Standardized flowcytometric MRD detection in BCP-ALL patients

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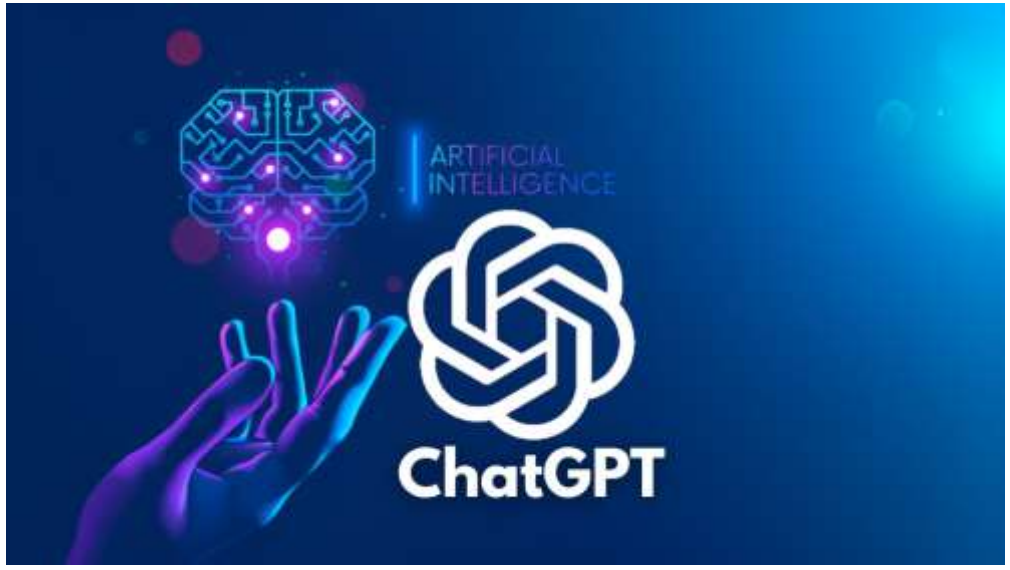
Conflict of Interest Disclosure

I hereby declare the following potential conflicts of interest concerning my presentation:

- Lab Service Agreements: BD Biosciences, Agilent, Navigate, Pfizer, Janssen
- Patents and Royalties: Co-inventor on EuroFlow patents (PCT/NL2010/050332 and PCT/NL2013/0505420); all income for institution

Standardized flowcytometric MRD analysis in BCP-ALL patients



- Standardized flow cytometric MRD analysis in BCP-ALL patients is essential for consistent and reliable monitoring of disease status and treatment response.

Standardized flowcytometric MRD analysis in BCP-ALL patients

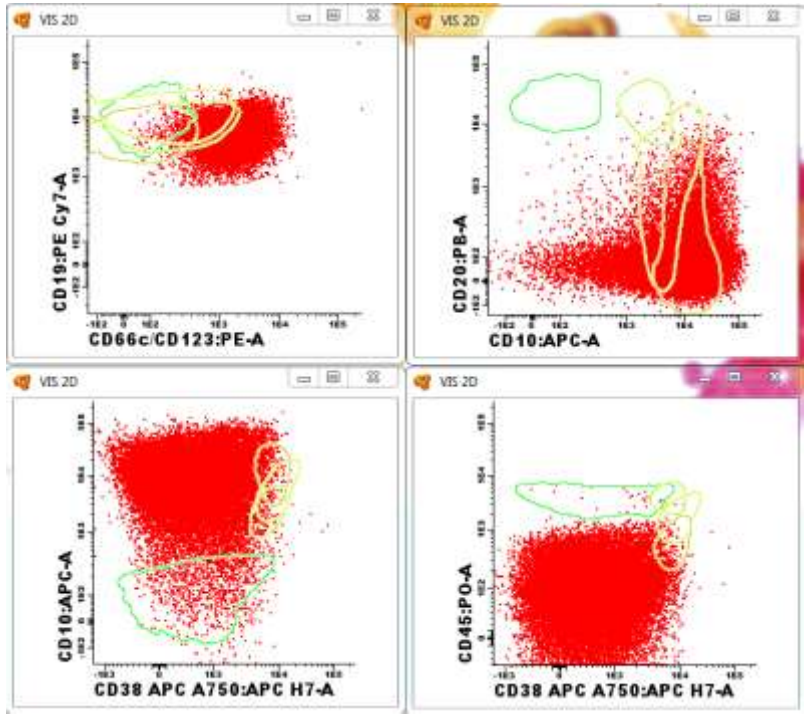
- Here are the key steps involved in standardized flow cytometric MRD analysis for BCP-ALL:
  1. Sample Collection
  2. Sample Preparation
  3. Antibody Panel Design
  4. Staining
  5. Flow Cytometry Acquisition
  6. Data Analysis
  7. Reporting
  8. Quality Control and Assurance
  9. Inter-Laboratory Standardization
  10. Clinical Interpretation
  11. Continued Education and Training
  12. Research and Development

Flow cytometry for MRD analysis in BCP-ALL patients

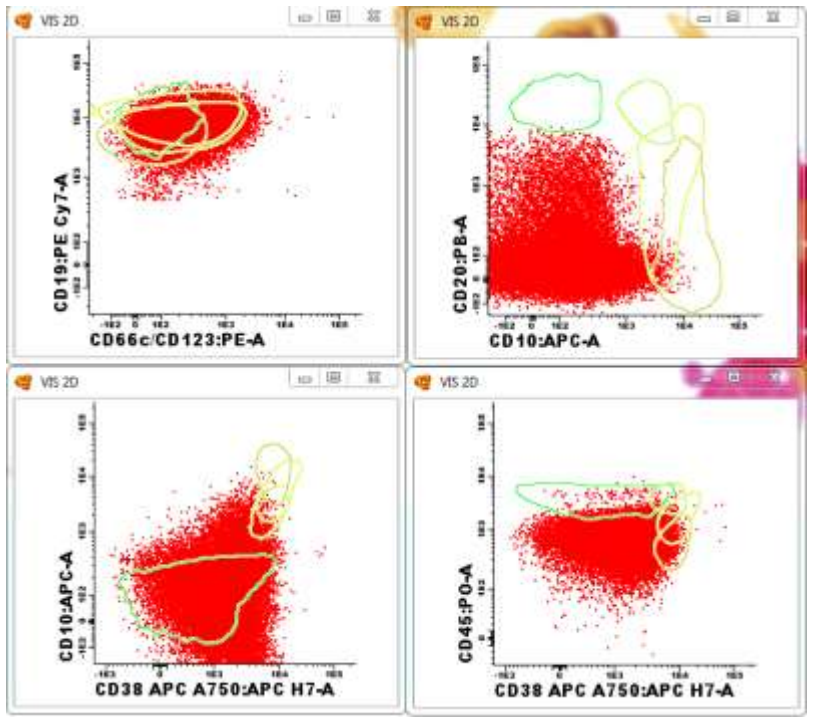
- Topics:
  - Current approaches
  - Possible impact of targeted therapies (especially CD19)

Flow cytometric MRD analysis in BCP-ALL patients

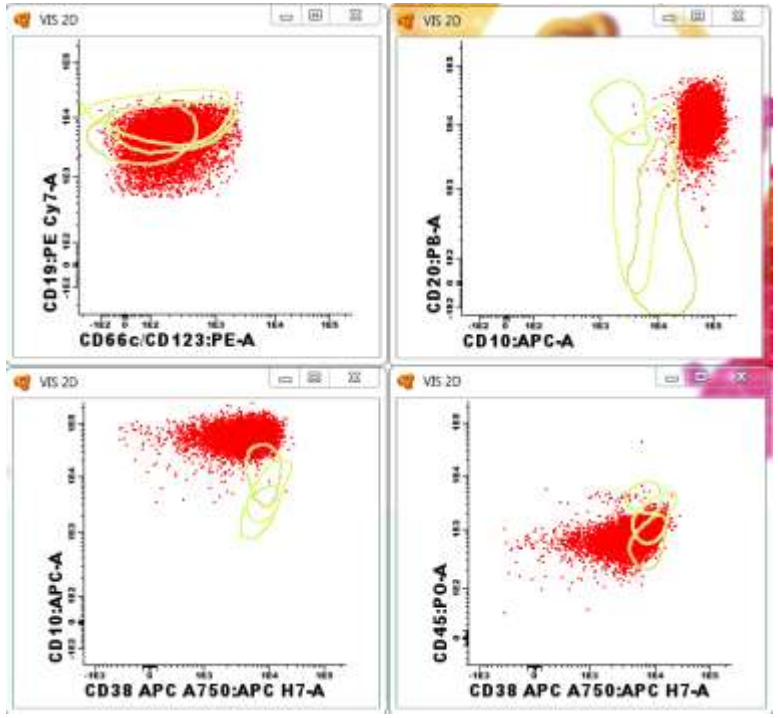
- Principle: BCP-ALL cells have an aberrant immunophenotype
- Focus on CD19+ B-cells:



Patient 1



Patient 2



Patient 3

ALL cells
(dots)

Normal cells
(2SD contours)

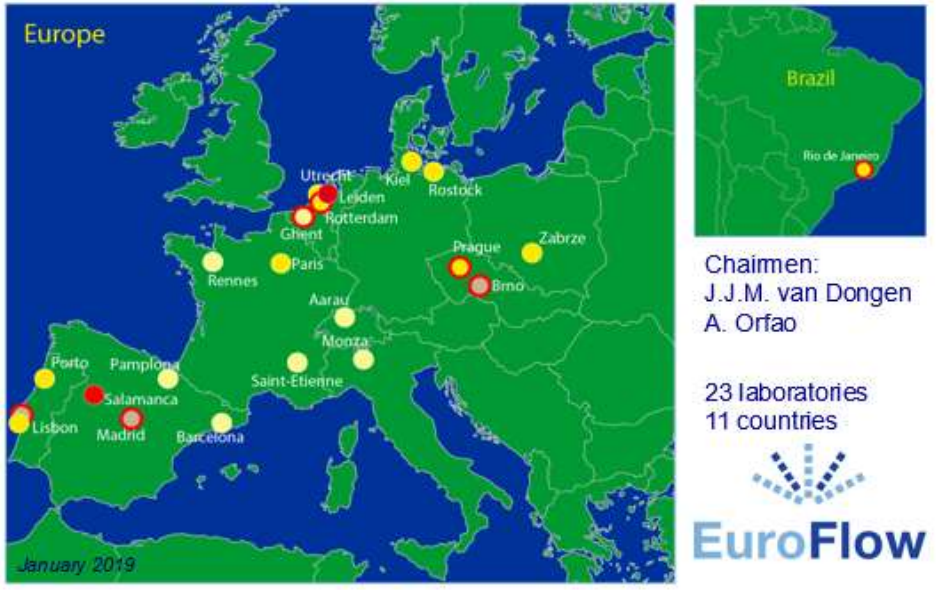
Different patients → different immunophenotypes

EuroFlow BCP-ALL MRD protocol

- Two 8-color BCP-ALL MRD tubes

PB	PO	FITC	PE	PerCP Cy5.5	PE Cy7	APC	APC C750
CD20	CD45	**CD81**	CD66c/CD123	CD34	CD19	CD10	CD38
CD20	CD45	**CD81**	**CD73/CD304**	CD34	CD19	CD10	CD38

- Applicable in >98% of patients (good separation between normal B-cells and ALL cells → high specificity)



Theunissen, et al. Blood 2017

Sensitivity – Optimization of protocol

- For a limit of quantitation, a cluster of 40 cells is needed
- Thus, at least 4 million cells shoud be acquired to reach a sensitivity of at least 10^{-5} (0,001%), comparable to RQ-PCR

- WBC counts are frequently low during follow-up

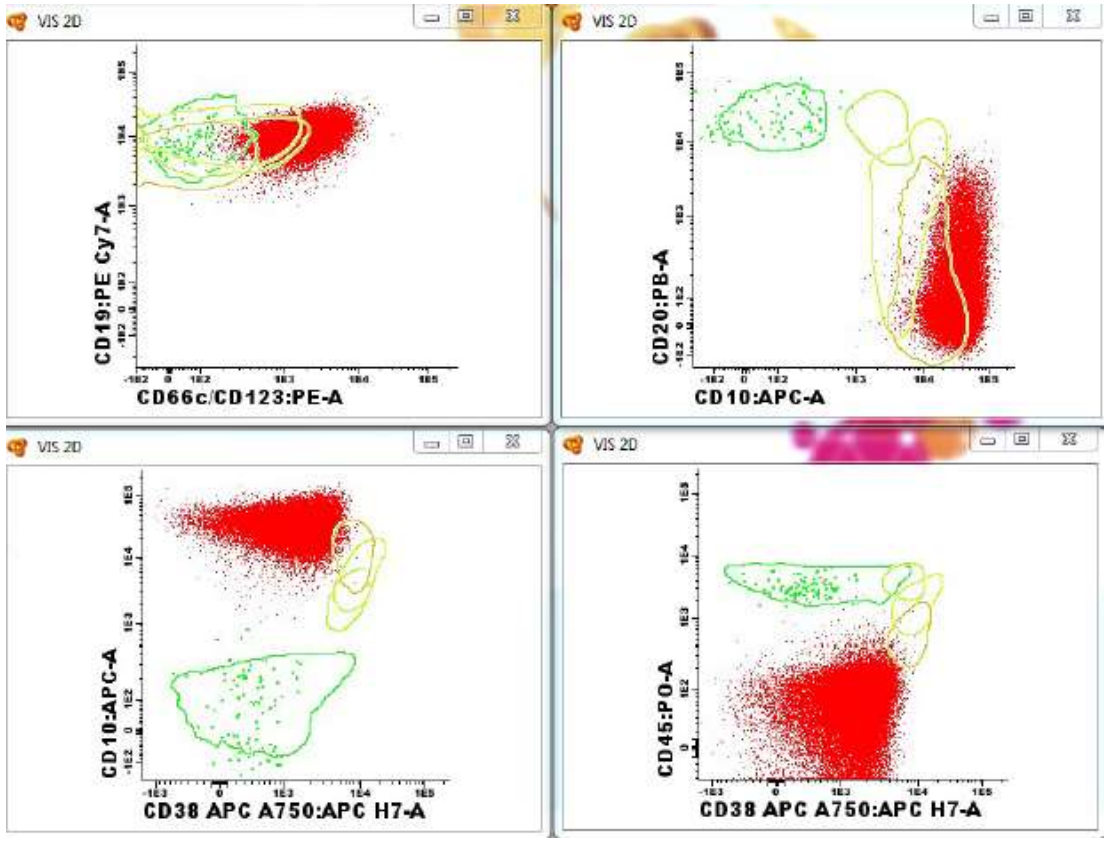
- Bulk lysis protocol adapted and optimized (www.EuroFlow.org)
  - Cell suspension $100x10^6$/ml, 100 µl/tube (10 million)

Theunissen et al, Blood 2017

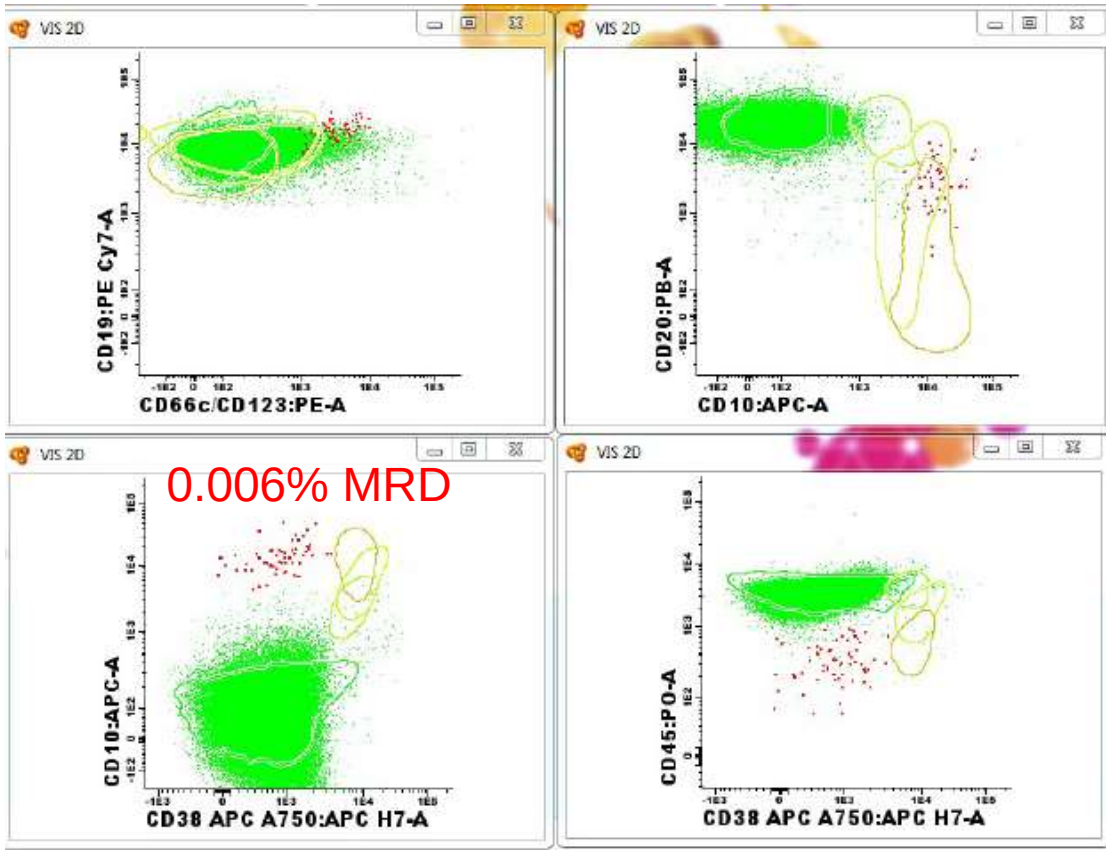
Flowcytometric MRD detection

- Gate on CD19+ B-cells:

Diagnosis



Follow-up



- Sensitivity 0,001% <u>if 4 million cells acquired</u> (98% concordance with RQ-PCR data)

Flow cytometry: standardization and QA

- Standardization
  - EuroFlow: full standardization of instrument settings, sample processing, antibody panels, staining protocol and acquisition

- Quality control
  - EuroFlow technical QA program since 2013
  - EuroFlow BCP-ALL MRD program opened in 2023
  - UK NEQAS ALL MRD program

plus workshops

→ Robust, highly applicable, sensitive standardized assay

- But….evaluated on “classically” treated patients

Kalina et al. Cytometry 2015

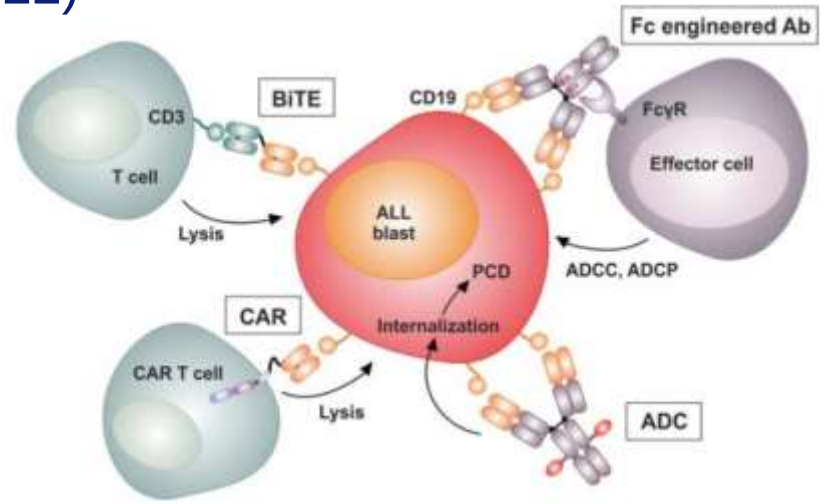
Flow cytometry for MRD analysis in BCP-ALL patients

- Topics:
  - Current approaches
  - Possible impact of targeted therapies (especially CD19)

Novel targeted therapies for BCP-ALL patients

- Antibodies
  - Naked antibodies: Rituximab (CD20), Daratumumab (CD38)
  - Toxin-conjugated antibodies: Inotuzumab Ozogamicin (CD22)
  - Bispecific T-cell engagers: Blinatumomab (CD19 x CD3)

- CAR-T cells
  - CART19
  - CART22
  - CART123



PB	PO	FITC	PE	PerCP Cy5.5	PE Cy7	APC	APC C750
CD20	CD45	**CD81**	CD66c/CD123	CD34	CD19	CD10	CD38
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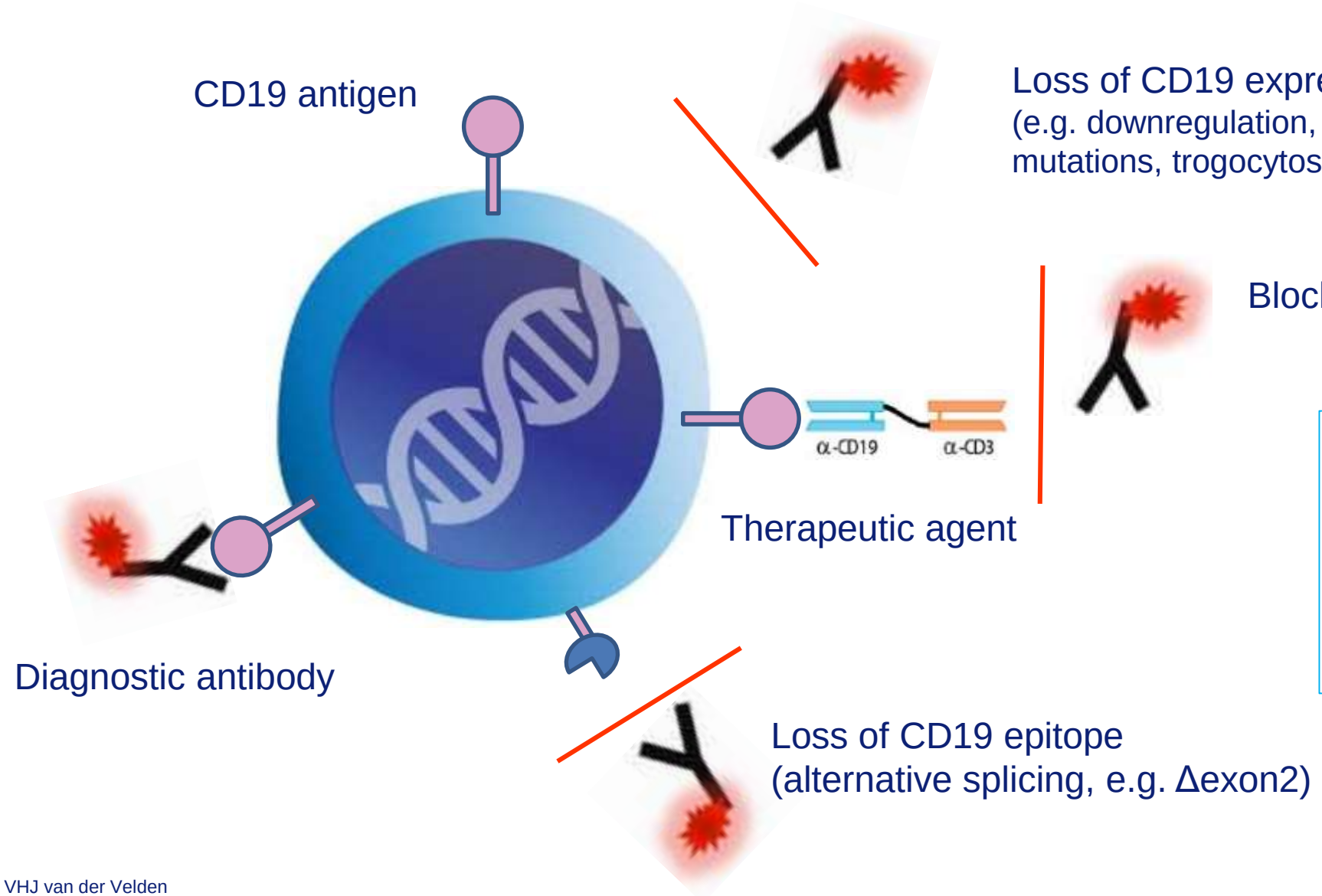
Kellner et al, OncoImmunology 2018

Diagnostic pitfalls of targeted therapies



Loss of CD19 expression
(e.g. downregulation, mutations, lineage switch, CD81 mutations, trogocytosis)

Blocking of CD19 epitope

Loss of CD19 epitope
(alternative splicing, e.g. Δexon2)

→ If CD19 gating not possible:

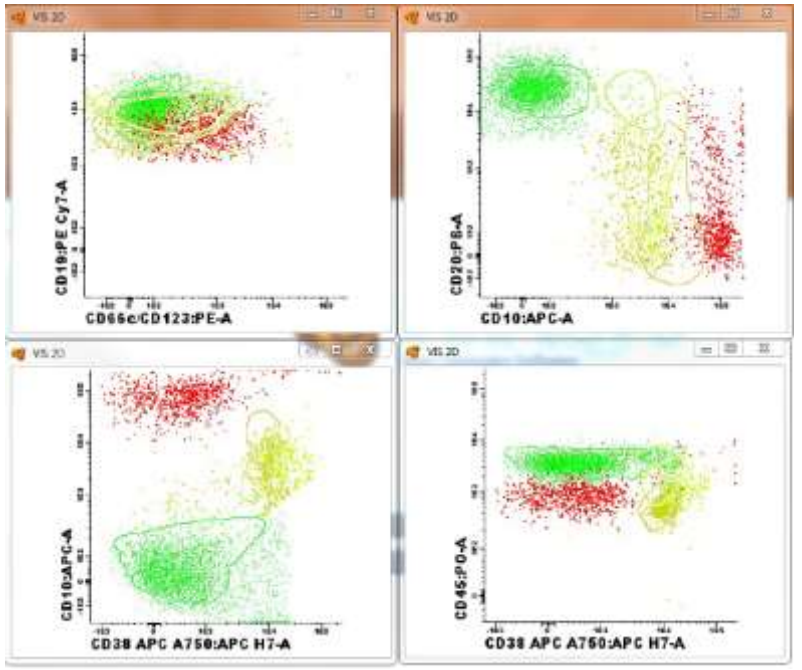
1. Alternative gating strategy?
2. Semi-automated analysis?
3. Alternative B-cell markers?

1. Alternative gating strategy?

- Data analysis in multiple phases
  - Patient files with high MRD levels
  - Patient files with low MRD levels

    → Design of common gating strategy (focus on CD10+ and/or CD34+)
  - Artificial CD19-negative files (without Dx information)
  - Artificial CD19-negative files (with Dx information)

    → Gating strategy adapted and reference images added
  - Validation using real life patient samples



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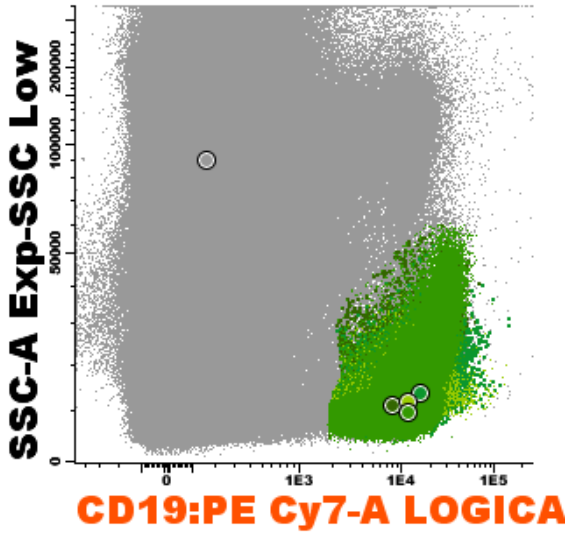
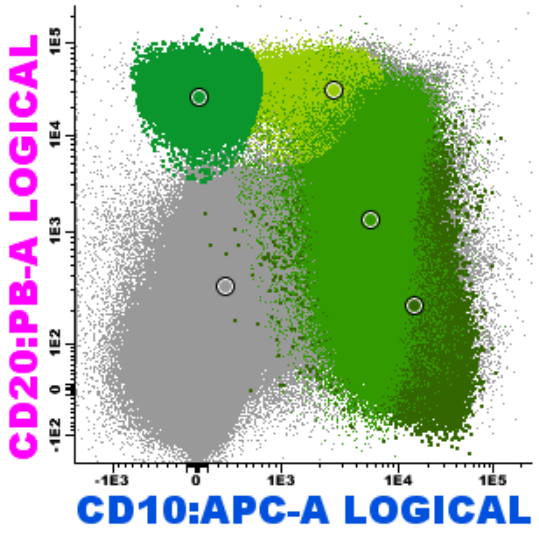
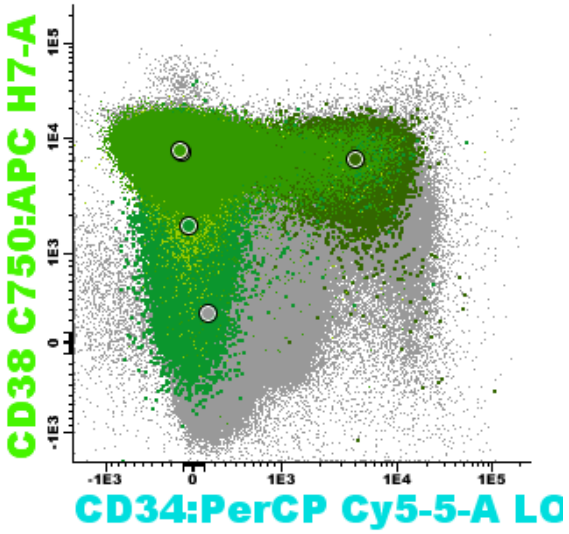
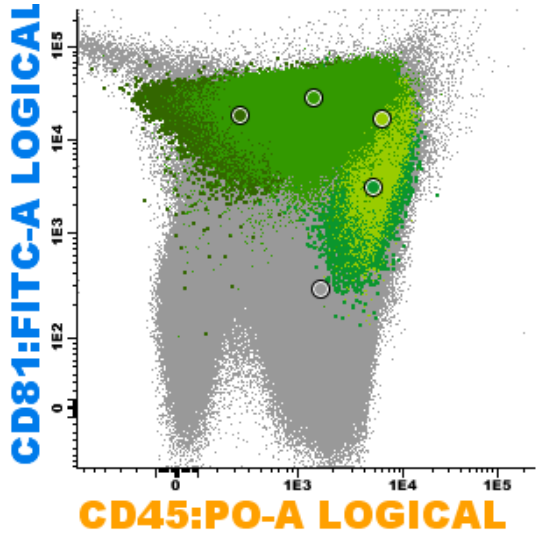
1. Alternative gating strategy?

CD19 gate

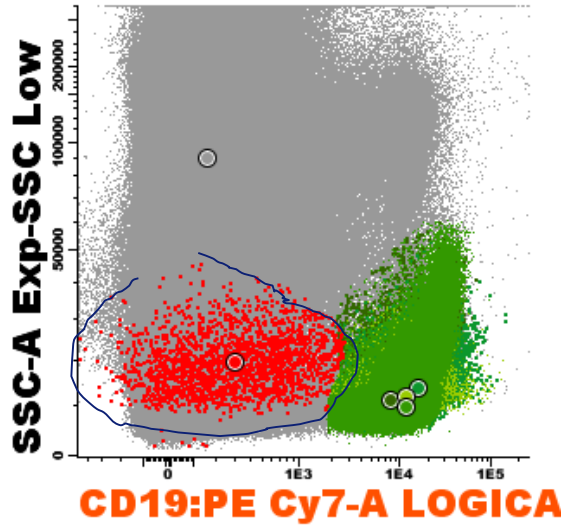


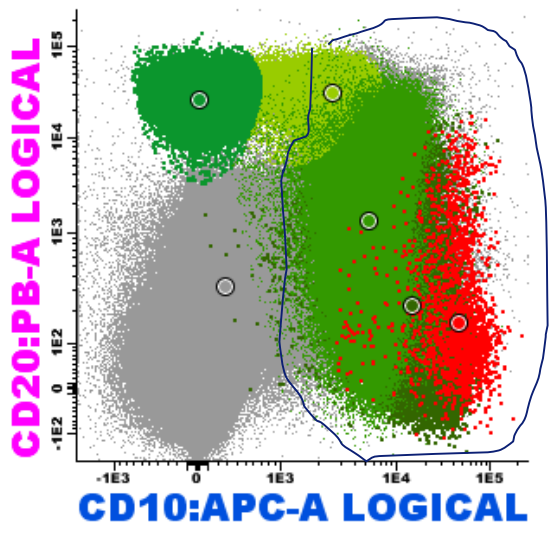


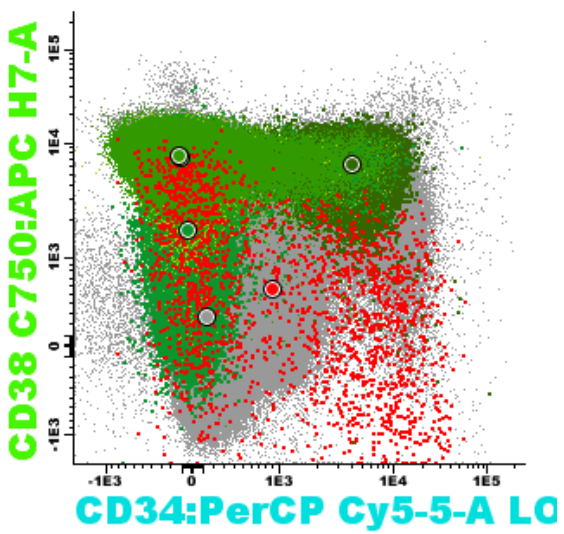


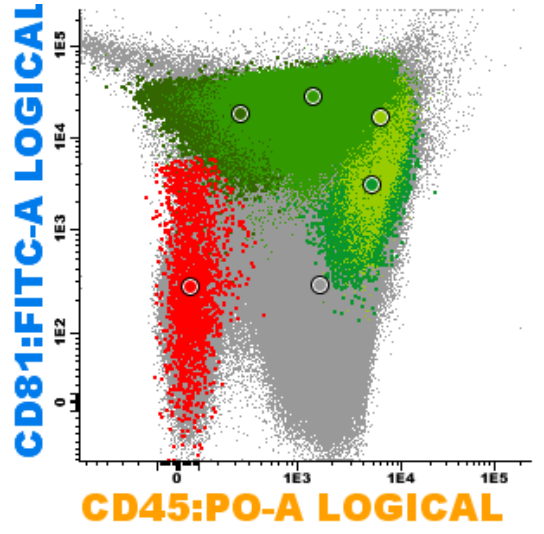


CD10+CD19- gate

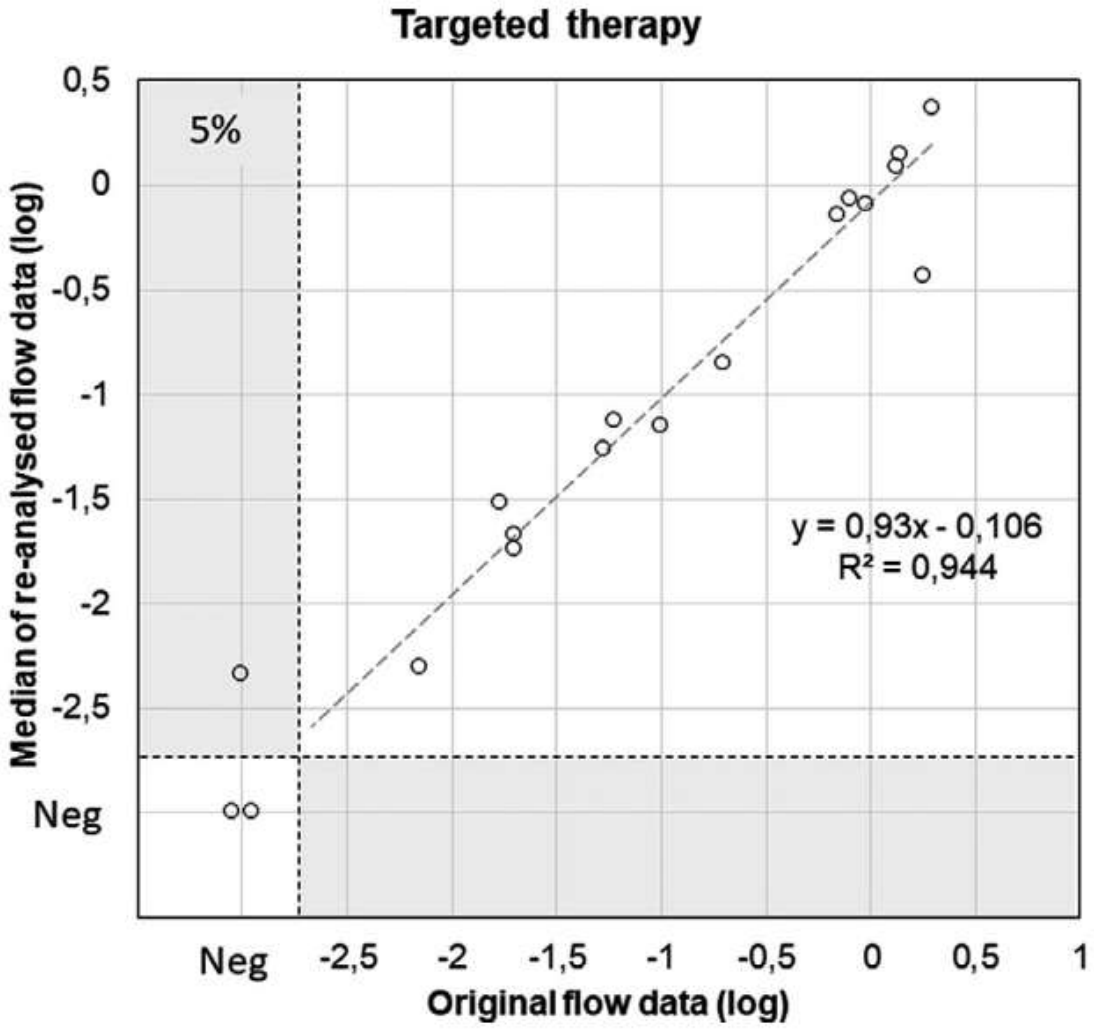


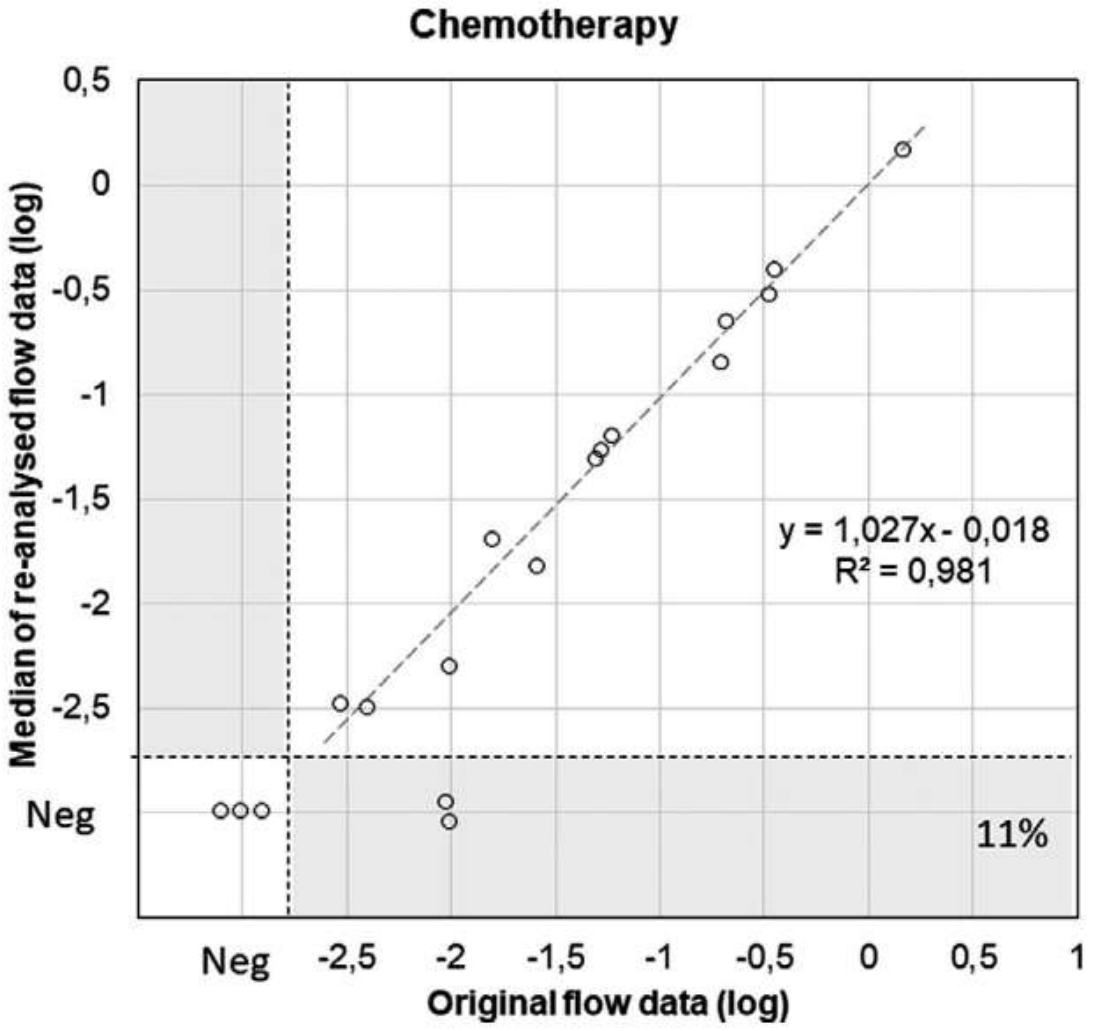






1. Alternative gating strategy – results





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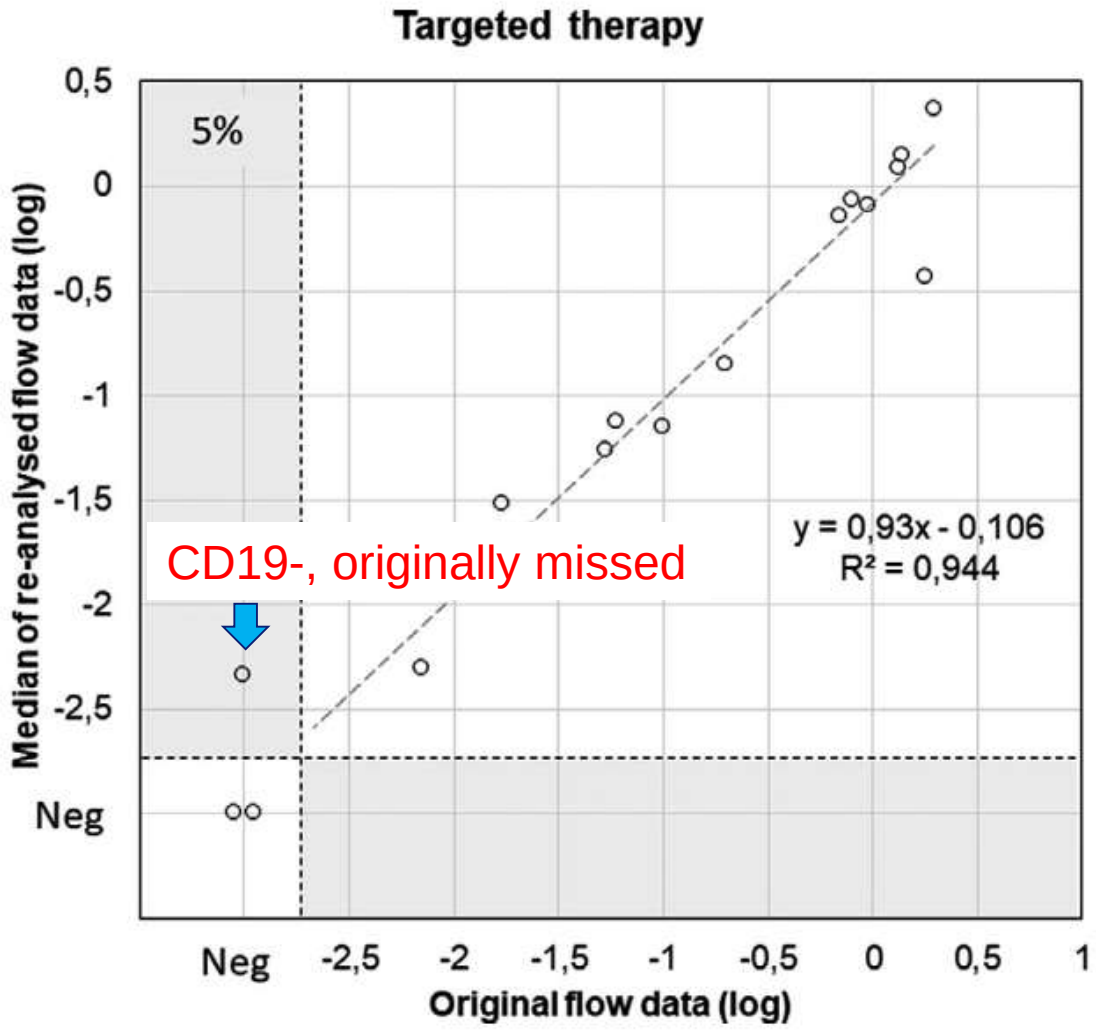

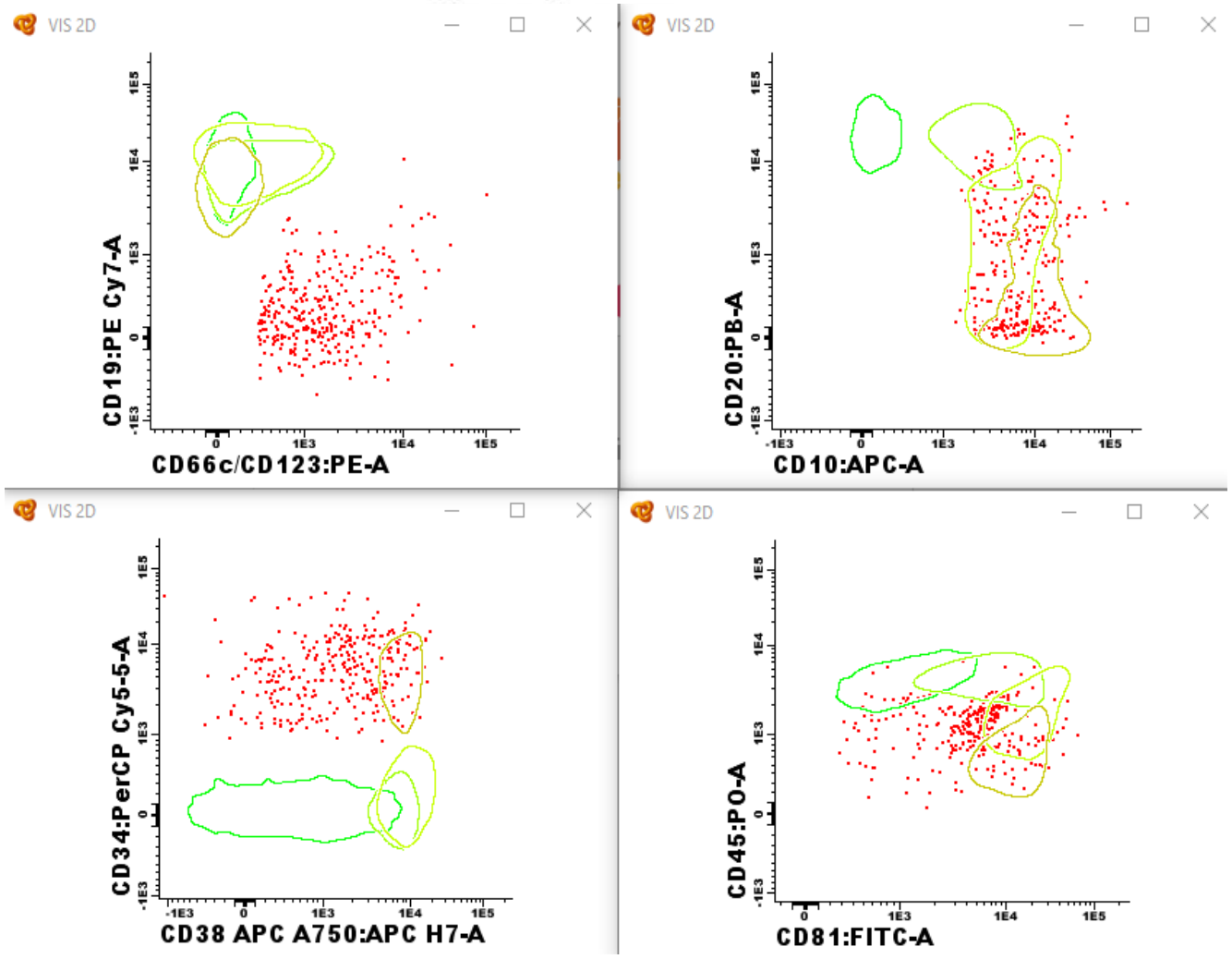

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Conclusions – 1

- MRD analysis in BCP-ALL patients using the eight-color EuroFlow tubes can reliably be done, both in patients treated with chemotherapy and in patients treated with CD19-targeted therapies

- It likely remains more difficult to assess MRD levels in CD10-negative BCP-ALL treated with targeted therapies, especially if these are also CD34-negative.

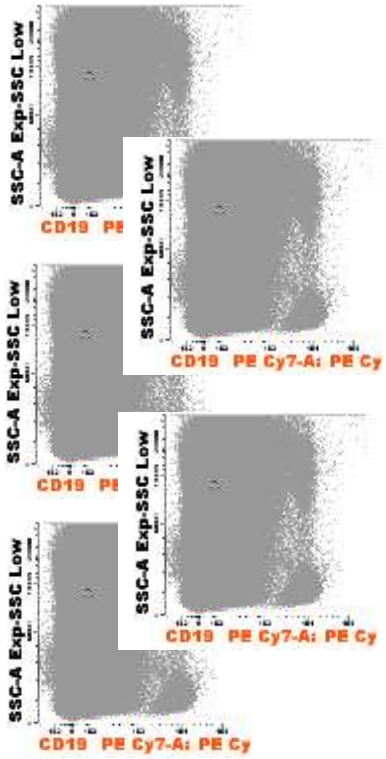
Conclusion – 1

If CD19 gating is not possible:

1. Alternative gating strategy? 
2. Semi-automated gating?
3. Alternative B-cell markers?

2. Semi-automated analysis?

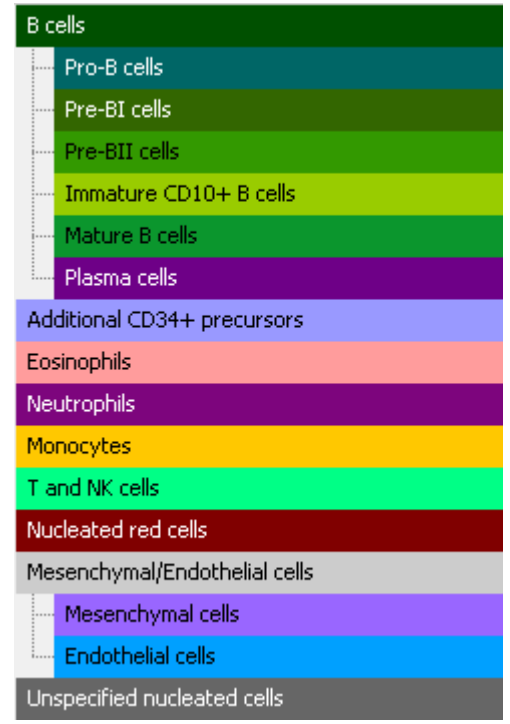
- Use database with immunophenotype of normal cells to allocate all normal cells



Normal bone marrow samples

clustering

Database of 15 normal cell types



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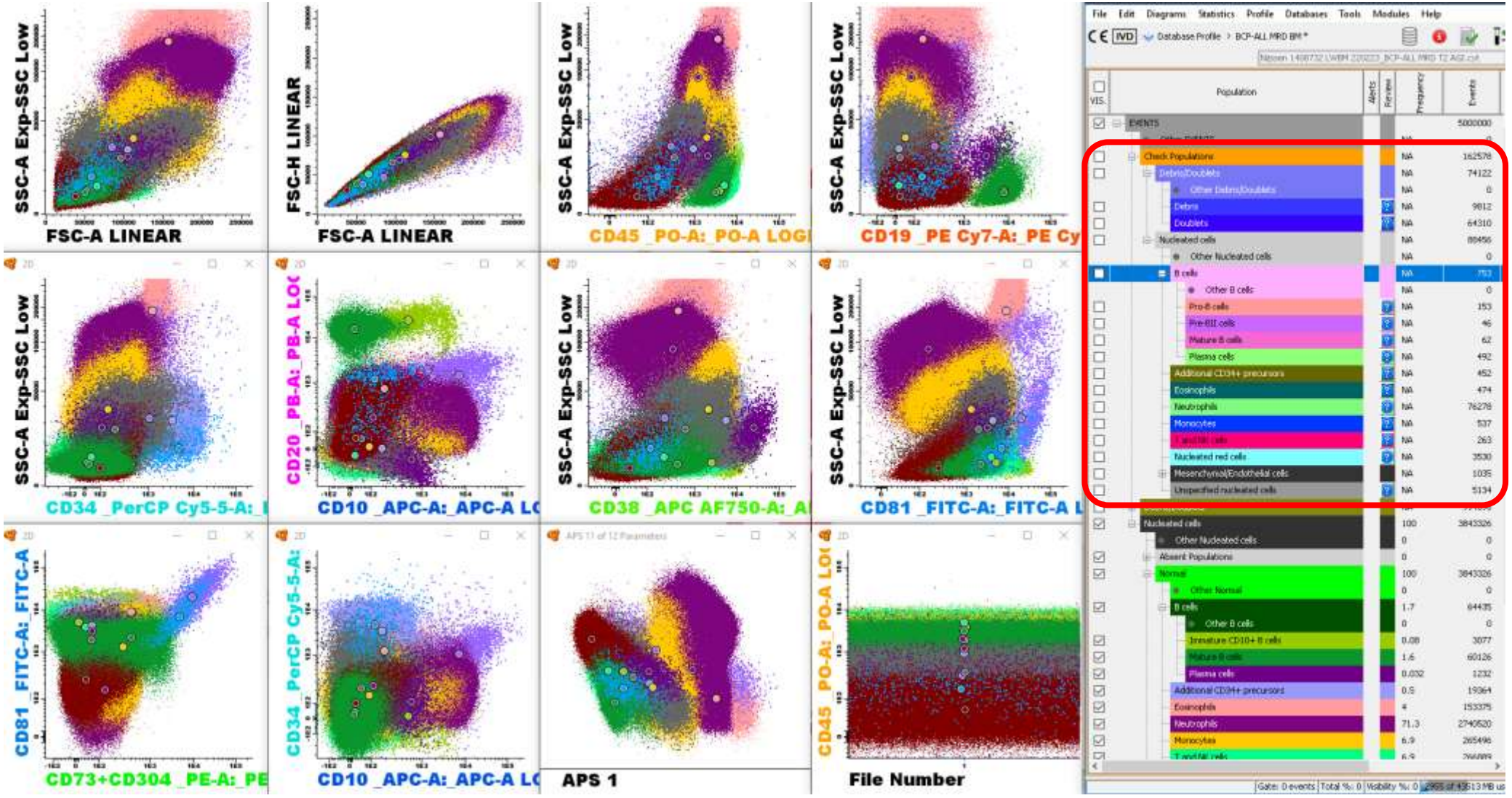
2. Semi-automated analysis – after AGI tool



3%

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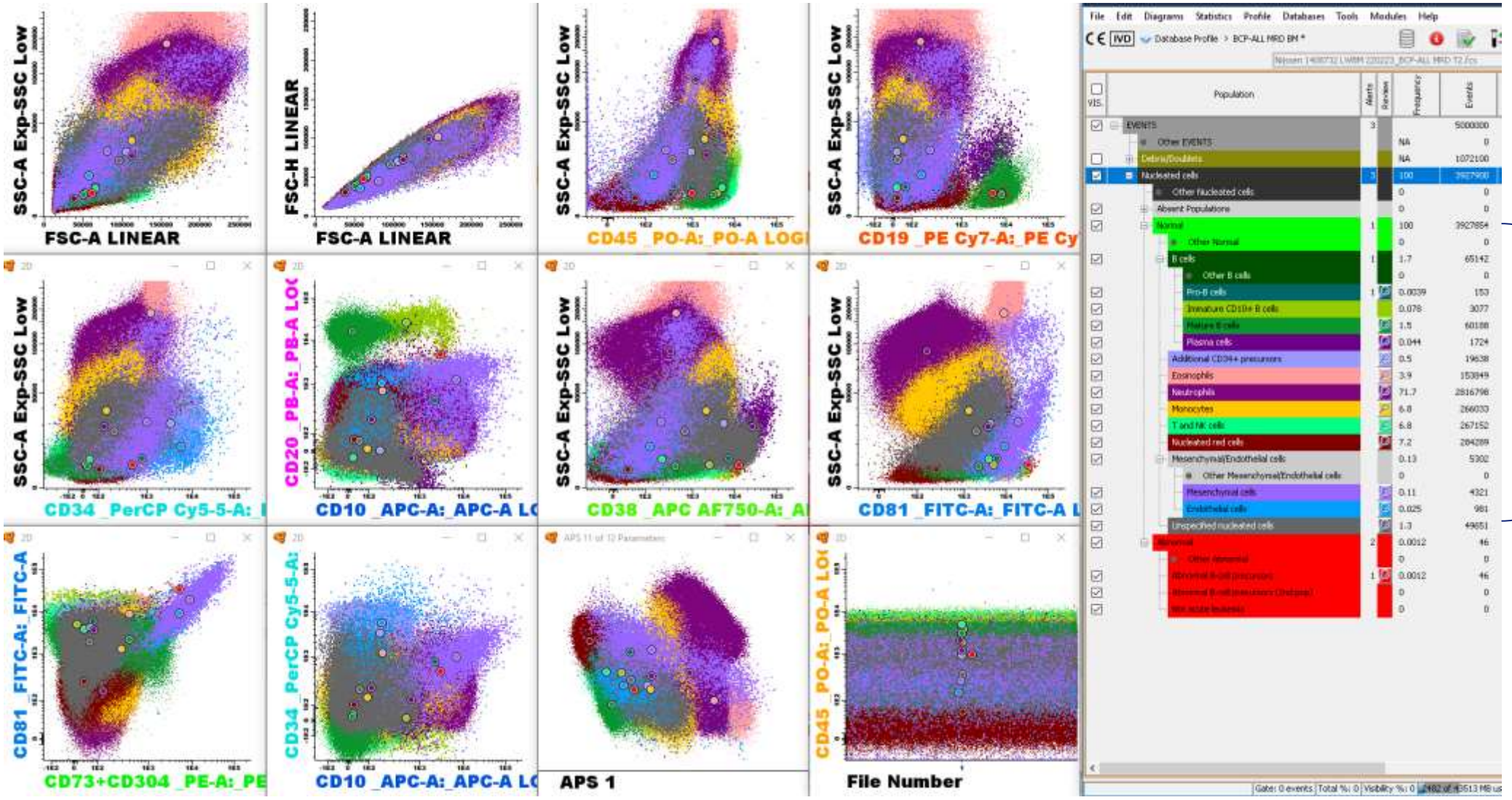
2. Semi-automated analysis – after review checks



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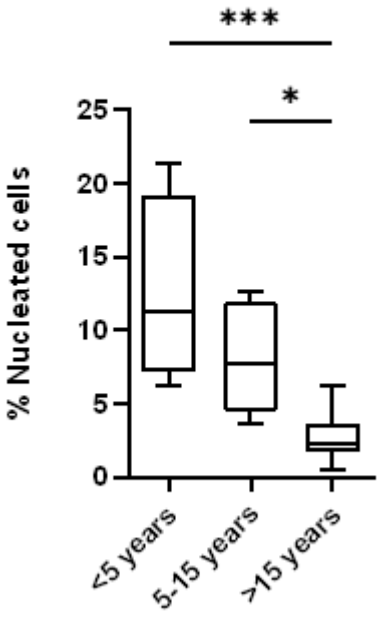
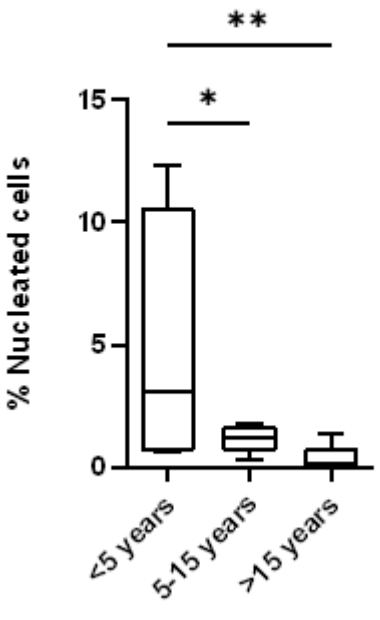
Age-related changes in cellular composition

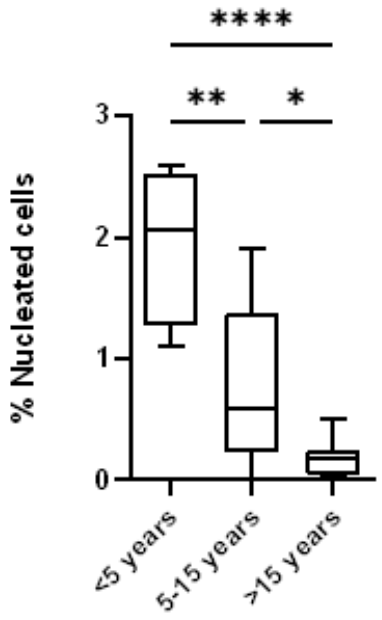
B-cells



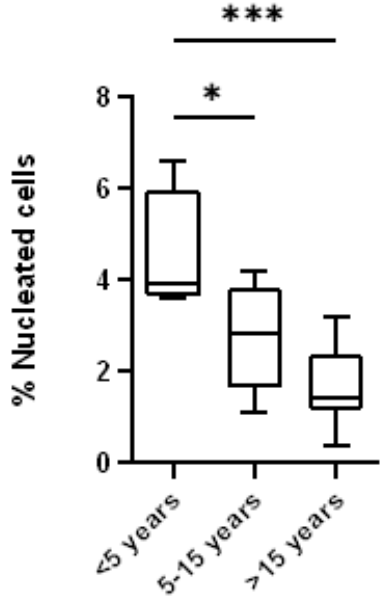
Pre-B-II cells



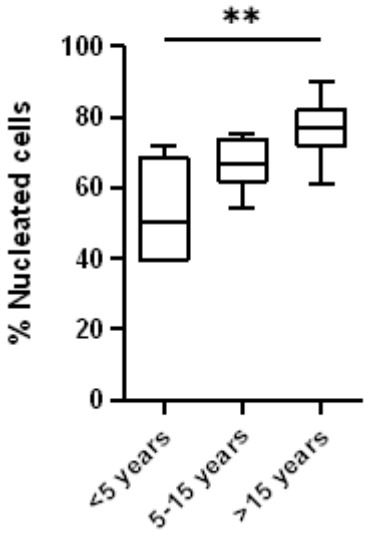
Immature CD10+ B-cells



Mature B-cells



Neutrophils



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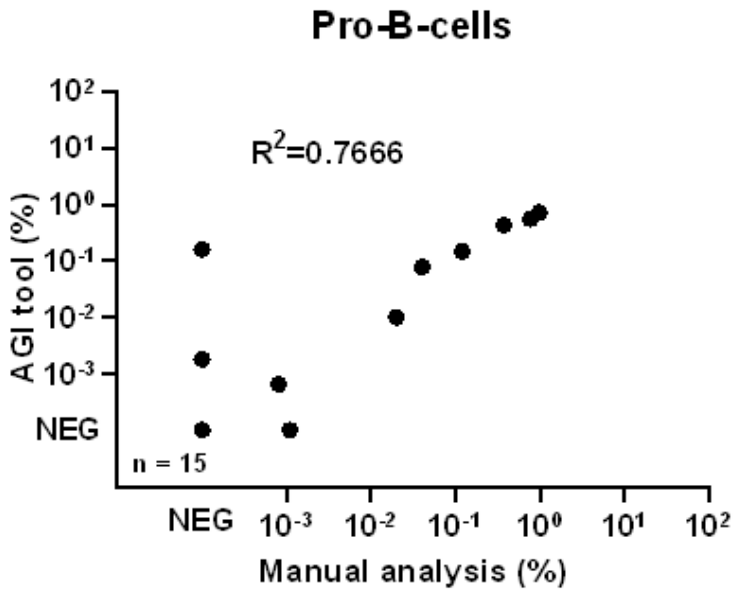
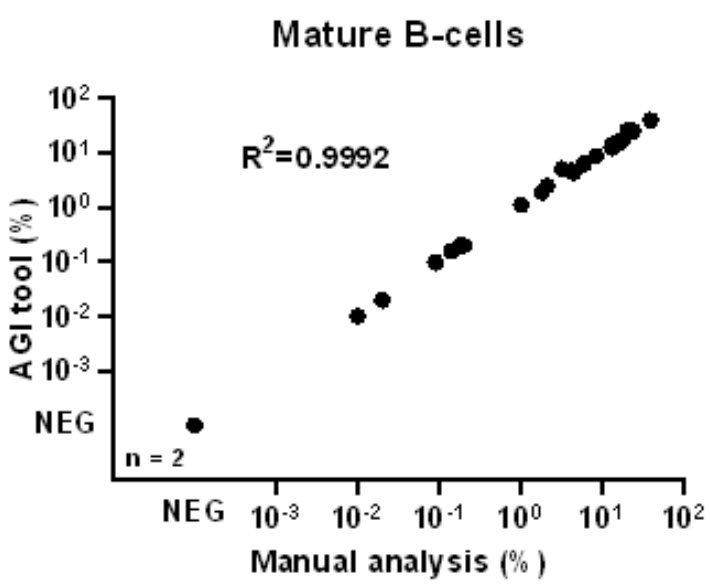
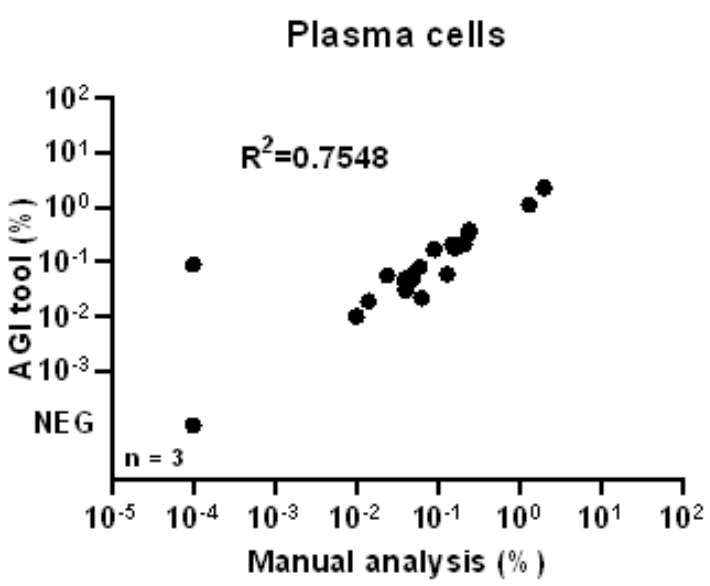
Correct assignment of normal cells by AGI tool







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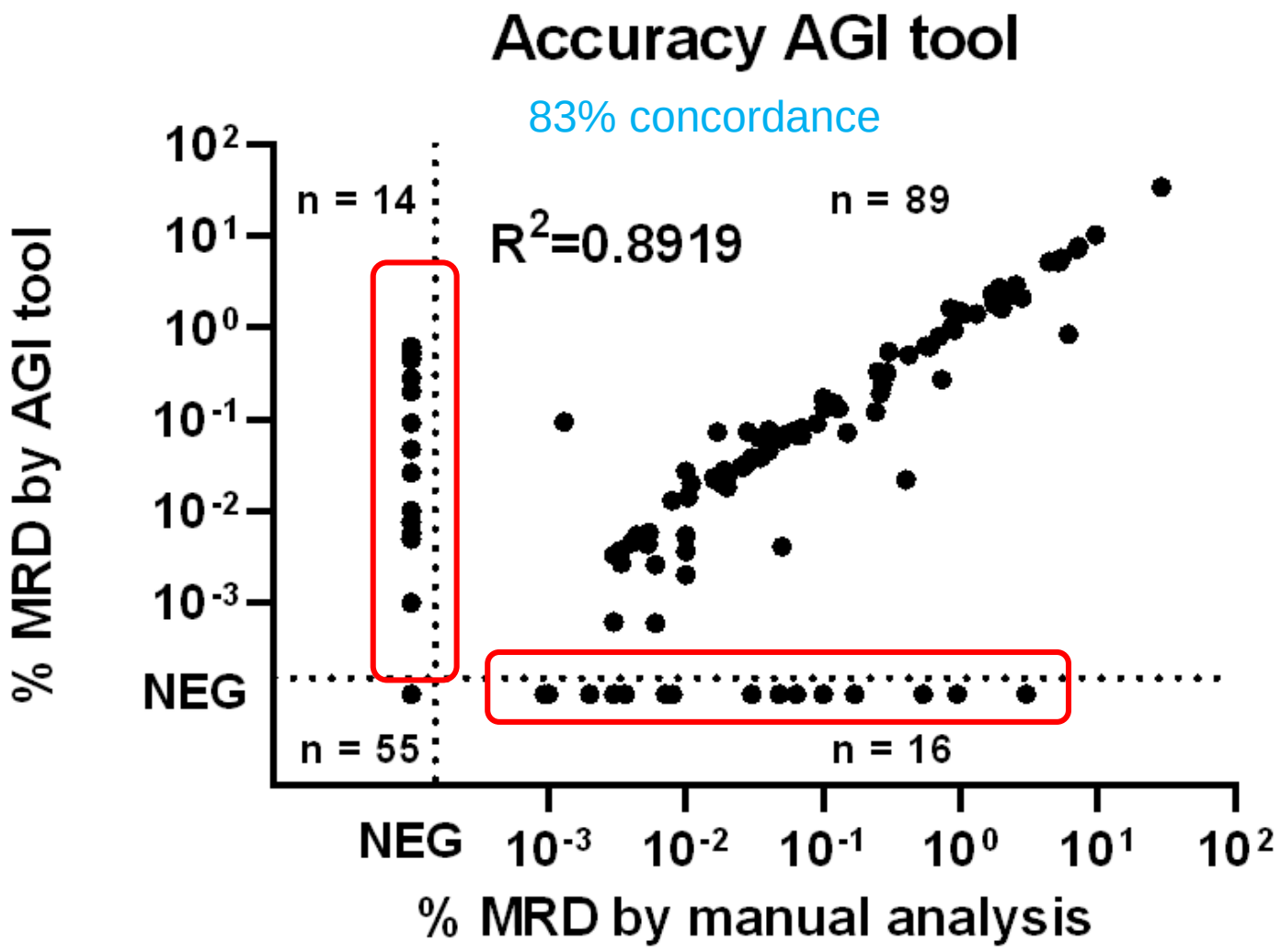
2. Semi-automated analysis – Results



Manual analysis and AGI tool by single but different expert

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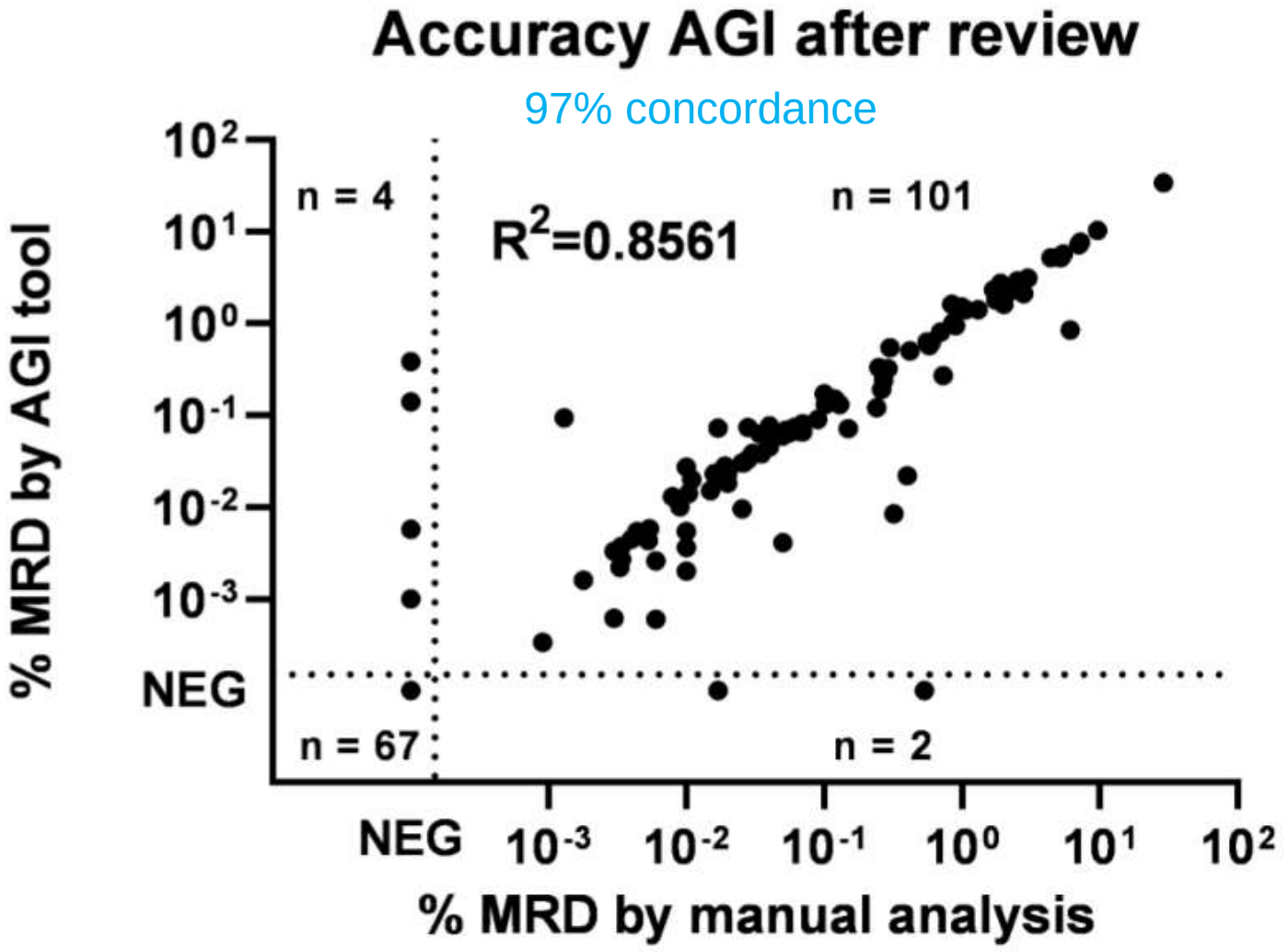
2. Semi-automated analysis – Results



Manual analysis and AGI tool both by multiple experts

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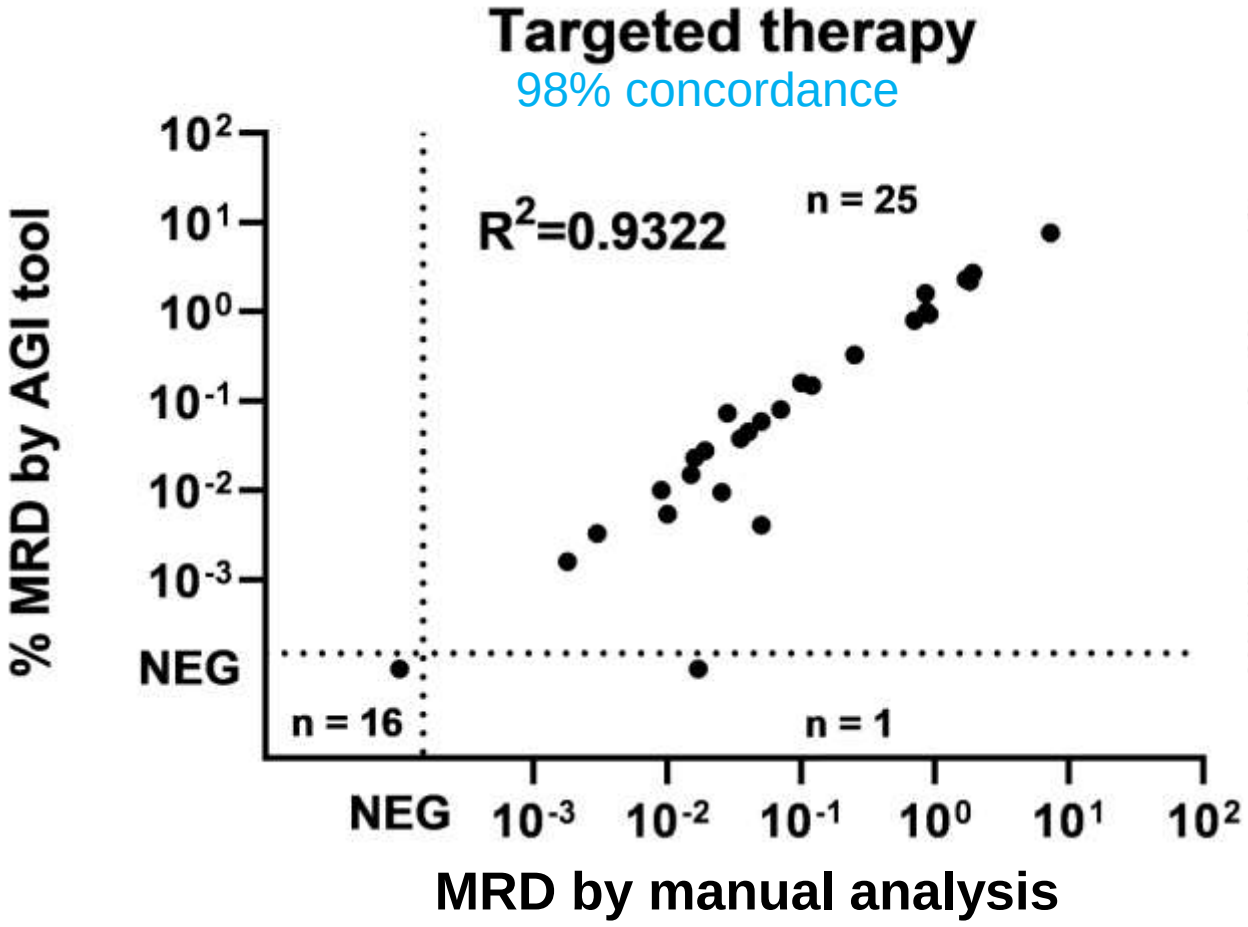
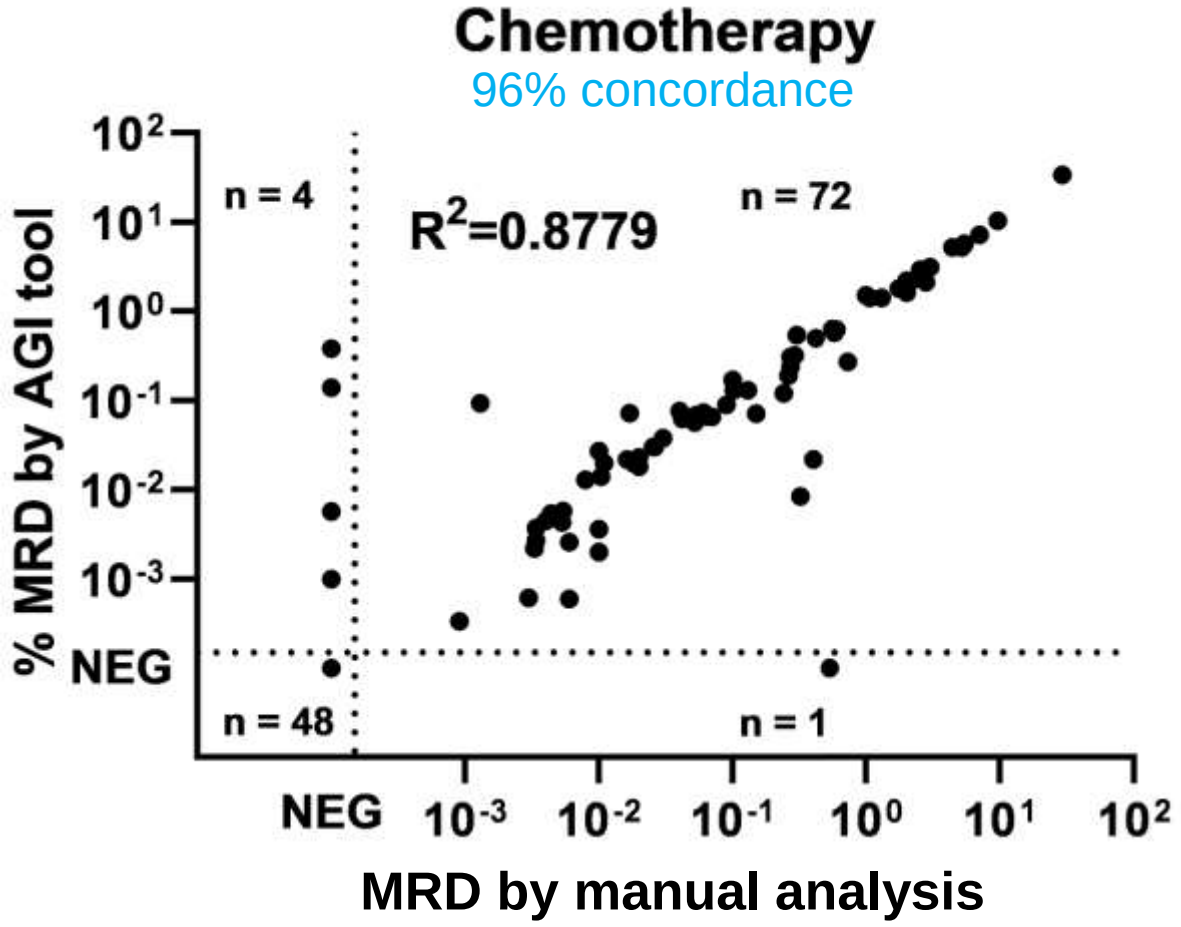
2. Semi-automated analysis – Results





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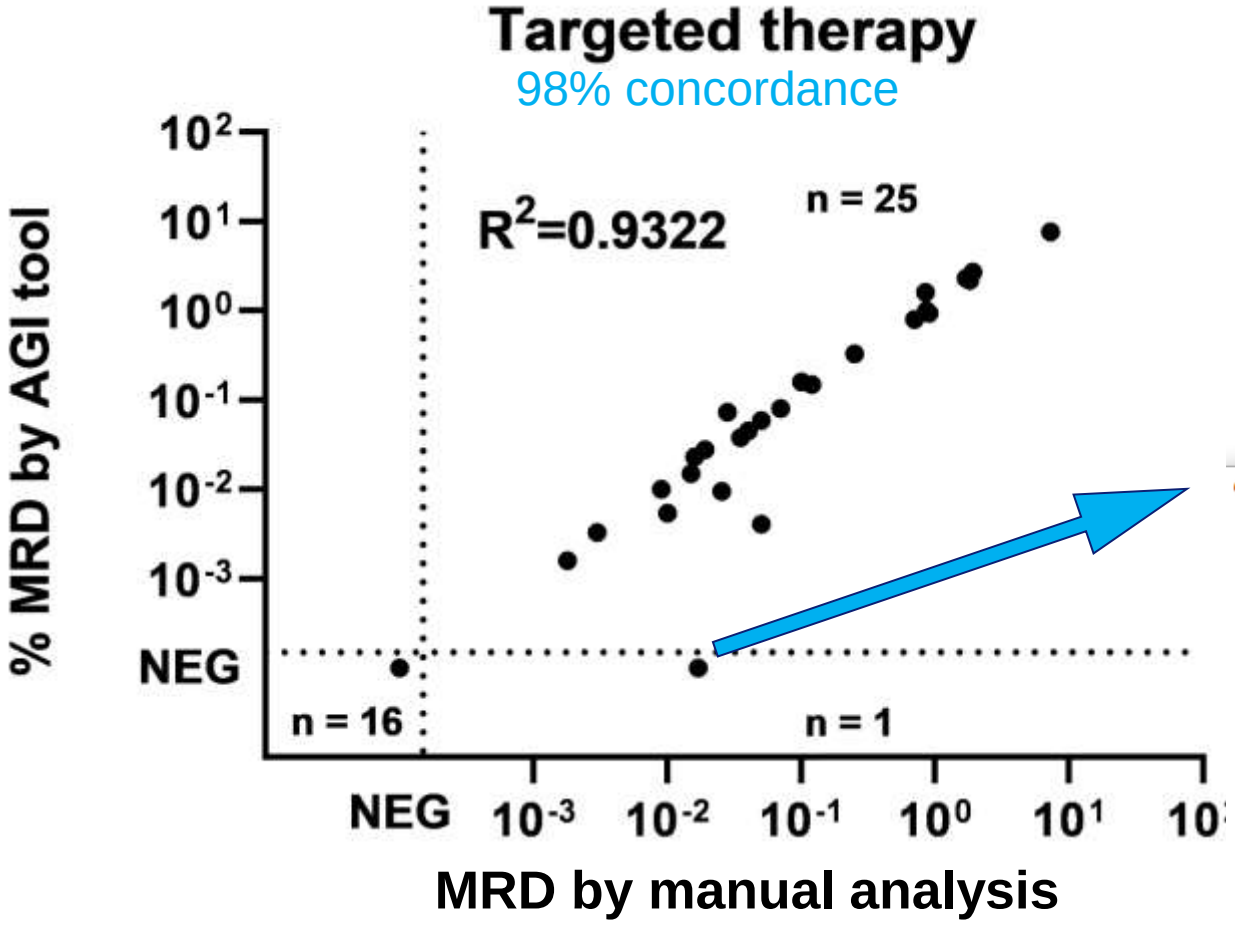
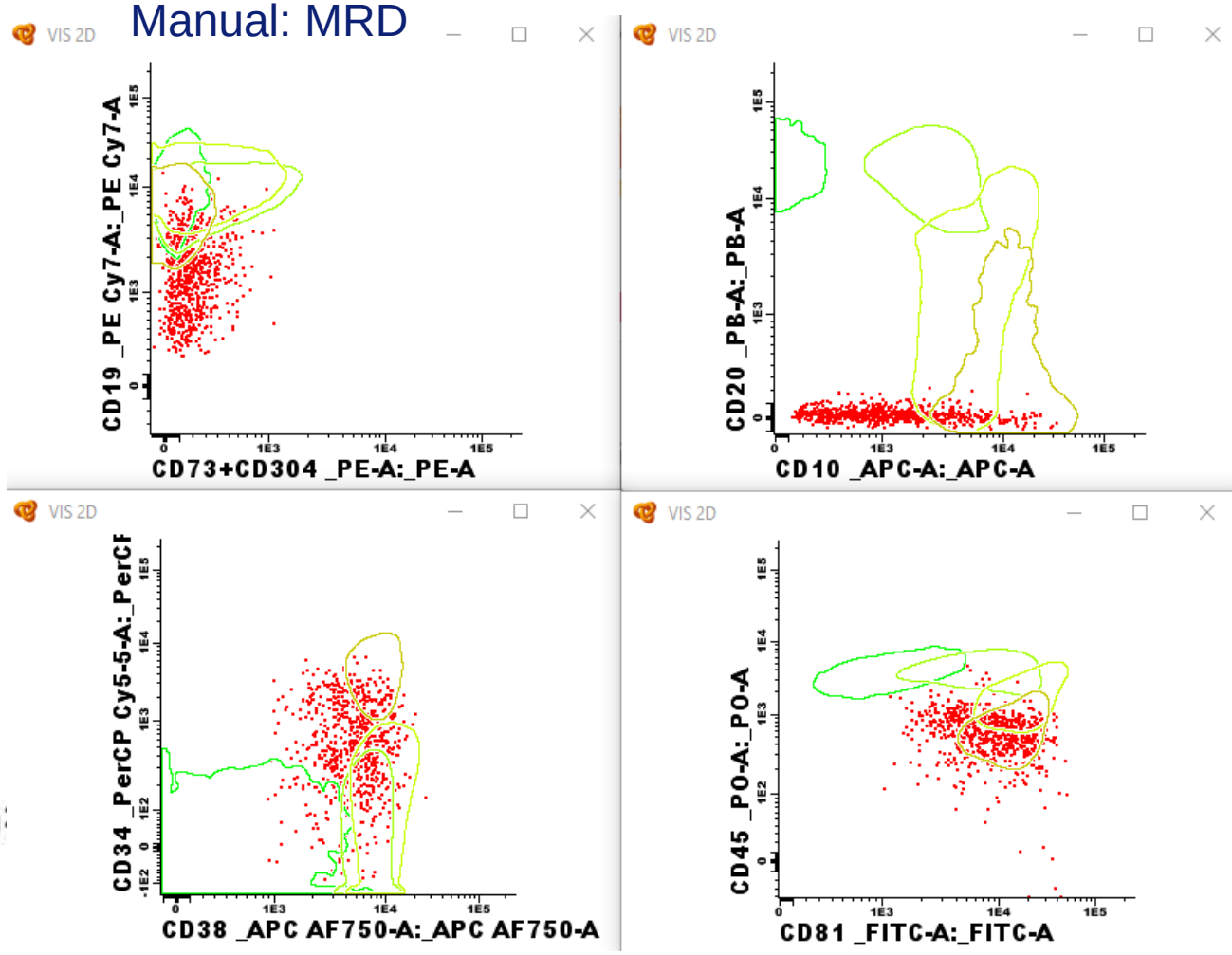


Manual: MRD



AGI: pro-B cells

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2. Semi-automated analysis – Automated report

CELLULARITY (estimated based on total nucleated cells analyzed)

Reference range: 0 - ≥ 70 years

Population	Frequency (%)	Reference (%)
B cells	2.9	(0.53 - 20.8)
Pre-BII cells ~	**< LOD**	(0.08 - 12.7)
Mature B cells ~	2.8	(0.37 - 6.3)
Plasma cells ~	0.15	(0.01 - 0.68)
Additional CD34+ precursors ~	0.76	(0.26 - 2.1)
Eosinophils ~	0.55	(0.18 - 4.8)
Neutrophils ~	59.4	(41.2 - 90.4)
Monocytes ~	4.6	(3.1 - 11.2)
T and NK cells ~	**29.7**	(3.8 - 26.8)
Nucleated red cells ~	0.89	(0.2 - 11)
Mesenchymal/Endothelial cells	0.22	(0 - 0.32)
Unspecified nucleated cells ~	0.84	(0.32 - 3)
Abnormal B-cell precursors ~	**0.021**	-

Absent populations: Pro-B cells, Pre-BI cells, Immature CD10+ B cells ~
~ Populations reviewed by user. Modifying events from the gates may influence the result of the analysis.
Sample with 6.1% of debris.

Limit of Detection (LOD):	0.00053	Lower Limit of Quantification (LLOQ):	0.0021

IMMUNOPHENOTYPE OF ABNORMAL B-CELL PRECURSORS

Abnormal B-cell precursors:
$CD81^{lo}$ CD66c/CD123^{-} CD34^{-} CD19^{+} $CD10^{lo}$ CD38^{-} CD20^{+} CD45^{+}.

lo: low; hi: high.

CONCLUSION

Bone marrow compatible with positive MRD (0.021%).

Verbeek et al. Clinical Cytometry (in press)

Conclusions – 2

- The AGI tool correctly identifies 15 normal BM subsets
- Bone marrow composition is age-dependent → age-dependent alerts
- The AGI supports MRD assessment with 97% concordance
  - Analysis is independent of tube, therapy or flow cytometer
- AGI tool supported analysis showed good intra- (100%) and inter-expert concordance (90%)

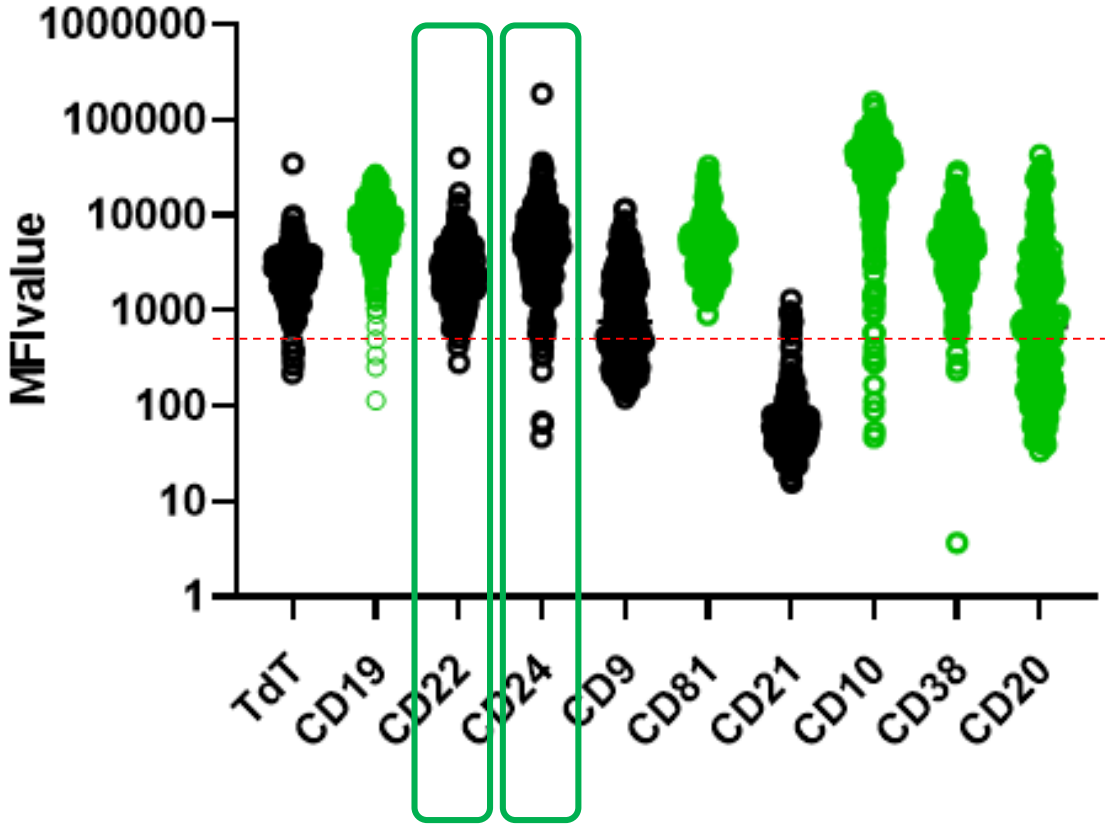
Conclusion – 2

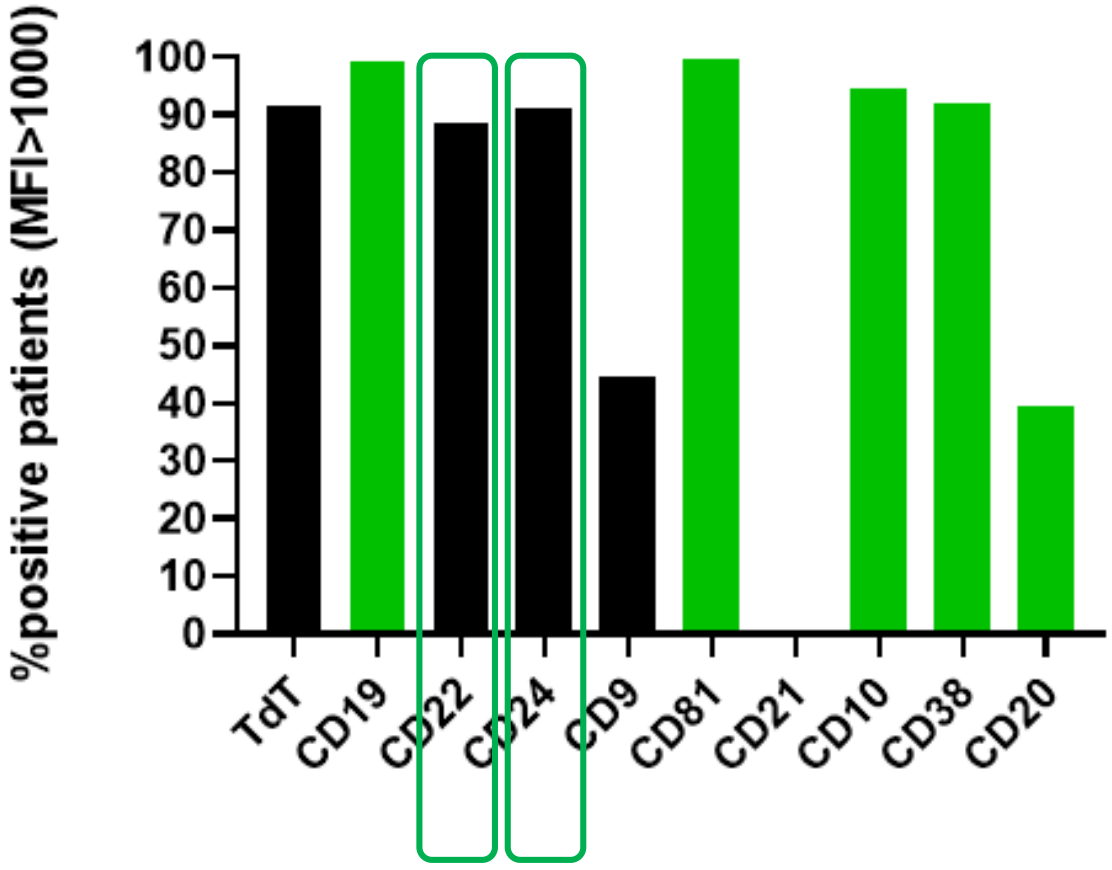
If CD19 gating is not possible:

1. Alternative gating strategy? 
2. Semi-automated analysis? 
3. Alternative B-cell markers?

3. Alternative B-cell gating markers?

- Retrospective analysis of B-cell markers (n=237 BCP-ALL patients at diagnosis)
  Green labeled markers: already present in BCP-ALL MRD panel

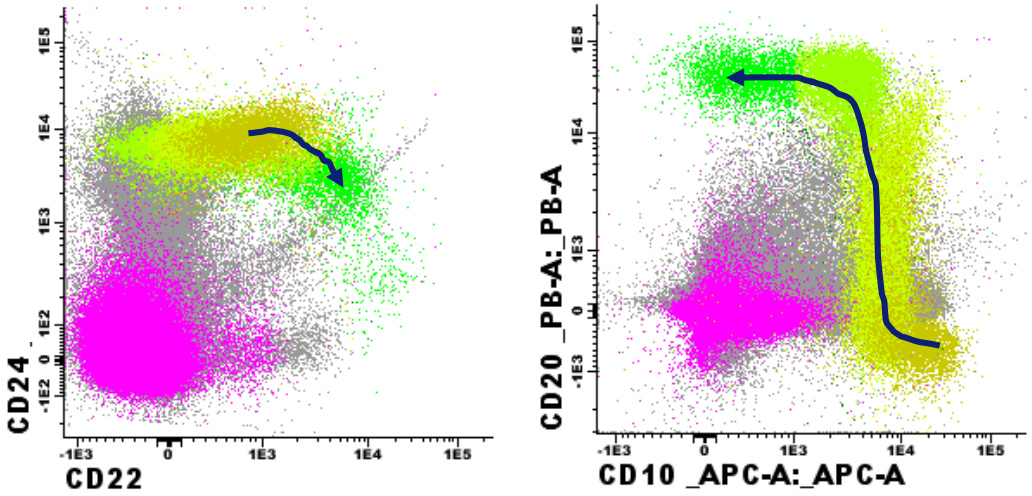




3. Alternative B-cell gating markers?

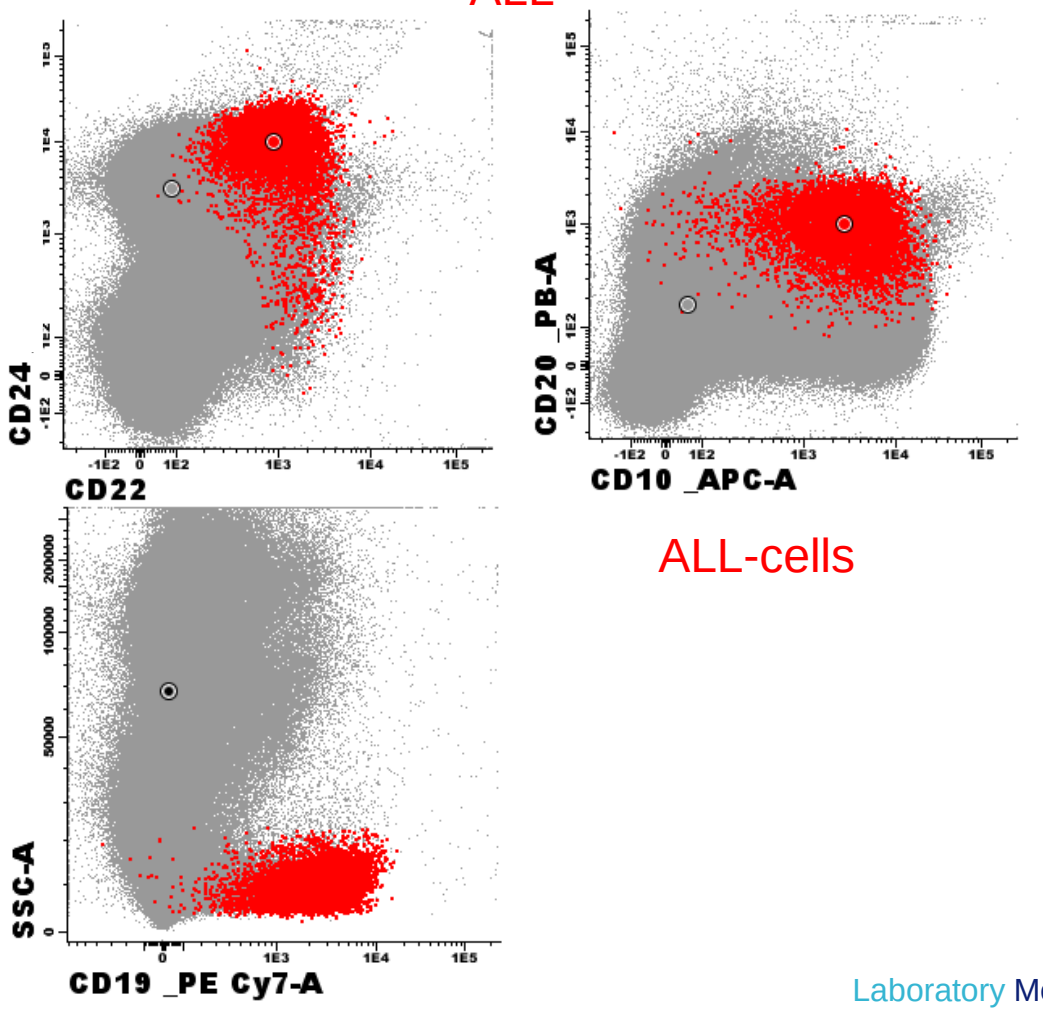
- 12-color stainings: EuroFlow 8-color MRD tube + CD22 + CD24 + …

Normal BM



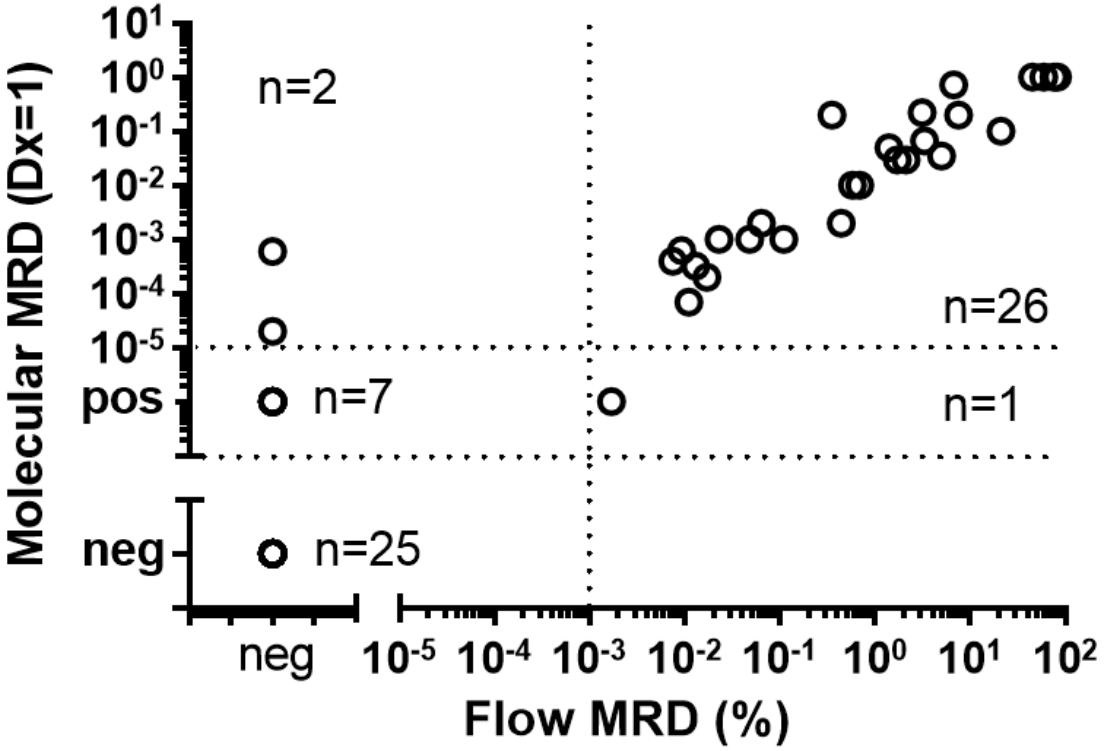
T/NK-cells B-cells

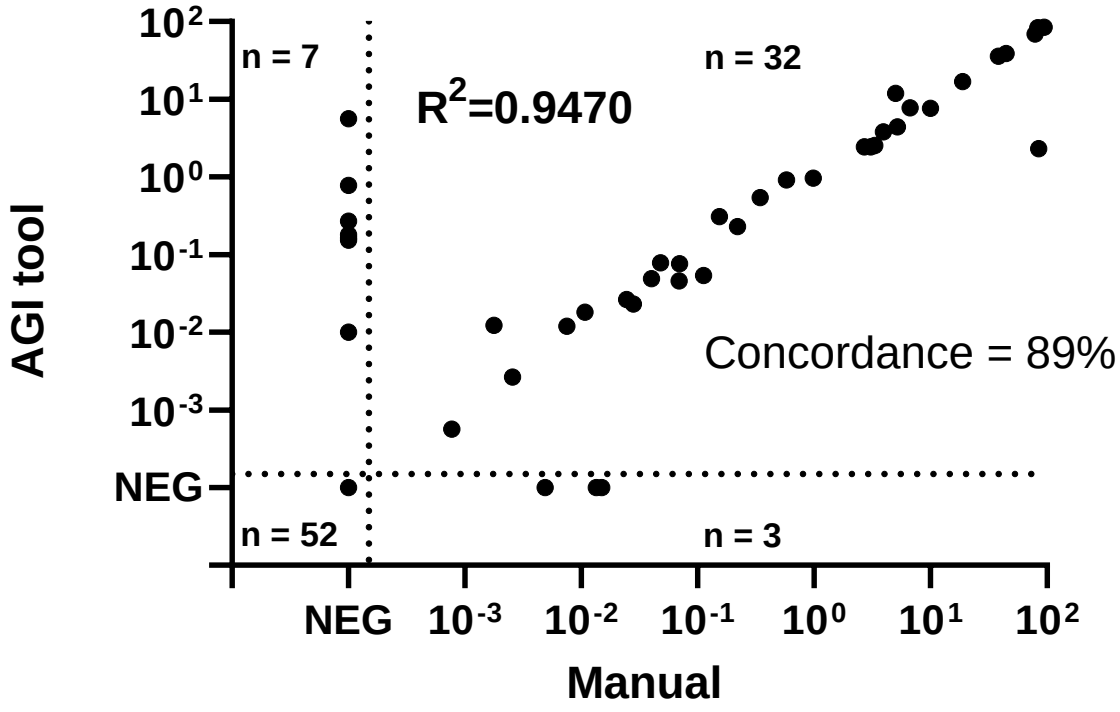
ALL



ALL-cells

Evaluation of 12 color tube





→Good correlation with molecular data

→ can be used with 8 color AGI tool

Further evaluation, focusing on (rare) CD19-negative cases, is ongoing

Standardized flowcytometric MRD analysis in BCP-ALL patients

- Here are the key steps involved in standardized flow cytometric MRD analysis for BCP-ALL:

1. Sample Collection → clinical protocols
2. Sample Preparation
3. Antibody Panel Design
4. Staining
5. Flow Cytometry Acquisition

→ EuroFlow

6. Data Analysis
7. Reporting

→ Infinicyte

8. Quality Control and Assurance
9. Inter-Laboratory Standardization

→ EuroFlow

10. Clinical Interpretation → clinical protocols
11. Continued Education and Training
12. Research and Development

→ EuroFlow

Flow cytometric MRD analysis – Conclusions

- Well established and standardized (EuroFlow) for “classically” treated ALL patients

- Next to MRD:
  - Presence or loss of therapeutic targets
  - Characterization of normal cells and other abnormal cells (e.g. switched acute leukemia cells)

- Flow cytometric methods will further be adapted to allow reliable MRD analysis in patients treated with targeted therapies as well → EuroFlow (12 color tubes)

- Data analysis should further be automated and standardized

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