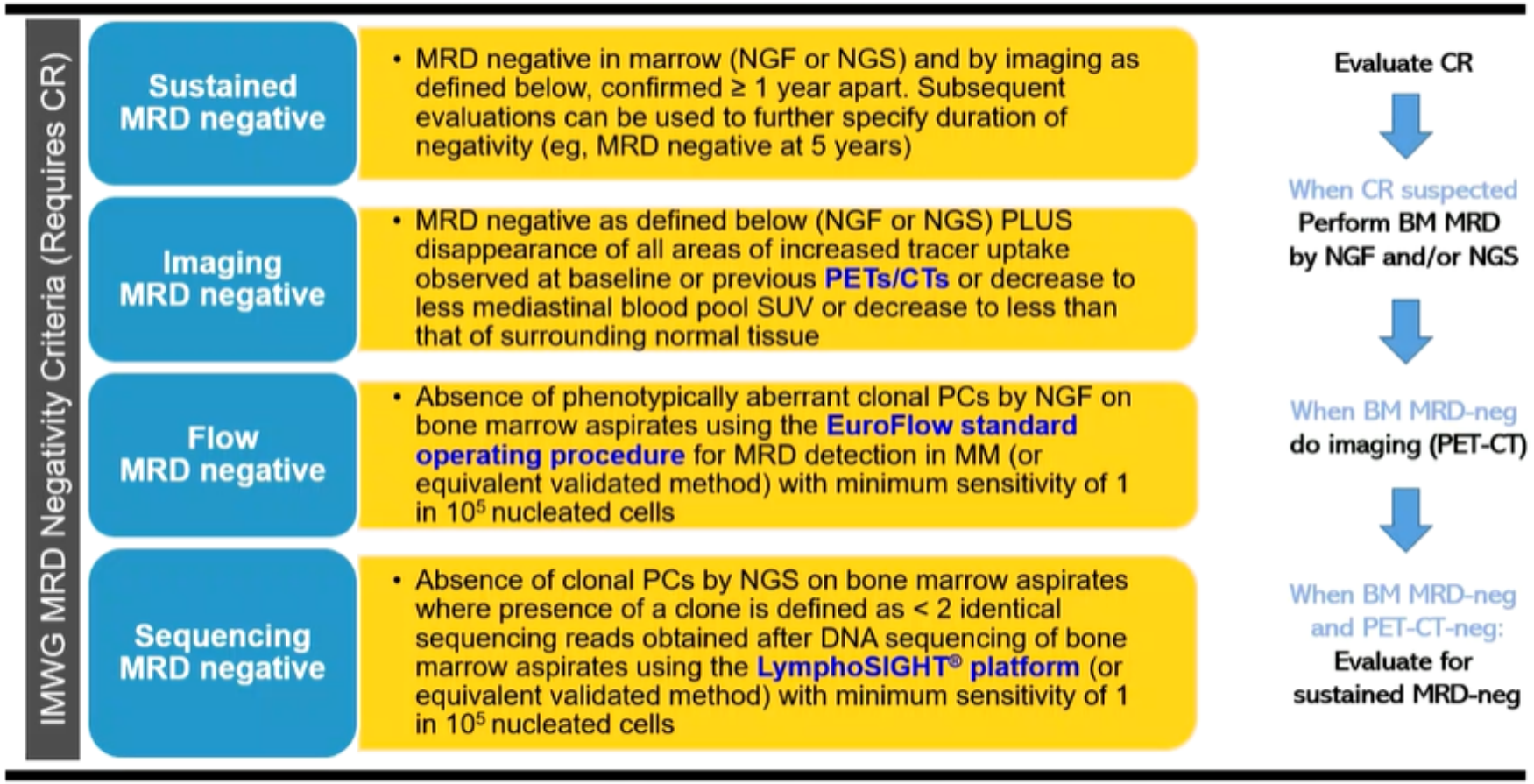
Flores-Montero, et al. Leukemia 2017

NGF MRD vs conventional 2nd generation (8-color) flow-MRD



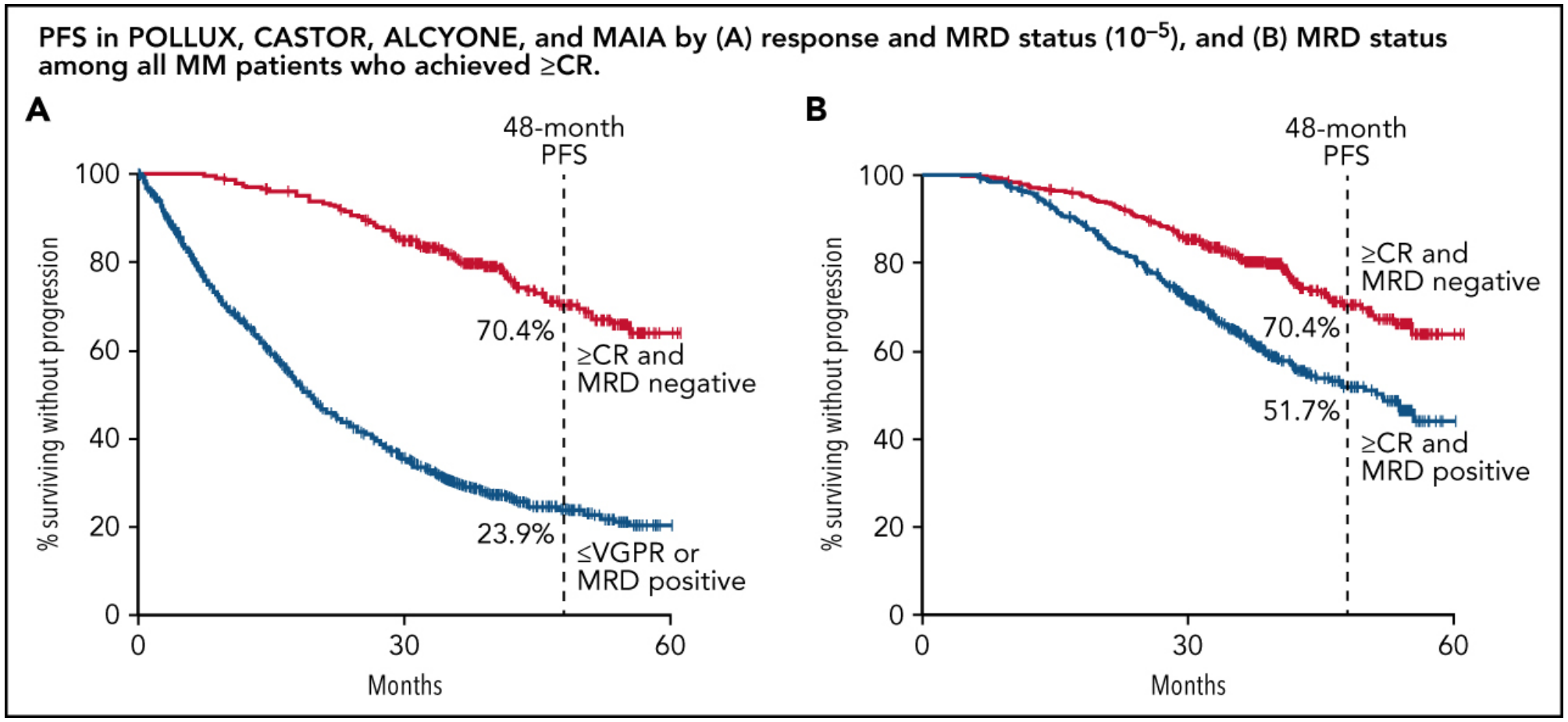
Case ID	MM Status	Conventional 8-color FCM				NGF (Tube 1 + Tube 2)			
		% tPC	% nPC	% aPC	# aPC	% tPC	% nPCs	% aPC	# aPC (Tube 1+ 2)
1	CR	0.01	0.01	0		0.0077	0.0073	0.0004	23
2	CR	0.013	0.013	0		0.018	0.017	0.0002	12
3	CR	0.0073	0.0073	0		0.0068	0.0068	0.0003	26
4	CR	0.04	0.04	0		0.02	0.02	0.0004	46
5	sCR	0	0	0		0.003	0.002	0.0006	43
6	CR	0.14	0.14	0		0.10	0.10	0.002	226
7	CR	0.21	0.21	0		0.24	0.22	0.02	1,866
8	sCR	0	0	0		0.002	0.0028	0.0002	13
9	CR	0.85	0.85	0		0.6	0.6	0.005	536
10	CR	0.19	0.19	0		0.06	0.06	0.0004	42
11	VGPR	0	0	0		0.01	0.01	0.0007	10
12	CR	0	0	0		0.01	0.01	0.006	476
13	sCR	0.23	0.23	0		0.17	0.17	0.005	450
14	sCR	0.03	0.03	0		0.03	0.029	0.002	133
15	CR	0.11	0.11	0		0.07	0.04	0.03	1,365
16	VGPR	0	0	0		0.009	0.008	0.001	92
17	CR	0.1	0	0		0.004	0.003	0.001	127
18	CR	0.04	0	0		0.01	0.01	0.0002	16
19	CR	0.09	0.079	0.011	113	0.05	0.05	0	%, percentage; #, number of dots; t, total; n, normal; a, abnormal; PC, plasma cells
20	CR	0.11	0.1	0.005	55	0.14	0.14	0	
21	CR	0.35	0.32	0.02	210	0.25	0.25	0	

2016 IMWG criteria for MRD in MM



CR, complete response; CT, computed tomography; IMWG, International Myeloma Working Group; MM, multiple myeloma; MRD, minimal residual disease; NGF, next-generation flow; NGS, next-generation sequencing; PC, plasma cell; PET, positron emission tomography; SUV, standardized uptake value.

MRD in MM



Cavo M et al. Prognostic value of minimal residual disease negativity in myeloma: combined analysis of POLLUX, CASTOR, ALCYONE, and MAIA. Blood 2022

Examples AG&I

- GHE_003
- GHE_004
- MM_MRD_2023_II_1

Conclusions

Next Generation Flow Cytometry (NGF)

Advantages

- ✓ **Fast** (within 3-4 h)
- ✓ **Highly Standardized** with automated gating
- ✓ Efficient data storage/management with easy data comparison and review
- ✓ Accurate **quantification**
- ✓ **Increased sensitivity** (10^{-5} – 10^{-6}
- ✓ **Information on normal and malignant cells** (sample quality and reconstitution of normal compartments)
- ✓ **Ready for IVD development**
- ✓ **Further purification/characterization of MRD cells**

Limitations

- **Education** and **training** still required
- **Many cells needed** to reach the required sensitivity, (e.g. 5 x 10^6, if quantification down to 10^{-5} is needed)
- **Fresh** (<24h) samples

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