Immunophenotyping in hematological malignancies

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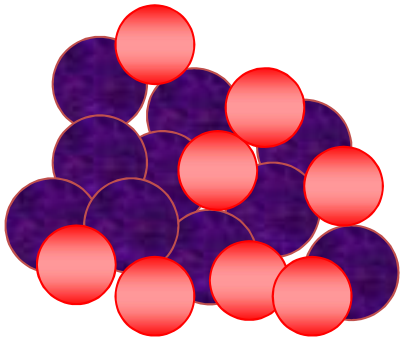
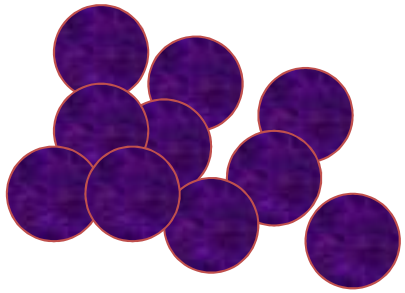
Unilab-Lg, Hematobiology

Blood
Marrow
Lymph node
CSF



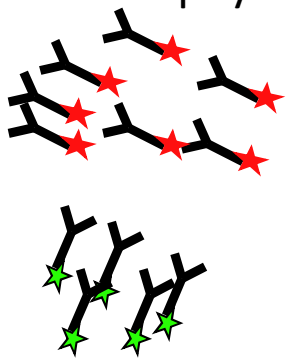
Dissociation
Red cell lysis



Incubation with fluorescence-tagged antibodies

(w or w/o permeabilization)

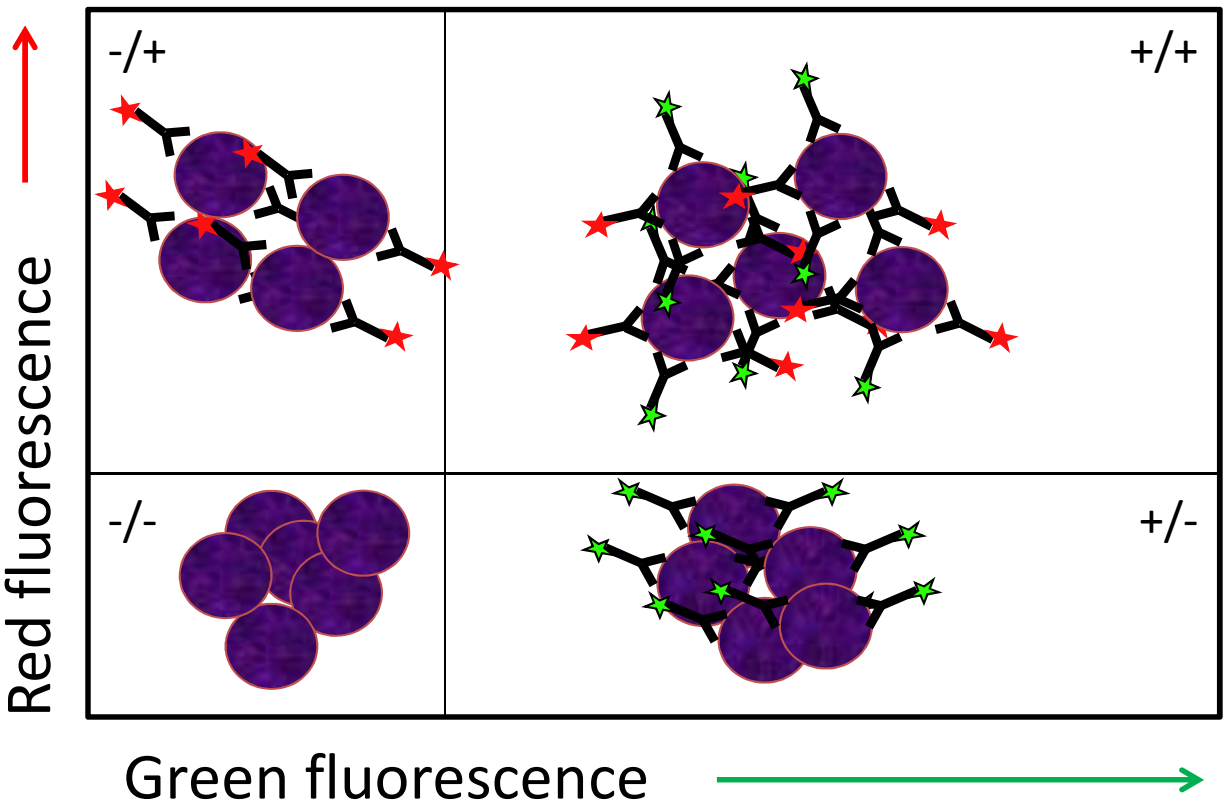
phycoerythrin



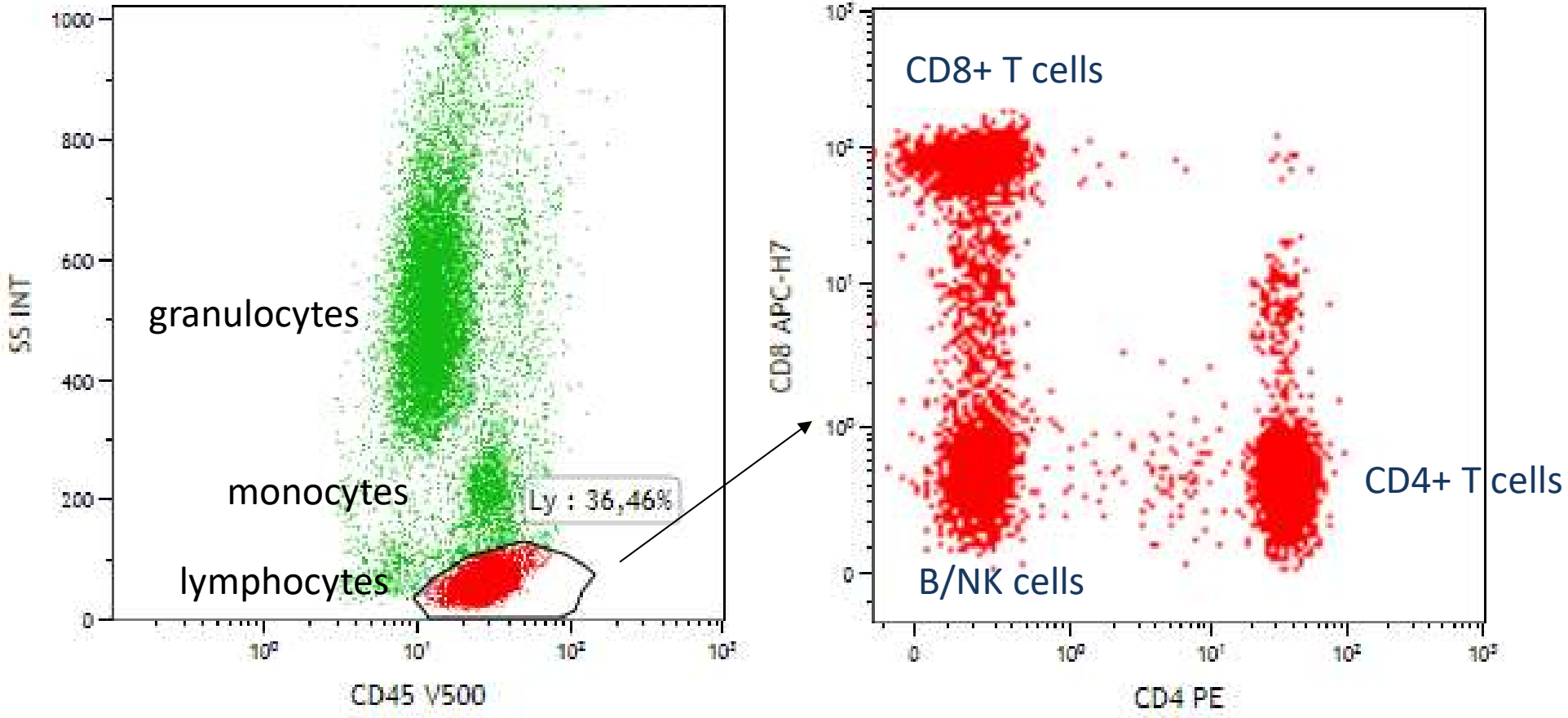
fluorescein

cytometer

Dot plot



« Gating » and « dot plots »



Fluorochrome	Abbreviation	Excitation max (nm)	Emisson max (nm)
Cascade blue		380, 401	419
Cascade yellow		399	549
Pacific blue		410	455
Alexa 488*		495	519
Fluorescein isothiocyanate*	FITC	494	519
Phycoerythrin*	PE	496, 546	578
Texas red*	ECD	595	615
PE-cyanine 5*	PC5/PE-Cy5	496, 546	667
PE-cyanine 5.5*	PC5.5/PE-Cy5.5	495, 564	696
PE-cyanine 7*	PC7/PE-Cy7	495, 564	767
Peridinin-chlorophyll*	PerCP	482	678
PerCP-cyanine 5.5	PerCP-Cy5.5	482	678
Allophycocyanin*	APC	650	660
APC-cyanine 7	APC-Cy7	650	785

What is your favourite colour?

In clinical flow cytometry (2020): standard = 8 to 12 colour combinations

Main indications for immunophenotyping in haematological malignancies

- Acute leukaemias
- Chronic lymphoproliferative disorders (B/T)
- Plasma cell disorders
- Minimal residual disease (ALL, AML, MM, CLL)

ACUTE LEUKAEMIAS

Acute leukaemias
Flow chart

Blast cells in leukocyte differential
Unexplained cytopenia

1. Is the abnormal cell population of a precursor cell type?

2. What is the lineage specificity?
   i.e., T, B, myeloid, mixed-type or undifferentiated

3. Is there aberrant antigen expression?
   Further assessment of minimal residual disease

Acute leukaemias: Identification of precursor cells

Precursor cell antigens	Normal expression	Hematological malignancy expression pattern
CD34	Hematopoietic stem cells Myeloid, B and T precursors	AML (70%) MDS blasts (50-100%) B-ALL (65-80%) T-ALL (30-50%)
CD117	Immature myeloid cells Mast cells Some plasma cells	AML (60-70%) Mastocytosis Multiple myeloma
TdT	Lymphoid precursors (B and T) Primitive myeloid precursors	ALL (90%) Undifferentiated AML
CD1a	Cortical thymocytes Immature dendritic cells	T-ALL (40-60%, indicative of cortical phenotype)
CD45	All leucocytes, brighter on lymphocytes and monocytes	Dim expression on precursor cells

Requirements to assign > 1 lineage to a single blast population

Lineage	Relevant antigen
Myeloid	- Myeloperoxydase (MPO) or - Monocytic antigens (two of CD11c, CD14, CD64, lysozyme) or - two of CD117, CD33, CD13
T-lineage	Cytoplasmic CD3 (cCD3)
B-lineage	- Strong CD19 + one of cCD79a/cCD22/CD10 or - Weak CD19 + two of cCD79a/cCD22/CD10

Acute leukaemias of ambiguous lineage

- < 5% of AL, poor prognosis

Diagnosis	Description
Acute undifferentiated leukemia	Often CD34+, HLA-DR+, CD38+ Sometimes TdT+, CD7+ No expression of myeloid or lymphoid specific markers
Mixed phenotype acute leukaemia (MPAL)	Co-expression of specific lymphoid and myeloid markers (mostly B/myeloid, T/myeloid)

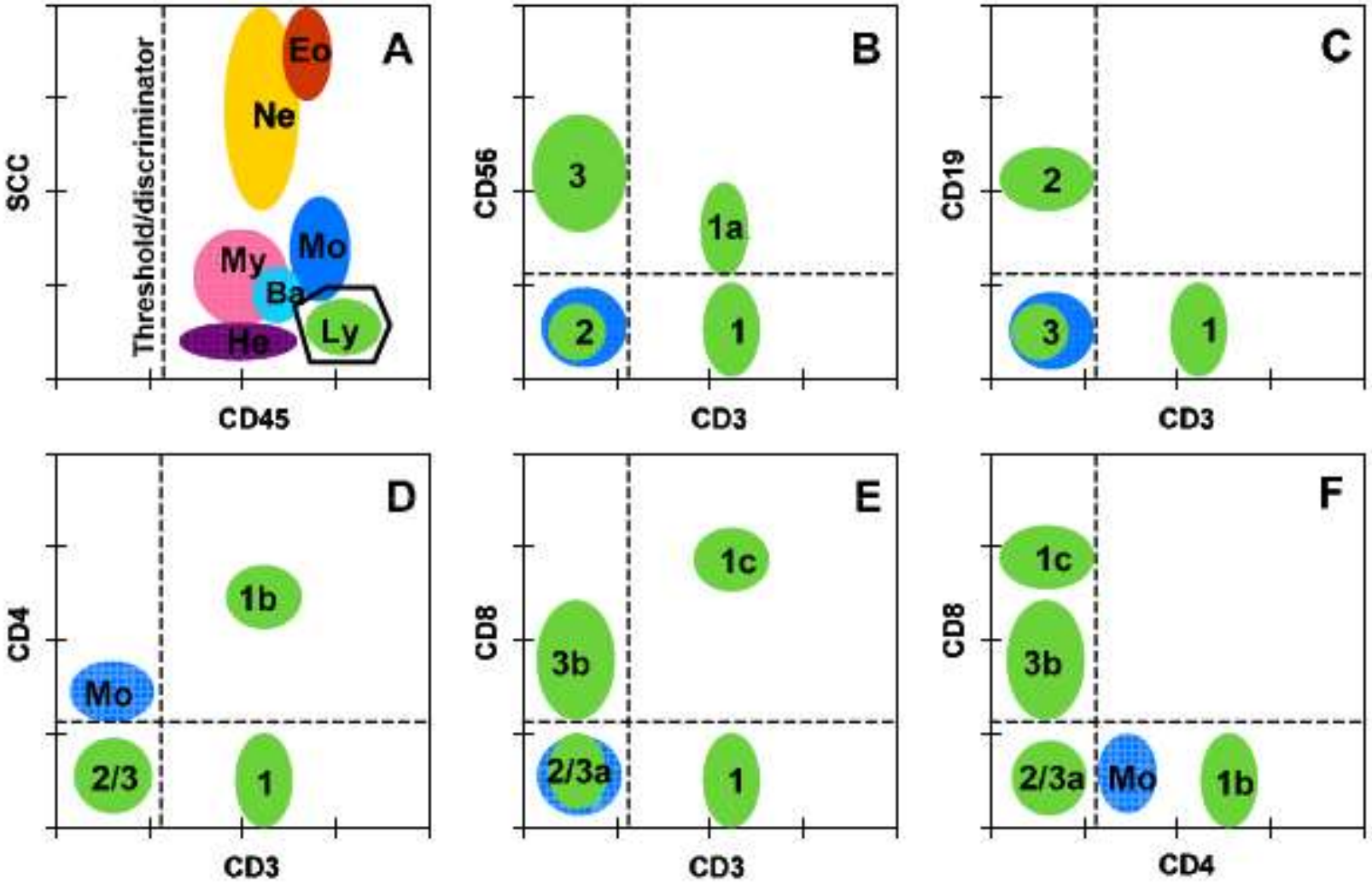
Acute leukaemias: aberrant expression – « lineage infidelity »

	AML	B-ALL	T-ALL
M		CD13, CD14, CD15, CD33, CD65	CD13, CD33
B	TdT		CD79a
T	TdT, CD7, CD2, CD4	CD4	
NK	CD56	CD56	CD56

Specific phenotype of tumor cells ≠ normal cells

CHRONIC LYMPROLIFERATIVE DISEASES

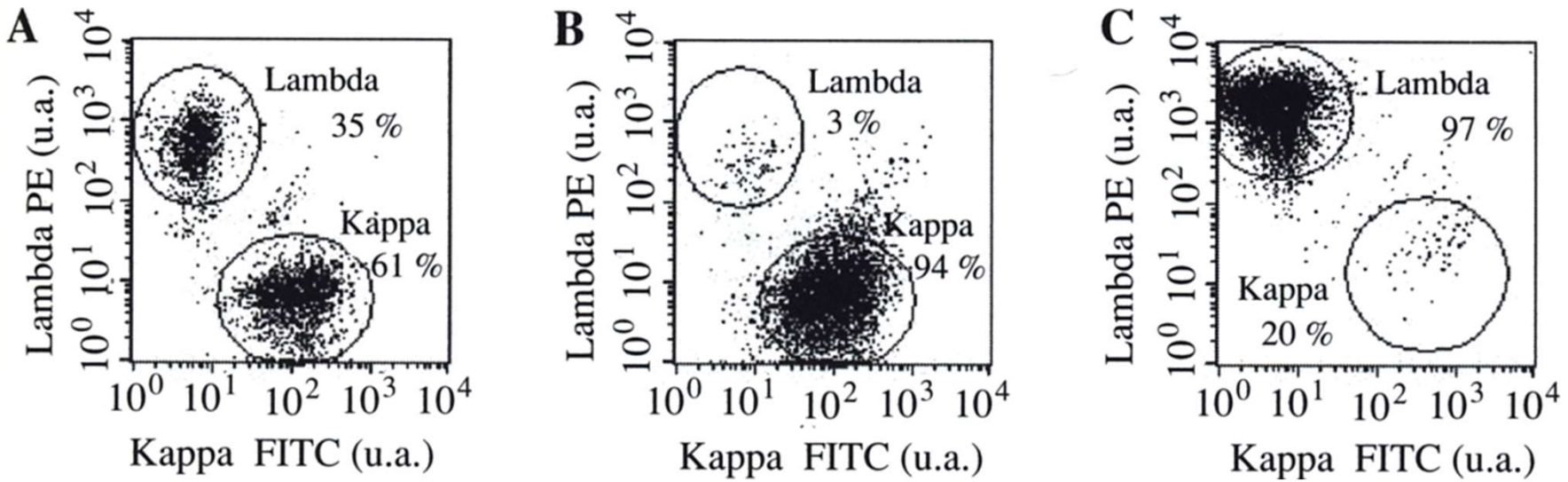
The basic « lymphocyte typing »



1. CD3+ T cells
1a. CD3+CD56+ NKT
1b. CD3+CD4+ T cells
1c. CD3+CD8+ T cells
2. CD19+ B cells
3. CD3-CD56+ NK cells

B-cell chronic lymphoproliferative diseases

- Identification of a clonal B-cell disorder
  - Clonality: skewing of kappa/lambda Ig light chain ratio > 3/1 or < 1/3
  - Weak or absent Ig light chain expression
  - Weak or absent markers expressed by normal B cells: CD79a, CD22, CD20



The Catovsky-Matutes score and differential diagnosis of B-CLPD

Markers	**Points**	
	0	1
CD5	Negative	Positive
CD23	Negative	Positive
FMC7 (CD20 epitope)	Positive	Negative
CD79a	Positive	Negative
Kappa or lambda	Moderate/bright	Weak

Score = 4-5 → **CLL**/MBL

Score = 3 → « atypical » CLL/MBL, assess CD43, CD200

Score = 0-2 → differential diagnosis of CD5+ LPD: → MCL, SMZL, B-PLL

→ differential diagnosis of CD10+ LPD: → FL, DLBCL, BL, B-ALL

→ CD11c+, CD103+, CD25+, CD123+: → **HCL**

Moreau et al., Am J Clin Pathol 1997;108:378-382.

CLL, SLL and monoclonal B cell lymphocytosis

- B cell reference range: 100-500 polyclonal B cells/µl,
- CLL = > 5000 circulating monoclonal B cells/µl
- < 5000 monoclonal B cells
  - With node/spleen involvement = SLL
  - Without node/spleen involvement = MBL
    - < 500/µl: low count MBL, no progression to CLL
    - > 500/µl: high count MBL, 1% progression to CLL/year

CLL: chronic lymphocytic leukemia
SLL: small lymphocytic lymphoma

Identification of clonal T CLPD

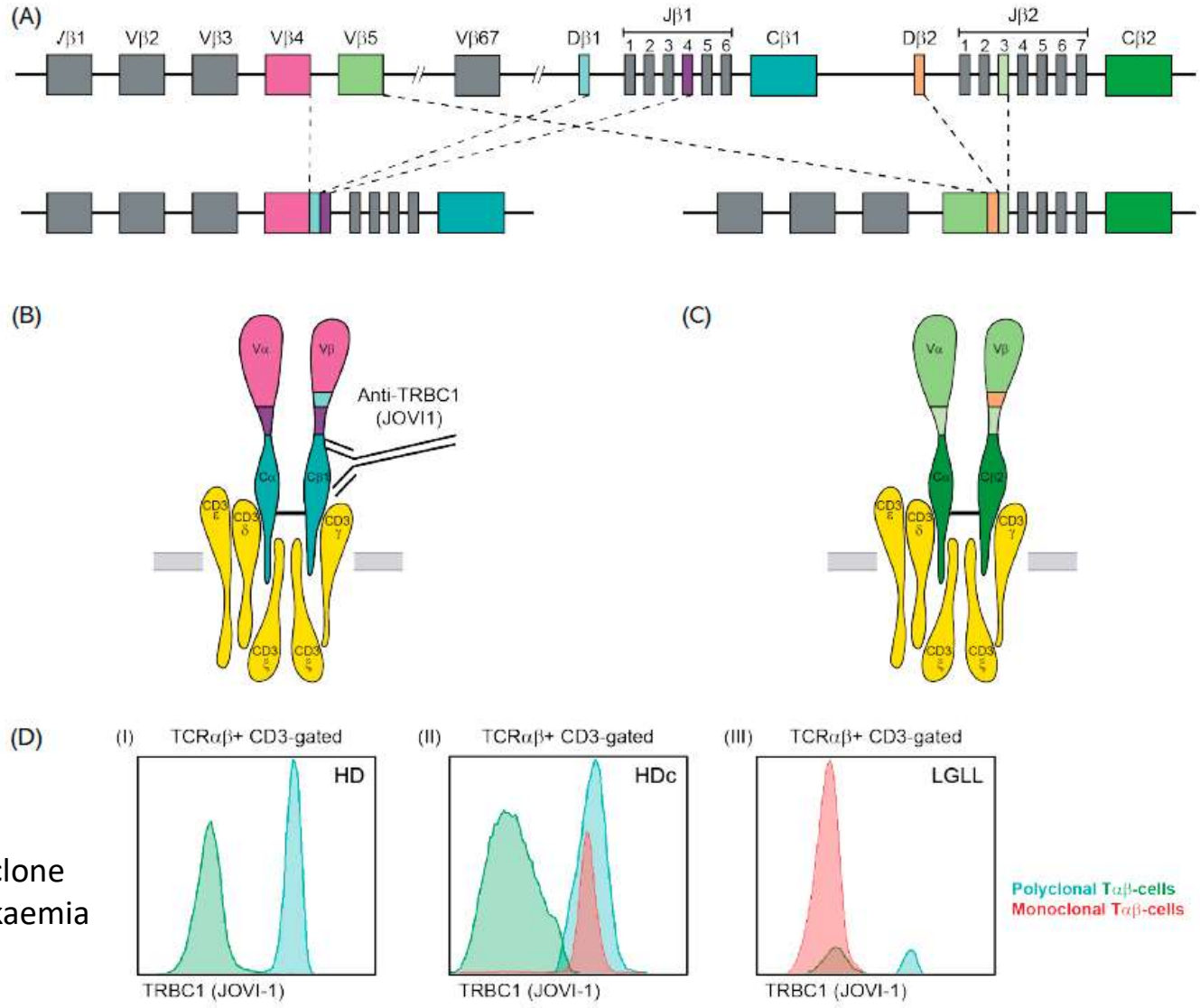
- Skewing of the CD4/CD8 ratio >10 or <0.1
- CD4+CD8+ or CD4-CD8- T cells
- Clonality: skewing of the TCR Vβ repertoire, TRBC1 inbalance
- Loss of normal T cell markers: CD5, CD7

Differential diagnosis

CD4+CD8- | CD4-CD8+ | CD4-CD8- | CD4+CD8+

See Craig FE, Foon KA. Blood. 2008; 111(8):3941-67

Identification of T clonality.
T cell receptor beta chain constant domain - TRBC1

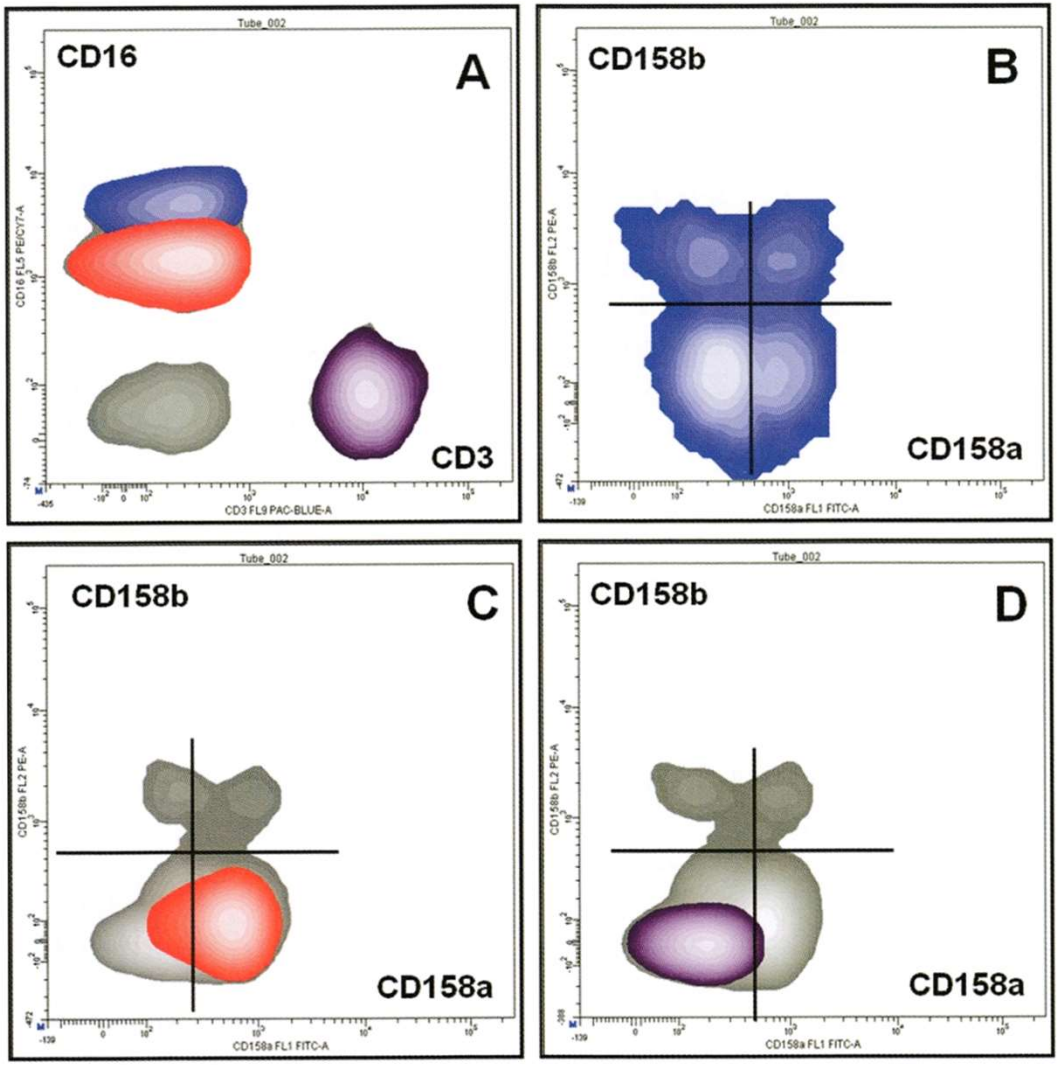


HD: healthy donor
HDc: healthy donor with indolent T clone
LGLL: large granular lymphocyte leukaemia

Munoz-Garcia, Cancers 2021.

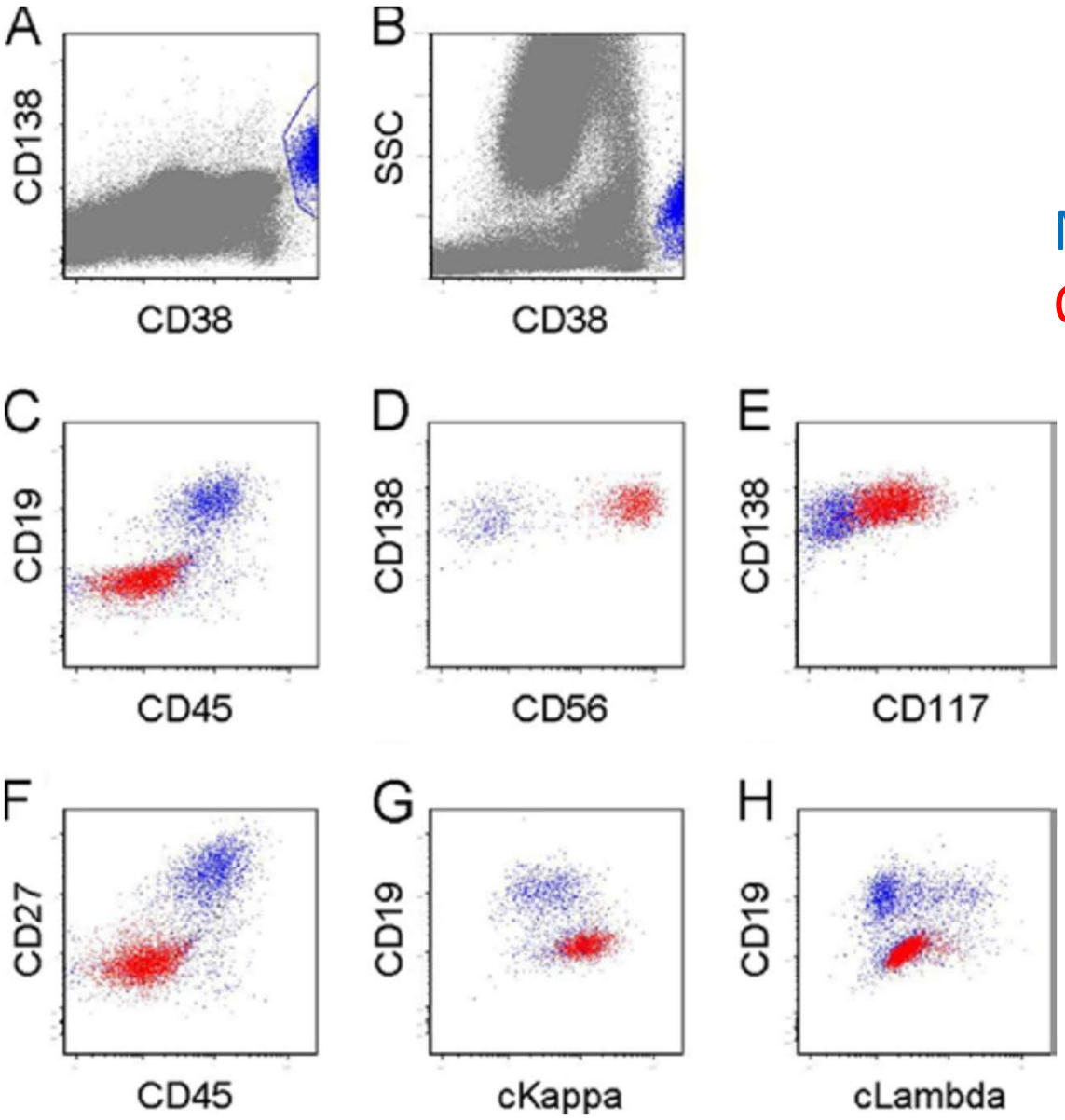
NK cells proliferative disorders. Clonality.

- Killer-cell Immunoglobulin-like Receptors (KIR):
  - NK cells
  - Some T CD8+ subsets
- Clustered to the CD158 family, 14 isoforms
- Indicative of clonality:
  - Restricted expression of a single KIR isoform



PLASMA CELL DISORDERS

Plasma cell disorders

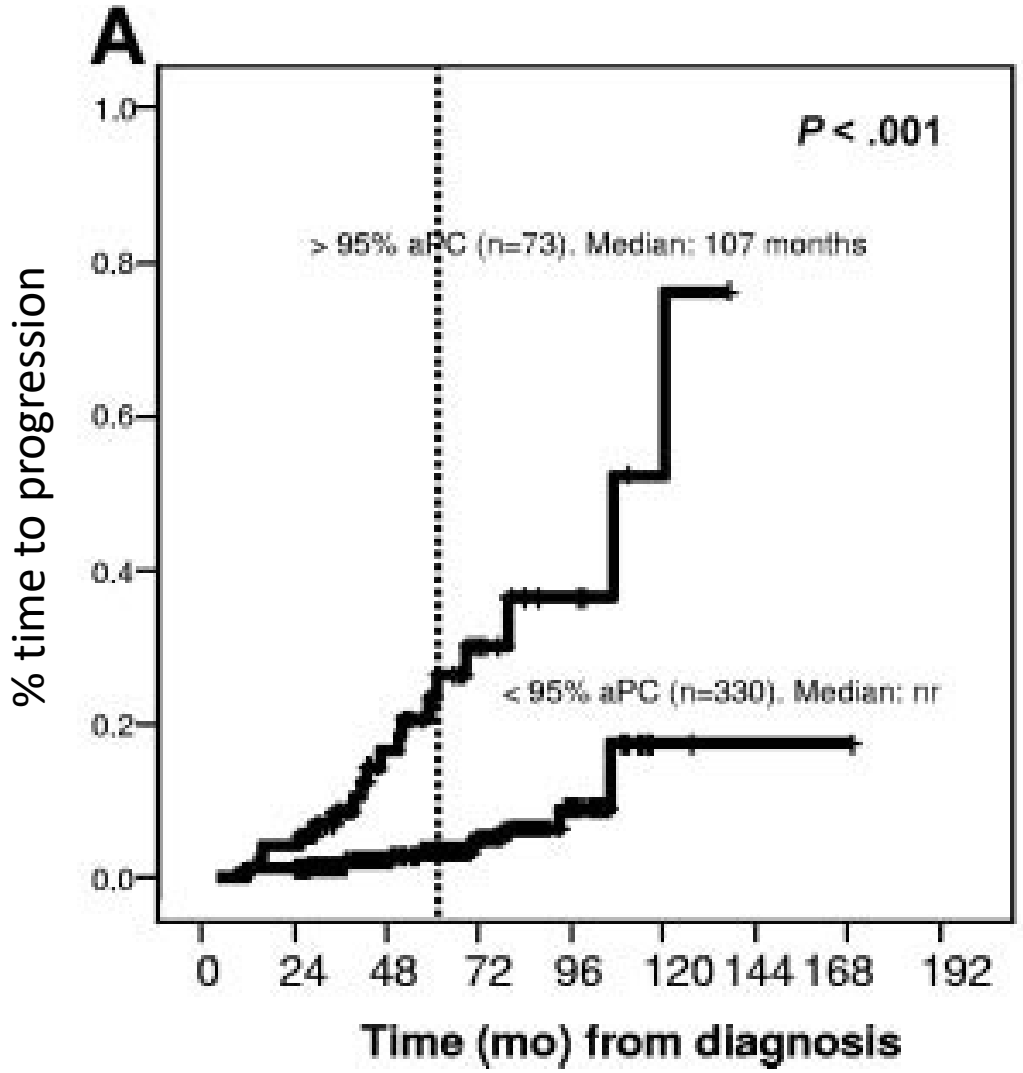


Normal plasmocytes

Clonal plasmocytes

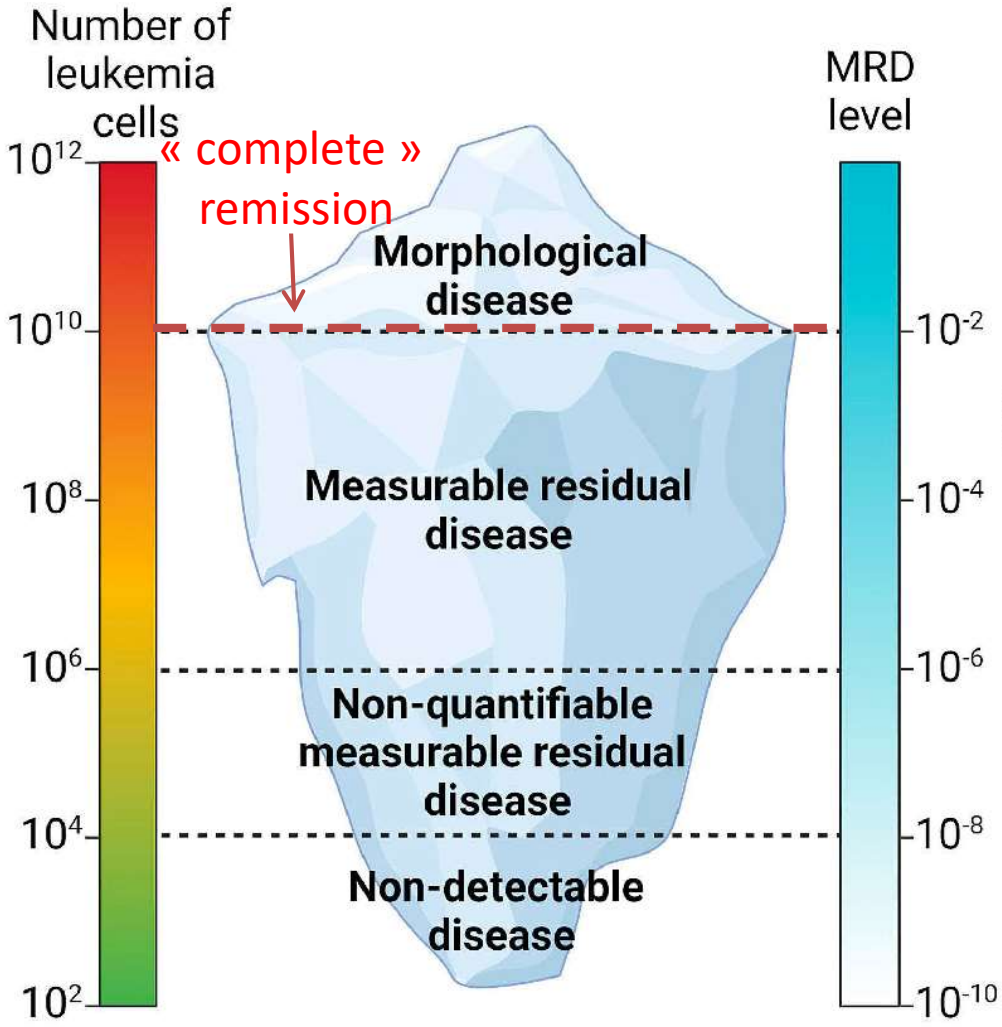
Residual normal plasmocytes and progression from MGUS to MM

MGUS

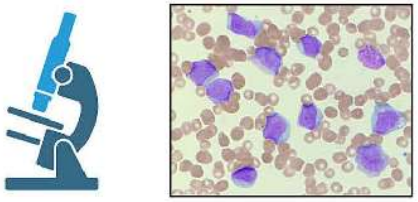


Perez-Persona et al., Blood. 2007;110:2586-2592

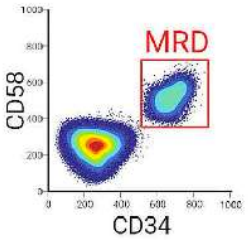
MINIMAL/MEASURABLE RESIDUAL DISEASE



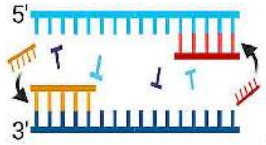
Methods of MRD detection



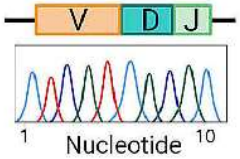
Light microscopic evaluation



Multicolor flow cytometry



Real time PCR



Next generation sequencing

MRD not quantifiable or non-detectable with current methods

CURE

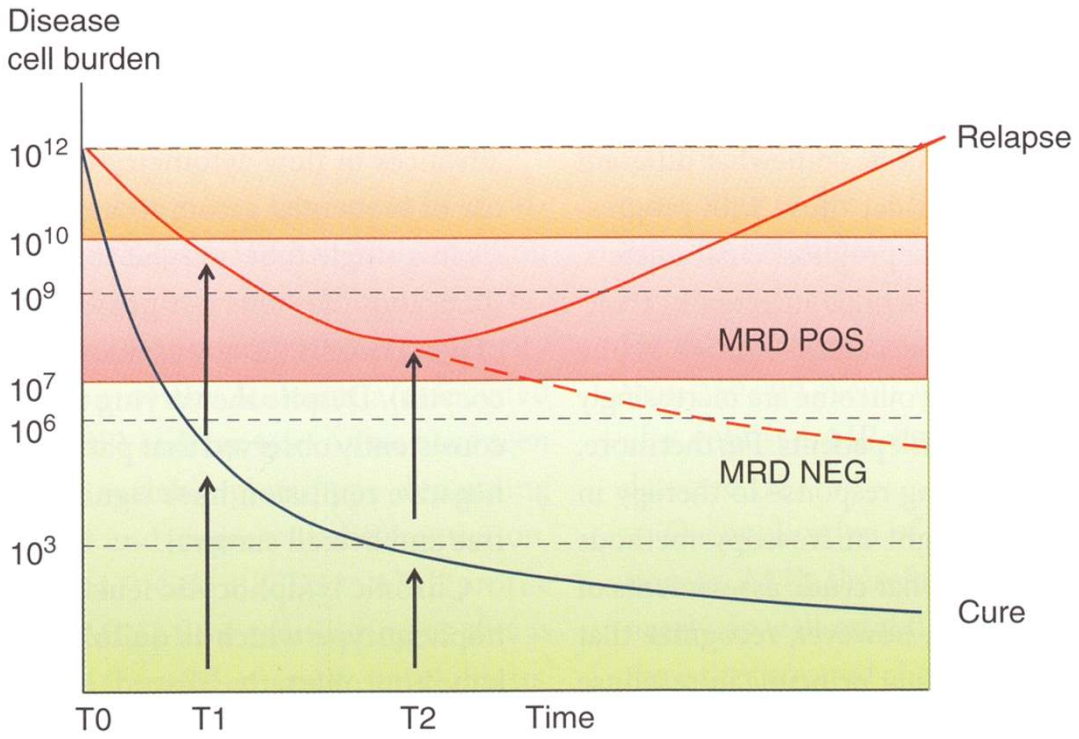
General principles for MRD quantitation by immunophenotyping

- Target disease ~
  - unique immunophenotype, at least two aberrant markers for discrimination from normal cells: « LAIP », **leukemia-associated immunophenotype**
  - Acquisition of lineage markers **different from normal** maturation pathways

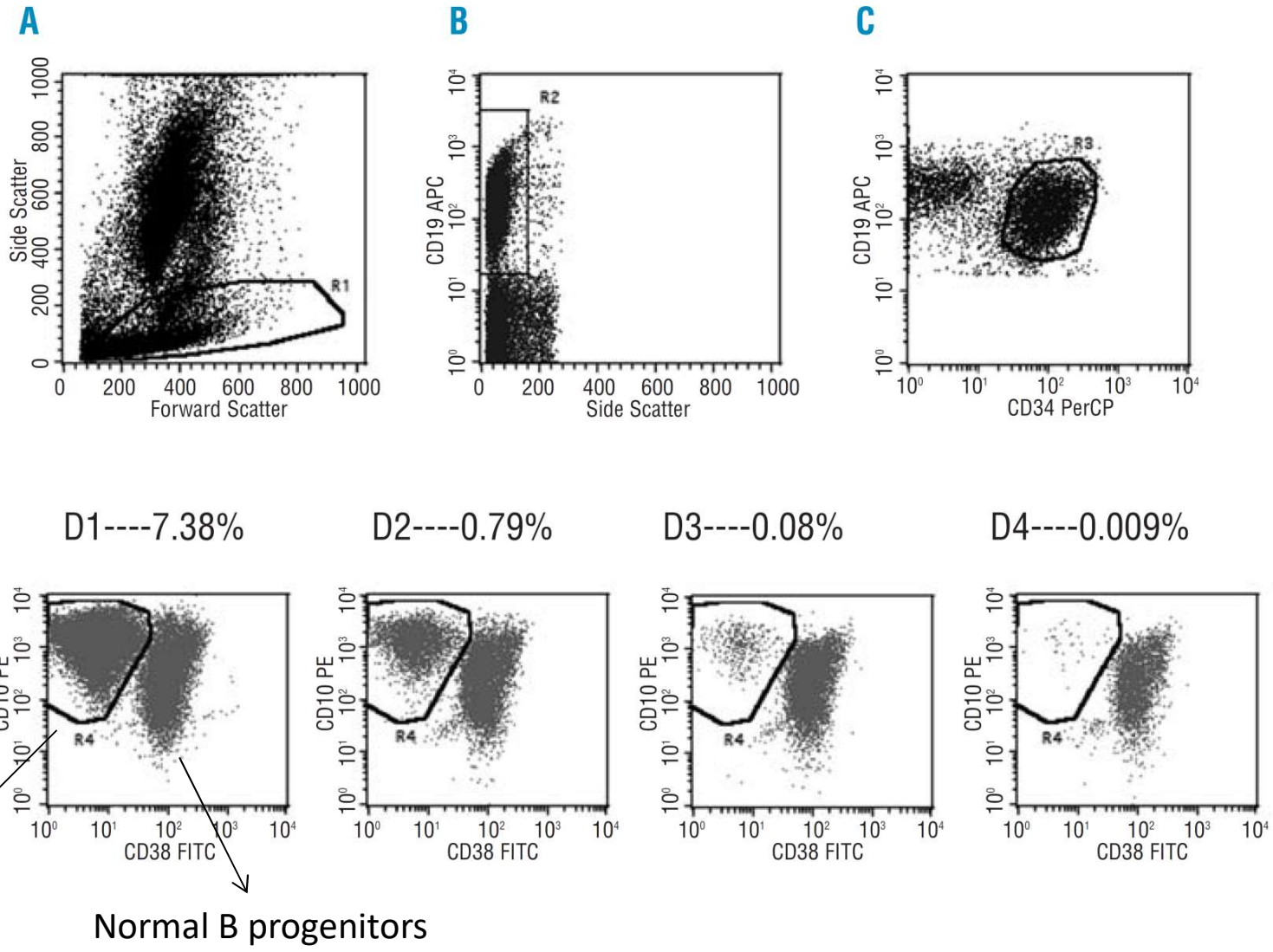
- High sensitivity → large number of cells analyzed
  - « rough estimate » = minimum cluster of 40 cells with a well-defined aberrant phenotype
  - $1*10^{-4}$ sensitivity → 400.000 cells to analyze
  - $1*10^{-5}$ sensitivity → $4*10^{6}$ cells to analyze

Main applications of MRD quantitation by flow cytometry

- Definition of deeper remission status than « complete » remission
- Estimation of risk of relapse post remission
- Early marker of impending relapse
- Surrogate end-point for drug development (vs « cure »)
- In clinical routine: ALL, AML, MM



B-ALL and MRD



UKALL Flow MRD Group, Irving et al., Haematologica 2009

REPORTING PHENOTYPIC DATA

Flow cytometry reporting

- Patient information: indication, previous FCM data, other lab results (WBC, differential)
- Sample information: sample type, anticoagulant, date collected/received
- Sample preparation: **antibodies used**, cell viability
- Data analysis:
  - Overall information on normal cells (B/T cells, CD4:CD8 ratio, NK, monocytes, granulocytes)
  - If present, % abnormal cells compared to a defined population (total leucocytes, total lymphocytes…)
  - Marker distribution on abnormal cells: +, –, partial; fluorescence intensity if relevant (dim, bright, heterogeneous, homogeneous)
  - ~~**List of % positive cells for each marker tested, relative to total cells**~~**: irrelevant, misleading!**
- Interpretation:
  - Differential diagnosis according to WHO defined subtypes
  - A definite diagnosis requires integration with relevant pathology/molecular biology/cytogenetic data

Recommendations of the Bethesda Consensus Conference, Wood et al., Cytometry, 2007, 72B-S14

References

- EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. Van Dongen JJ et al. Leukemia 2012;26:1908–1975.
- Validation of cell-based fluorescence assays: practice guidelines from the ICSH and ICCS – part V – assay performance criteria. Wood B et al.; ICSH/ICCS Working Group. Cytometry B Clin Cytom. 2013 Sep-Oct;84(5):315-23. Review.
- Minimal residual disease:
  - ALL: Theunissen P. et al. Blood 2017; 129:347
  - MM: Flores-Montero J. et al. Leukemia 2017; 31:2094
  - AML: Heuser et al., Blood 2021; 138-2753